Developmental biology IV

Twinning

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Twinning has fascinated human beings over the centuries. New technologies and large study groups have led to improved documentation of frequency and complications in twin pregnancies and long-term outcomes. Artificial reproductive technologies have led to a pronounced rise in numbers of dizygotic and monozygotic twins. Although spontaneous dizygotic twinning is clearly associated with increased concentration of follicle-stimulating hormone and ovulation of more than one egg, causes of monozygotic twinning remain illusive. Twin studies are used increasingly to study complex traits and disorders: however, caution is suggested, since twins might not be representative of a typical singleton pregnancy. Monozygotic twinning seems to represent an anomaly in itself, with an increased number of spontaneous abortions and structural congenital anomalies. Both monozygotic and dizygotic twins have growth rates that slow at 30 weeks in utero and might be programmed both developmentally and biochemically earlier in pregnancy to have different responses at birth and after birth compared with singletons.

Twins have intrigued civilisations across the centuries. With developments in obstetrics, developmental biology, clinical genetics, and molecular genetics, it might be possible to understand why twinning takes place in human beings, and to use the processes of twinning for insights into early human development.

Twins are not rare: the spontaneous rate of twinning is about 1 in 80 livebirths, which means that about 1 in 40 babies born would be a twin.[1] The rate of monozygotic twinning has been fairly constant around the world, with variability being attributable to the rate of dizygotic twinning, so that the ratio of monozygotic to dizygotic twinning varies strikingly.[1–3] The prevalence of spontaneous twinning in livebirths ranges from about 6 in 1000 in Asia, about 10–20 in 1000 in Europe and the USA, to about 40 in 1000 in Africa. In Japan, only 1 in 250 newborn babies is a twin, whereas in Nigeria, 1 in 11 is a twin. In African-Americans, only 26 in 1000 neonates are twins, indicating admixture and different geographical localities.

In the 19th century, the Scottish obstetrician J Matthews Duncan recognised that there were two types of twins, but Francis Galton is usually credited with suggesting the possibility of using twins to compare the contributions of nature (heredity) with those of nurture (environment).[4,5] This method has been expanded greatly, particularly in the past few decades, to study the genetic basis of complex traits and diseases.[6] These studies depend on the notion that MONOZYGOTIC TWINS come from one fertilised egg (zygote), and thus their genetic makeup would be expected to be identical, hence the lay use of the term identical twins when referring to monozygotic twins. Thus, differences between twins of a monozygotic pair (discordance) would be expected to be attributable to environmental factors—since their genetic inheritance for practical reasons is the same—whereas DIZYGOTIC TWINS (with two separately fertilised eggs) would be expected to be different on the basis of both genetic and environmental factors.

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Differences in concordance rates between monozygotic and dizygotic twins for traits and disorders have been used in many studies, from congenital anomalies to adult-onset diseases and psychological traits, to identify heritability of particular traits and disorders. This approach has been very useful, and can deal with many variables simultaneously, and many twin registries have been set up to use this method.[6]

This report will not discuss medical complications of mothers and children associated with twinning, but rather will focus on some biological and genetic aspects of twinning.

Zygosity

Zygosity refers to whether twins arose from one fertilised egg—ie, monozygotic—or from two eggs fertilised by two different sperms, ie, dizygotic. Early in the 20th century, Weinberg[7] proposed a method to obtain a rough population estimate of the number of monozygotic versus dizygotic twins. He assumed all monozygotic twins and half of dizygotic twins would be of the same sex, and the other half would be of unlike sex. He multiplied the number of unlike sex twins by two, which he suggested would provide a good estimate of the number of dizygotic twins: the excess of same sex twins would be the number of monozygotic twins. In other words, you could subtract the total number of unlike sex twins from the number of same sex twins to provide an estimate of the number of monozygotic twins. This calculation has been the process by which population data on twinning frequency has been developed over the years, and continues to be in many jurisdictions.

However, although this method gave an estimate of the number of twins, it was based on several incorrect assumptions, including the rare occurrence of monozygotic twins who are of unlike sex (one 46,XY and one 45,X),[8] the fact that when monozygotic twins are obviously discordant they are judged dizygotic,[9] and the

Search strategy and selection criteria

This review involved systematic review of published work on twins, including reference textbooks, Twin Research, and a 5-year MEDLINE review with the keywords 'twins', 'twinning', 'monozygotic', 'dizygotic' and 'mechanisms of twinning'.

GLOSSARY

CHIMERISM
Presence of cells from two genetically distinct sources—eg, transplantation, graft, or embryonic fusion.

CLONES
Individuals derived from one somatic cell rather than by fusion of gametes.

DIZYGOTIC TWINS
Two individuals born together derived from two separate eggs fertilised by two separate sperm.

MONOZYGOTIC TWINS
Two individuals born together derived from one sperm and one egg.

MOSAICISM
The presence in an individual or tissue of at least two cell lines differing in genotype or karyotype but derived from one zygote.

ZONA TAMPERING
Term to describe when the zona is punctured, traumatised, or split artificially.

suggestion that the actual frequency of same sex twins is higher in dizygotic twins than expected.[10,11] Later twin studies were based not on population frequencies but rather on carefully gathered and documented twin pairs.[6]

Type of chorion

Early in human development (usually by day 4), placental membranes begin to form. Benirschke[12–14] suggested that examination of placental membranes might be useful in distinguishing zygosity, and indeed today, twins are classified by their membranes, with monochorionic twins at increased risk of various complications (figure 1). Dizygotic twins have separate placentas and membranes (dichorionic diamniotic), although they might be fused and even have vascular connections. 70–75% of liveborn monozygotic twins share one placenta with monochorionic diamniotic membranes, whereas 25–30% have completely separate placentas and membranes (figure 2). 1–2% of liveborn monozygotic twins have one set of membranes and one placenta (monochorionic monoamniotic); these twins are at risk of vascular compromise because of twisting of their umbilical cords around each other.

The membranes and placenta are also a good way to estimate the timing of twinning events (table 1). In monozygotic twins with separate placentas and membranes, separation is thought to take place early—ie, between day 0 and day 3. In usual embryogenesis, the chorion begins to form at about day 3. Monozygotic twins who have a monochorionic diamniotic placenta are thought to arise after the chorion has formed. This type of monozygotic twin development would be expected to take place between day 4 and day 7 in normal embryonic development since the amnion begins to form between day 6 and day 8. Monozygotic twins who have monochorionic monoamniotic placentas probably arise between day 7 and day 14. The number of monozygotic twins actually conceived is not clear because it is probable that many abort. Conjoined twins must arise after the primitive streak begins to form, theoretically after day 14. However, how long after and by what mechanism or mechanisms is not clear.

Figure 1: **Monochorionic diamniotic placental membranes in monozygotic twin pregnancy**
Note the placental septum indicating two amniotic sacs. Since there is one placenta, it is most probably a monochorionic diamniotic placenta, but microscopic studies of the membranes as they connect to the placenta are needed to be sure. Courtesy of Dagmar Kalousek.

Monochorionic placentas of all types are prone to have vascular connections that can lead to vascular compromise or imbalance—ie, twin reverse arterial perfusion sequence or twin-twin transfusion syndrome—and to disruptive congenital anomalies. The vascular connections can also be expected to lead to sharing of haemopoietic stem cells, microchimerism between dizygotic twins, and MOSAICISM if genetic discordance arises between the monozygotic twins. The placentas of dizygotic twins and monozygotic twins who have separate placentas seem to be similar.

Recognition of zygosity

Up to now, monozygotic twins with separate placentas have been recognised to be monozygotic on the basis of physical characteristics after birth and biochemical characteristics such as blood types, enzyme polymorphisms, and HLA types.[12–15] DNA typing has been the most useful and reliable way of defining zygosity;[16] however, since 70% of monozygotic twins share vascular placental connections, DNA in blood cells will probably seem to be identical even if the twin pair could be genetically or epigenetically discordant.[9,15,16] Furthermore, several examples of dizygotic twins with monochorionic diamniotic placentas are now being recognised (Langlois S, University of British Columbia, personal communication). In at least one case, the bone marrow of one twin also filled the marrow of the other, so unless skin or some other non-haemopoietic tissue was tested, the twins would seem to be monozygotic. Nevertheless, a monochorionic diamniotic placenta is usually a very helpful way of recognising monozygosity.

Examination of the placenta of twins at birth is very important to establish the type of chorion and whether any anomalies are present. For practical reasons, a monochorionic placenta is a monozygotic twin pregnancy until proven otherwise. In twins who appear to be discordant at birth, blood studies to establish zygosity should be done only when separate placentas are present. Ideally, a skin biopsy specimen, umbilical cord tissue, or a buccal smear should be used instead of blood for DNA studies to establish zygosity and the basics of discordance.

Growing evidence suggests that dizygotic twins might also carry haemopoietic stem cells derived from their twin, even with no obvious vascular connections.[17] Whether this CHIMERISM arises because of small, non-obvious placental vascular connections or via fetal-maternal-fetal microchimerism is not yet clear. Results of most studies of dizygotic placentas show no obvious vascular connections.[16] Thus, cells from the other dizygotic twin might come via the mother, who very clearly experiences

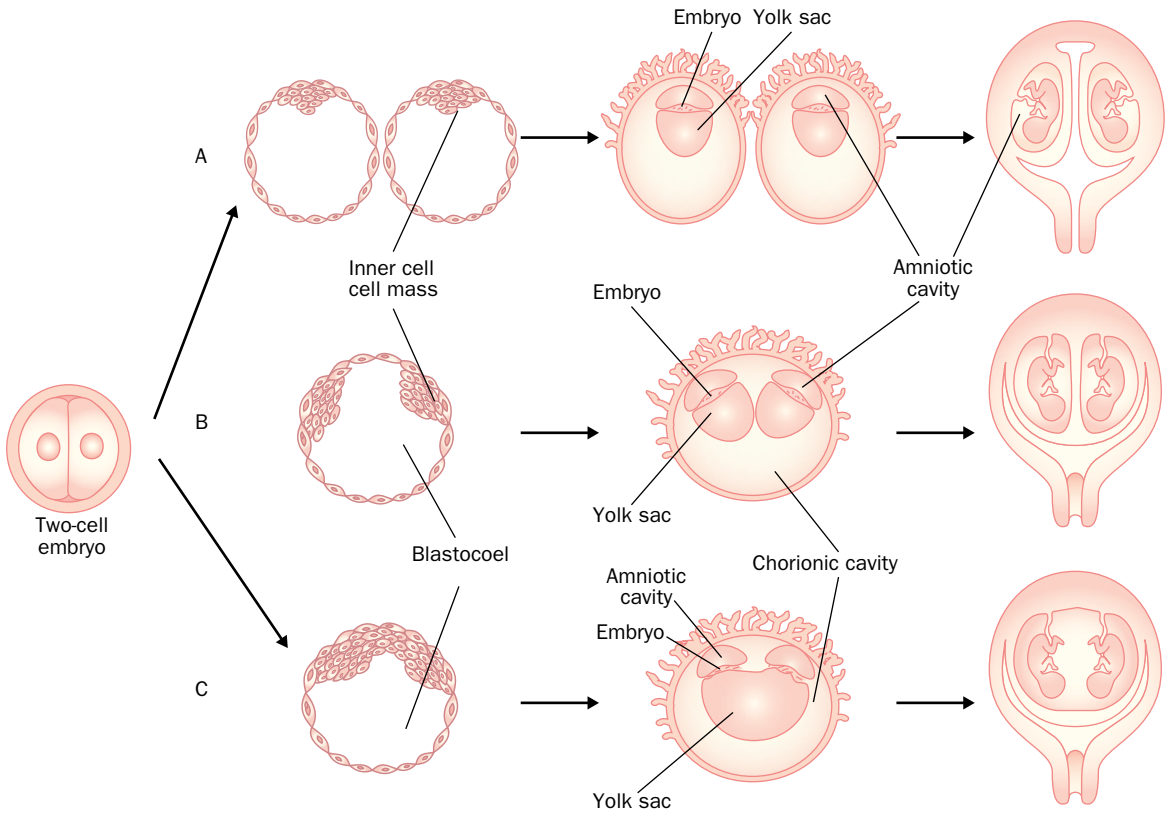

Figure 2: **Three types of monozygotic placenta and membranes**
A=dichorionic diamniotic pregnancy. B=monochorionic pregnancy. C=monochorionic monoamniotic pregnancy. Adapted from reference 13 with permission of the Massachusetts Medical Society.

fetal-maternal microchimerism during pregnancy.[18] More fetal-maternal microchimerism takes place with complications of pregnancy, such as pre-eclampsia or bleeding, which are typically seen in twin pregnancies.[18,19]

Dizygotic twinning

Dizygotic twins result from two eggs fertilised by two sperm. Genetically, dizygotic twins are as alike (or unlike) as any other pair of siblings—ie, they would be expected to share, on average, 50% of the same genes from each parent and as a result to be identical at 25% of their loci. Furthermore, dizygotic twins would be expected to be slightly squashed because of having two babies in a space usually occupied by one, as is the case for monozygotic twins. This compression is reflected in the slowing of weight gain in both types of twins compared with singletons, starting at about 30 weeks,[20] and by an increase in compression-type congenital anomalies, such as greater moulding of the head, clubfoot, bowing of legs, and dislocated hips.[9,16]

Dizygotic twinning arises because more than one dominant ovarian follicle has matured during the same menstrual cycle. Most animals are polyovulatory; however, humans are usually not. The spontaneous prevalence of liveborn dizygotic twinning in North America and Britain is almost 1 in 100 births.[2,19,21] However, the frequency of naturally occurring dizygotic twins varies widely depending on the population.[1] Over the past century, a gradual reduction in dizygotic twinning was noted in developed countries (thought to be associated with decreasing maternal age and urbanisation). However, with increased use of fertility drugs and in-vitro fertilisation since the 1980s, there has been a pronounced rise in dizygotic twinning rates in livebirths.[3,16,22]

The cause of spontaneous dizygotic twinning seems to be associated with an increased concentration of follicle-stimulating hormone (FSH) in the mother.[1,21] These amounts vary with geography, season, ethnic origin, and increasing parity, and are raised in tall, heavy, and old mothers, although there is a peak at about 37 years of age.[23] Spontaneous dizygotic twinning has been noted to fall with both urbanisation and starvation.[21] Dizygotic twinning varies seasonally and geographically in direct relation to the amount of seasonal light:[24] an increase in dizygotic conceptions is seen during the summer months in the north of Finland and Japan, and dizygotic twinning happens at a high frequency in equatorial Africa.[1,21] FSH concentrations have been increased when studied in all the groups with which dizygotic twins are associated, and this rise fits with the increased occurrence of dizygotic twinning with use of fertility drugs, which mimics naturally occurring high amounts of gonadotropins.[2,22] With the development of ultrasound, residual corpora lutea can be seen in dizygotic pregnancies, and two are present in one ovary in about half of cases.[25] Dizygotic twinning takes place, of course, in in-vitro fertilisation because more than one fertilised egg is placed into the uterus.

Spontaneous dizygotic twinning runs in families and is associated with raised concentrations of FSH. This type of

Type of monozygotic twin	Time of division into twins from fertilisation	Percentage of all twin pregnancies surviving to birth
Dichorionic diamniotic	0–3 days	25–30%
Monochorionic diamniotic	4–7 days	70–75%
Monochorionic monoamniotic	Up to 14 days	1–2%

Table 1: **Subtypes of monozygotic twins according to placentation**

twinning has been described as having both autosomal recessive and autosomal dominant patterns of inheritance, and even as having paternally derived inheritance patterns.[26–28] Researchers have postulated that there is a dominantly inherited gene carried by 7–15% of the population, which leads to dizygotic twinning.[26,27] Linkage to chromosome 3 has been reported.[29] Such a gene could be expected to have a selective advantage by producing more offspring, but this occurrence would have to be balanced in evolution against the risk to the mother during a pregnancy with more than one offspring.

Dizygotic twinning is a type of superfecundation—ie, more than one fertilised egg is present in the uterus. Usually, dizygotic twins have the same father, but many well-documented cases of dizygotic twins with different fathers have been reported.[30] Superfetation is implantation of a second zygote in a uterus in which a pregnancy is at least 1 month along in development. Although obviously rare, more than 1 month's difference in maturation could arise between the twins of a pair because of the timing of conception, and as a result, the twins could be quite different in birth sizes and maturation.[31] Spontaneous dizygotic twins can even be born on different days; however, this occurrence is quite rare.

In cows, vascular communication between the placentas of unlike sex dizygotic twins leads to abnormalities in the female calf, which is sterile and called a freemartin. Male hormones circulating in utero to the female calf are thought to have a damaging effect. No such effect has been seen in unlike sex dizygotic human twins. However, cerebral asymmetry (a male characteristic) has been reported in females from unlike sex paired dizygotic twins.[32] Furthermore, birth size might be affected by an unlike sex dizygotic twin.[33]

Finally, polar body twinning has been suggested to be a type of dizygotic twinning that might rarely arise with fertilisation of the egg nucleus and a polar body by different sperm. This occurrence has been reported only once, and no viable offspring were produced, so it seems to be a theoretical rather than a frequent cause of dizygotic twinning.[34]

Monozygotic twinning

Monozygotic twinning entails one zygote (fertilised egg) splitting into two separate individuals and represents about a third of all spontaneous twins. About 1 in 330 spontaneous livebirths is a monozygotic birth, which means about 1 in 160 babies is a monozygotic twin.[1–3]

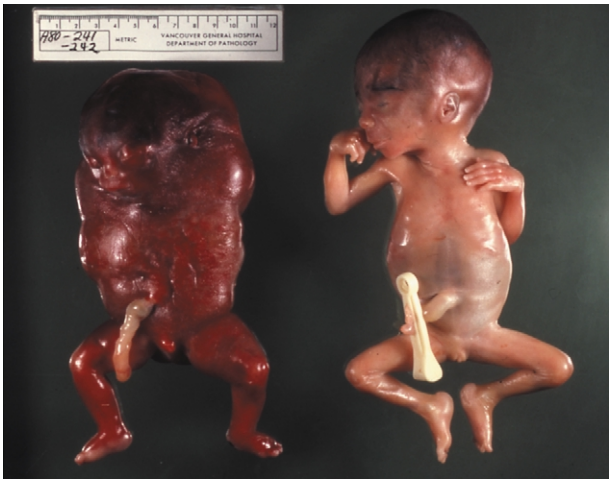

Figure 3: **Acardiac monster with normal monozygotic twin**
Courtesy of Dagmar Kalousek.

These twins are said to be natural CLONES. However, in the course of development of every large multicellular organism, cells will arise with somatic mutations and differential epigenetic control,[35] not to mention the stochastic processes that are part of the generation of immunological and neurological responses. Monozygotic twin pairs show a high degree of discordance for complex genetic traits and disorders. Furthermore, Humpherys and colleagues[36] have shown most mouse clones do not have normal control of epigenetic processes during development. It seems possible that some monozygotic human twins might also lack normal control of epigenetic processes.

No naturally occurring animal models of monozygotic twinning exist, apart from armadillos, in which identical quadruplets or octuplets arise depending on the strain.[37] Spontaneous twinning is very rare in animals. Monozygotic twinning can be produced in animals by toxic or mutagenic exposure and by so-called ZONA TAMPERING.[4,38–40] A rise in monozygotic twinning has been induced in mice by exposure to hypoxia in early development, and by exposure to changes in temperatures in minnows. Monozygotic twinning can be induced by delayed fertilisation in rabbits, and has been noted when crossing strains of mice with different structures of their chromosomes. In mice, spontaneous monozygotic twinning conception seems to be very rare, with fewer than 1 in 1000 twinning pairs actually surviving to be born.[41] Double-yolked eggs are seen very occasionally in birds, but rarely survive to birth.

Monozygotic twinning can be divided by placental types[13,14,16] or by presence or absence of abnormalities.[15,35,42] Of liveborn monozygotic twins, more than 70% are apparently concordant healthy monozygotic twins. Obviously discordant monozygotic twins, in whom an anomaly or disease is present in only one twin, and monozygotic twins in which abnormalities are present in both twins, occur in about 10% of liveborn monozygotic twins.

Mirror-image twinning happens in about 10–15% of otherwise healthy monozygotic twins. Mirror-image monozygotic twins have inverse laterality (but not usually including situs inversus), which suggests that the twinning event took place after the cells of the embryonic plate were beginning to lateralise but before formation of the primitive streak.[16,43,44] In mirror-image twins, minor features are on opposite sides, such as whether the first tooth erupts on the right or left side and on which side cowlicks or hair whorls are present. Handedness, wrinkles, tooth loss, position of warts, and type and presence of neuralgias may all be seen on the opposite side in mirror-image twins.

Furthermore, several abnormalities are only seen in monozygotic twin conceptions. One such example of abnormal monozygotic twinning is the acardiac monster (figure 3). About 1 in 35 000 births produces monozygotic twins of which one is an acardiac monster. There is an excess of female acardic twins. These monozygotic twins have placental anastomosis, with reverse flow (twin reverse arterial perfusion) in the acardiac monster.[4,45] In these pregnancies, at least 20% of the monozygotic co-twins can be expected to have congenital anomalies, and in fact, 55% of co-twins die in the newborn period.

Fetus papyraceous happens when one twin has died in utero and has not been completely reabsorbed (figure 4).[46] How many of these twins are monozygotic versus dizygotic is not clear since DNA studies have not been done, and an assessment of the corpus luteum number has not been

reported. If one of the twins dies during the first trimester it is usually completely reabsorbed, whereas if it dies in the second or third trimester, careful examination of the placenta usually still has evidence of the dead twin. The surviving twin is often smaller than usual for age compared with a singleton baby, suggesting secondary vascular compromise has taken place in the surviving twin or that small birth size of twins is determined early in pregnancy.[20] Careful examination of all placentas for evidence of a fetus papyraceous might indicate abnormalities that help to predict outcomes for the surviving twin.

Conjoined twins are always monozygotic.[47] They result from an incomplete monozygotic twinning event and arise in about 1 in 100 000 births or about 1 in 400 monozygotic twin births. There is an excess of females among conjoined twins. They are classified by site of attachment.[4] Conjoined twins are thought to develop after 14 days of embryonic development, because at about this time the primitive streak sets up lateralisation, assuming monozygotic twins follow the typical human developmental schedule. This explanation assumes that incomplete separation of the two embryonic plates has occurred with two centres of embryonic growth arising. However, because some eccentric attachments incompatible with abnormal splitting have been recorded, early reattachment of the twins has also been suggested as a mechanism in conjoined twins.[48,49] In the past, 40–60% of conjoined twins have been stillborn: improved prenatal diagnosis and perinatal care seems to be improving this rate. 10% of conjoined twins are unequal in their distribution—eg, an embryo begins to develop, and after this embryo is well established, a second (fetus in fetu) arises or is attached. 50% of conjoined twins have structural anomalies of major organs. A great deal of publicity, public interest, and ethical debate takes place around surgical attempts to separate conjoined twins.

The different types of monozygotic twins and birth defects that occur with monozygotic twinning suggest that there may be several different processes during early human development that can give rise to monozygotic twinning. Some might be variations of normal processes, whereas others might not be entirely representative of the normal developmental process in human singletons.

Why monozygotic twinning happens in human beings is not clear.[9,35,50] Spontaneous monozygotic twinning has been suggested to arise because of damage to the inner cell mass, and subsequently two points of regrowth might take place, each giving rise to a separate individual, which seems to be the case in some animals.[4,40] However, there is no evidence for this occurrence in human beings.

Monozygotic twinning is increased two to five times in babies born by in-vitro fertilisation.[38,51] This increased frequency could relate to breaks in the zona pellucida, which in turn could be associated with handling, could be because of intentional holes being placed in the zona, as happens in intracytoplasmic sperm injection, or could be because of zona hardening in relation to ageing or the drugs and media used in artificial reproductive technologies. The earliest daughter cells of the zygote (blastocytes) can fall apart without the zona pellucida to hold them together—ie, before the eight-cell stage tight gap junctions have developed between cells. In monozygotic twins produced by in-vitro fertilisation, dichorionic diamniotic twins are present more frequently than expected, which also suggests that the monozygotic twinning process and subsequent separation of twins seen with in-vitro fertilisation happens at a very early stage.[16,51] These findings also fit with the suggestion that if the zona of a fertilised ovum hardens prematurely then blastocytes could split apart and even be separated by the edge of the broken or hardened zona.[35]

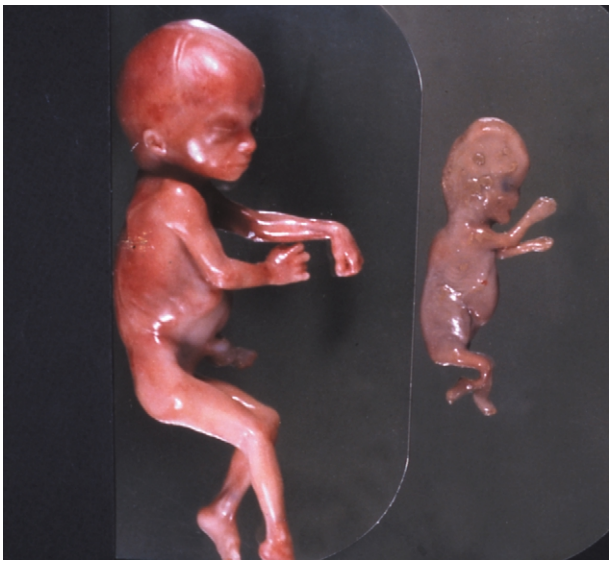

Figure 4: **Normal twin with fetus papyraceous**
Courtesy of Dagmar Kalousek.

Very rarely, familial monozygotic twinning is seen, which can be transmitted by both the father and the mother.[16,33,52] This observation suggests that in familial monozygotic twinning, there may be an inherited defect—eg, in one of the zona pellucida proteins, which could lead to a fragile zona pellucida that allows early separation of clumps of blastocytes—or failure or delay of cell-to-cell recognition and adhesion (again due to a defective protein).

There may be other reasons why monozygotic twinning happens more frequently in human beings than in other mammals. For instance, skewed X-chromosome inactivation has been suggested as a mechanism for the excess of monozygotic female twins.[15,53,54] Also, some part of the monozygotic twinning process could be associated with the cyclic oestrus type of reproduction of human beings. This type of reproduction carries the risk that timing of fertilisation and of embryonic development could be variable and as a result not follow the usual time schedule thought to take place in human singletons. The suggestion is that fertilisation of an old (as far as the oestrus cycle goes) ova might lead to a zona that will fracture and even cut the blastocyst in two. Furthermore, the cytoplasm of an old ovum may not have important nutritional and energy sources, which can result in developmental delay and risk for errors in programming.

Congenital anomalies

In monozygotic twins, congenital anomalies occur frequently, both in liveborn babies and miscarriages.[42,55–59] These anomalies are generally separated into malformations, disruptions, and deformations.[60] Malformations—including cloacal anomalies, neural-tube defects, and congenital heart defects—occur when normal development fails. In particular, malformations involving the midline may be part of the twinning process—eg, conjoined twins, acardiac twin.[4,42] Congenital heart disease seen in monozygotic twins could be associated with placental vascular connections, leading to fluctuations in flow early in cardiogenesis. Disruptions in monozygotic twins—including, hemifacial microsomia, limb reduction defects, and amyoplasia—are probably related to the shared placental circulation unique to monozygotic twins,

Type of twin	Sex ratio (male/male+female)
Dizygotic and singletons	0·514
All monozygotic	0·484
All monoamniotic twins	0·231
Conjoined twins	0·230
Sacral teratomas	0·250

Data from James[70] and Derom.[71]

Table 2: **Sex ratios in twins**

which is associated with vascular connections, leading to secondary disruptions, and occasionally the intrauterine death of one twin. Deformations associated with constraint and intrauterine crowding—including, clubfeet, dislocated hips, and cranial synostosis—are apparently a function of two babies growing in space where one usually grows.

The rate of congenital anomalies in monozygotic twins is difficult to establish. 2–3% of singleton neonates have an obvious major congenital anomaly;[56] however, with follow-up, this rate doubles. Results of several studies suggest there is a 2–3-fold increase of congenital anomalies in monozygotic twins. Thus, probably 10% of monozygotic twins are born with a congenital anomaly.

The vanishing twin

Researchers have noted that many twin pregnancies are lost or convert to singleton pregnancy.[61–65] Most twins are lost very early in pregnancy. New techniques of imaging and hormone assay have enabled recognition that many twin pregnancies convert to singleton pregnancy. Theoretically however, the singletons that started out as monozygotic twins are still at risk for the congenital anomalies that are frequent in monozygotic twins. Thus, singletons that are born with these types of anomalies might have started as a monozygotic twin.

Boklage[66] has estimated that only one in eight individuals originating as a twin actually goes on to be born as a twin. Many twins are aborted spontaneously; the abortion rate is three times higher than that for true singletons.[67,68] It is noteworthy that if fewer than a third of twins actually come to term, and 10% of liveborn monozygotic twins have a congenital anomaly at birth, then more than 70% of monozygotic twin conceptions have some type of problem.[69] Tong and colleagues[25] monitored the number of corpora lutea present in the ovaries of recognised pregnancies between 5 and 9 weeks, and reported that the probability of a double ovulation resulting in a singleton birth was 80%. This probability accords with the expected loss of each member of a dizygotic twin pair as independent events.

Sex ratio

Sex ratio is the ratio of males to total births. The ratio for dizygotic twins is the same as for singletons—ie, slight male excess (table 2). James[11,70] noted that there is an excess of females in spontaneous monozygotic twins, which is also the case in spontaneous triplets and quadruplets. However, there does not seem to be an excess of males in aborted twins. The increase in females is smallest in dichorionic monozygotic twins and greatest among monochorionic diamniotic monozygotic twins, thus seeming to rise with later twinning events. The increase is even greater in monochorionic monoamniotic twins, and still greater in conjoined twins.[71] Furthermore, X-inactivation patterns—eg, the percentage of paternal X chromosomes inactivated versus maternal X chromosomes inactivated—are usually quite different in dichorionic diamniotic monozygotic female twins, but are similar in monochorionic diamniotic twins and are almost identical in monochorionic monoamniotic twins, suggesting the timing of monozygotic twinning events is in and around the timing of X inactivation.[72–74]

Females seem to be more susceptible to monozygotic twinning than males. Early in embryonic development, females are delayed in their development,[75] which could be related to X inactivation, since until females undergo this process, the cells of the developing embryo might act aneuploid and grow more slowly.[76] This delay could account for the excess of female monozygotic twins[54] and the increase in certain types of congenital anomalies—eg, conjoined twins, anencephaly—seen in these twins. In other words, with delayed development there could be a

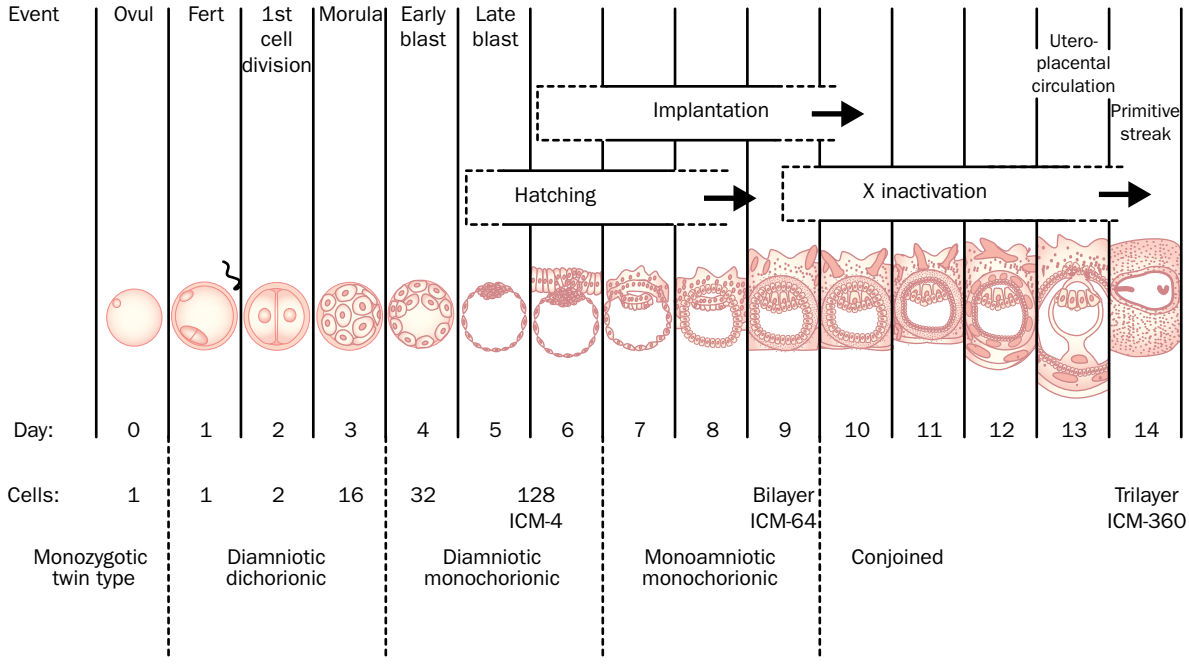

Figure 5: **Schematic drawing of normal human embryonic development with timing of monozygotic twinning superimposed**
Adapted from reference 35 with permission of Elsevier Science.

risk of missing certain critically timed events and thresholds and of skipping parts of the normal processes involving methylation, such as genomic imprinting and X inactivation (figure 5). The delay and retarded growth at this early stage could even lead to positive outcomes, such as female longevity.

Folic acid

Results of studies suggest that adequate maternal folic acid consumption could affect the number of twins coming to term.[77–79] In several studies looking at the role of folic acid supplementation, findings have shown that more twins come to term and are born than would be expected. These twins do not seem to show the usual excess of congenital anomalies generally seen in twins. Results of other analyses are less convincing,[80,81] but might relate to populations that are at reduced risk of folic acid-dependent congenital anomalies.

Discordance

Most, if not all, monozygotic twins are discordant in some aspect, despite the fact that most are very similar in appearance. Many mechanisms have been identified, both genetic and stochastic, that could lead to discordance in the twin pairs.[82] Discordance between monozygotic twins has been noted for chromosomal anomalies,[8,35] single gene disorders,[35] skewed X inactivation,[15] genomic imprinting defects,[83] mitochondrial disturbances,[84] and minisatellites.[85] These factors must be kept in mind during prenatal diagnosis, since monozygotic twins could be discordant for chromosomal abnormalities and chromosomal mosaicism. Discordance between monozygotic twins has even been used to identify the gene causing a monogenic disorder, which once identified, was then found to be mutated in 45 unrelated affected families with the disorder.[86] Molecular subtraction studies could prove very useful in the future.

In addition to genetic discordance, Machin[16,82] has suggested that many physical and physiological reasons would be expected to lead to discordance in monozygotic twins—eg, initial differences in the number of cells at the time of separation, differences in vascular flow, differences in attachment to the placenta, type of chorion, and total number of cell divisions. Differences in vascular flow leading to vascular compromise[82] might lead to an enhanced risk of disruptive types of congenital anomalies, such as hemifacial microsomia,[87] amyoplasia,[88] and bowel atresia.[89]

Growth

Both monozygotic and dizygotic twins have compromised growth starting early in the third trimester at about 30 weeks of gestation. On average, compared with singletons, their individual growth falls off while their combined weight greatly exceeds the normal for a singleton.[20,90] Discordance in size is typically seen at birth in both types of twins. A higher proportion of twins has intrauterine growth retardation compared with singletons, which obviously affects the average birthweights for twins. About 10% of monozygotic twins are more than 10% discordant in weight at birth, with more than expected being small for gestational age.[90]

Monozygotic twins live off the cytoplasm of one egg until they implant. Generally, implantation is thought to take place at about 8 days of embryonic development (22 days after the last menstrual period), but in monozygotic twinning, embryonic development could happen with slightly different developmental timing, or it could be delayed because both twins are developing off a cytoplasm that is intended for one zygote. Important nutritional requirements for growth and gene regulation at these early stages might slow down the rate of normal development. These changes would not account for the sudden decrease in dizygotic and monozygotic twin growth noted at 30 weeks' gestation. Thus, some type of signalling must take place between mother, placenta, and twins that regulates twin growth in a different way to singletons. Maternal physiology changes during pregnancy more strikingly in twins than in singletons,[91] and thus could play a part in downgrading growth physiologically, so that the long-term negative effects of intrauterine growth retardation are avoided.

What causes monozygotic twinning?

The causes of monozygotic twinning in human beings are unknown. Skewed X inactivation, with one cell mass inactivating the paternal X chromosome and one inactivating the maternal X chromosome,[15,54] has been suggested to account for the excess of monozygotic twinning seen in females. Damage to the inner cell mass could also contribute to twinning in human beings.[40] From in-vitro fertilisation work in human beings, a break in the zona, an old egg, and delay in fertilisation or ovulatory drugs that lead to the hardened zona pellucida could all produce monozygotic twinning by physically separating the conception into two cell masses.[35] Familial monozygotic twinning could be associated with an inherited abnormality of the zona pellucida or some other mechanism, leading to failure of early blastocytes to stay together.[53,92] Zona abnormalities could account for 5–10% of spontaneous monozygotic twinning in human beings and for the excess of twinning seen with artificial reproductive technologies.

However, most monozygotic twinning is probably not accounted for by these mechanisms. A possible explanation is that cells within the blastocyst could develop in such a way as to become discordant, recognising each other as foreign, and use cell-recognition mechanisms to set up two separate cell masses.[33] The ways by which cells develop a change (mutation, chromosomal number, change in gene control as in imprinting, etc) of any type, compared with the original cells, could lead the original cells to recognise the new cells as different and establish separate cell masses.[9,35] This possibility would suggest that most monozygotic twins, although having most of their genetic information in common, would have minor but recognisable differences (perhaps in mosaic form), which could be recognised by new molecular techniques.

Use of twins to study complex disease

Comparisons of monozygotic and dizygotic twins are frequently used to estimate the environmental and heritable factors that contribute to complex diseases. This comparison assumes that twin pregnancies, births, and early childhood are representative of singleton pregnancies, births, and early childhood. Growth of twins in utero is not typical of singletons. The effect of intrauterine growth on later adult diseases could also be atypical. All twins can be expected to have many kinds of in-utero differences, such as placental flow in monozygotic twins and the amount of microchimerism in dizygotic twins.

Findings of twin studies need to be viewed with some reservation. Twins may not be representative of singletons in relation to some complex diseases. Furthermore, increasing use of artificial reproductive technologies, presence of high concentrations of gonadotropins, intentionally broken or abnormal zona pellucidas, and physiological abnormalities related to culture media might have effects on childhood or adult disorders not yet

recognised or not even present in spontaneous twinning. Long-term studies and outcomes must record whether twinning was spontaneous, the type of artificial reproductive technology used, complications of twin pregnancies, placental flow, changes in maternal physiology, type of chorion, and microchimerism during pregnancy to establish whether twin pregnancies can be representative of singleton pregnancies.

The in-utero adjusted growth rate of both monozygotic and dizygotic twins could also alter other pathways that protect against certain diseases and predispose to others. Differences in maternal metabolism during twin pregnancies versus singleton pregnancies—with lower packed-cell volumes, more gestational diabetes, greater cardiac output, etc—could also alter the metabolism of the embryo and fetus.

These observations have interesting ramifications for the Barker hypothesis,[93] which suggests that diminished intrauterine growth predisposes to early onset of hypertension, cardiovascular disease, and diabetes in later life. However, the smaller twin at birth in a twin pair does not seem to have the same type of risk for these diseases as is seen in singletons.[94–96] An increase in breast cancer[97,98] and testicular cancer[94,99,100] has been noted in twins. Further, risk of autism has been reported to be increased in both monozygotic and dizygotic twins.[101,102]

Conclusion

Human twinning is a fascinating experiment of nature from which a great deal can be learnt about early human development and predisposition to disease. However, the more that is learnt about these early stages of development, the more caution is suggested in interpreting findings of twin studies used to understand complex diseases. Documentation of zygosity at birth is strongly recommended for all same sex dichorionic twins, since DNA testing can easily be done on cord blood at that time. The long-term implications for predisposition to disease, transplantation, and inclusion in twin studies demand that this type of testing becomes part of routine care in the developed world.

Conflict of interest statement

None declared.

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