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Father Filice, who is affiliated with the department of biology at the University of San Francisco, gave this address at the 1978 NFCPG annual meeting.

A. INTRODUCTION

A number of authors have raised the possibility that the ease with which twinning can take place in humans during the first week after fertilization might offer data that would allow the theologian to argue that the conceptus, during that period, is really not yet a human individual. [2] As supportive evidence, the apparent existence of individuals whose genetic structure suggests the fusion of two distinct twins has been offered as indicating how little "hominization" is present at this stage.

In this paper I would like to review the pertinent portions of development during the period of fertilization, cleavage, and implantation in humans; then I would like to review what is known of twinning and discuss our present knowledge about recombinant humans.

B. FERTILIZATION AND EARLY DEVELOPMENT

As the graffian follicle enlarges in the ovary, the oogonium undergoes the first meiotic division. One set of chromosomes is gathered into the diploid first polar body, which then can be seen as a small bit of protoplasm lying next to the ripe ovum. Ovulation takes place as the result of an increase of Leutinizing hormone. Afterwards, the oocyte and its first polar body are free but still enclosed in the thick (20-25 u) zona pellucida, an albuminous membrane around which cling the nurse cells of the cumulus oophorus which make up the corona radiata.

Fertilization normally takes place in the upper end of the Fallopian tube. Although the details from human material are very sketchy due

to a laudable reluctance hitherto to experiment with humans, what we have substantiates the picture drawn from lower organisms.[3] The cells of the corona radiata are dispersed, in part due to their own activity and in part due to lytic enzymes released from the acrosomes of a number of sperm cells. One sperm works its way through the zona pellucida with the aid of acrosomal enzymes and finally attaches to the plasma membrane of the oocyte by the post-acrosomal membrane. A peri-vitelline space forms by shrinkage of the vitellus; this is presumed to accompany the elaboration of chemicals that prevent polyspermy. Dispermy, thus, should be an extremely rare event. The sperm is engulfed by the vitellus through a phagocytic action and the second meiotic division takes place in the egg nucleus. This is a reduction division and a haploid pro-nucleus is retained while a haploid second polar body is formed with the other set of chromosomes. This polar body can also be seen within the zona pellucida. Both polar bodies normally disintegrate with time. [4, 5]

The entire process of fertilization involves a complex series of interactions between the sperm and the egg. The mechanism is initiated by the linkage of the sperm cell membrane to that of the egg and it is completed by the intermingling of the chromosomal material from the male and female pro-nuclei.

With no apparent pause, the zygote begins the first cleavage division. At first, the protein apparatus that carries out cleavage is that laid down in the egg by the genetic code of the mother, but early in the cleavage divisions this is replaced by a protein structure genetically determined by the zygote.[6]

At present, the timing of subsequent events in humans is uncertain. The information derived from in vitro fertilization experiments seems abnormal and is considered unreliable.[7] Most observations seem to confirm — at least in general — the timetable deduced in the macaque by Lewis and Hartman in 1941. Streeter correlated this data with what information was available from humans to derive the following timetable given by Blandau with modifications. [8, 9, 10]

Stage	Day
1 cell	1
2 cells	1.5
4 cells	2
8 cells	3
16 cells	4
Blastocyst	4–9

In any event, cleavage continues as the embryo is being transported slowly down the Fallopian tube. The rate of transport is modified by the relative amounts of progesterone, secreted by the developing

corpus luteum, and estrogen. These hormones also affect the permeability of the zona pellucida and hence govern the rate of absorption of the materials used by the embryo as energy sources during this migration. [11]

The first cleavage plane yields two cells of different chemical composition and is meridional. One cell divides rapidly and will surround the offspring of the slower cell and form an epithelium — the trophoblast (or trophoderm). The slower cell yields an enclosed inner cell mass (ICM). The zona pellucida becomes more permeable and the imbibing of fluid results in the formation of a cavity and the embryo becomes a blastocyst. By this time the embryo is in the uterus.

One of the earliest human blastocysts collected after a natural fertilization is that described by Hertig and Rock. [12] This is estimated to be four to five days old and consists of 58 cells. Fifty-three are in the trophoblast and five in the ICM.

The change in permeability of the zona pellucida is accomplished by changes in progesterone concentration. It begins to thin down around the sixth day as the blastocyst enlarges and soon disappears. Now the inner surface of the trophoblast delaminates a loose, amoeboid, extra-embryonic mesoderm into the cavity of the blastocyst. This spongy network forms a loose inner membrane that lines a cavity (the primary yolk sac). This can be seen in the seven-day blastocyst.

Implantation takes place around the seventh day. This is accomplished by the trophoblast which goes on to form the fetal portion of the placenta and thus, with the maternal contribution, provides the embryo with the energy sources and waste-disposal outlet it needs for the intense activity that takes place during organogenesis.

About this time, the lower surface of the ICM (or, perhaps, in humans, the inner surface of the trophoblast) [13] delaminates a cuboidal epithelium (the hypoblast or endoderm) which grows down the inner surface of the primary yolk sac to form the true (or secondary) yolk sac. In an implanting embryo studied by Hertig and Rock [14] the endoderm is formed but the secondary yolk sac is not yet completed. This embryo is estimated to be seven and one-half days old. The secondary yolk sac is completed by the 15th day.

The amniotic cavity is formed in that part of the ICM in contact with the trophoblast. It is a space lined with a columnar epithelium. Now the amniotic cavity is floored by a columnar epithelium, the epiblast, that lies directly on the endoderm. This bilaminar disc is the embryonic disc.

While the extra-embryonic coelom is being formed by cavitation in the extra-embryonic mesoderm, a circular patch of cells becomes apparent at one edge of the embryonic disc in the hypoblast. These become elongated and vacuolated; they form the pre-chordal plate, which marks the anterior end of the embryo and establishes bilateral

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symmetry. Immediately posterior to this pre-chordal plate, the linear midline cells of the epiblast begin to pile up to form the primitive streak. This produces the lateral mesoderm that grows from the streak, laterally, between the two layers of the embryonic disc, out to meet the extra-embryonic mesoderm.

At the same time, Hensen's node appears. This is a small region of intense mitotic activity that is found at the anterior tip of the primitive streak. It involves the upper layer of cells and produces a solid, midline column of cells between the two layers of the disc. This grows forward toward the pre-chordal plate as the notochord.

Hensen's node seems to be the seat of the substances responsible for the primary organization of the embryo. Extrapolating from lower organisms, these substances are present in the zygote from the time of fertilization, and they are sorted out in the cleaving embryo to wind up in the cells of Hensen's node where they become activated at this time. In some manner not yet understood, they act on a receptive structure in the cells of the embryo in such a fashion as to cause them to become determined. In the process of twinning, one or a group of cells separates from the embryo; if these chemicals are present in each group then each will re-organize so as to form a complete embryo. If, by chance, the substances are isolated in one group of cells, then that will form the embryo and the other will remain a disorganized mass.

When Hensen's node appears, most of the primary tissues very soon have their fates fixed.[15] Prior to this time, only the cells of the trophoblast have lost the capacity to re-organize and serve different functions.[16] The cells of the ICM have a *presumptive* fate that can be changed by changing their relative positions.[17] However, after notochord formation, if the position of the cell changes, it will form in the adult, cells characteristic of its position at the time of notochord formation. Thus the possibility that accidentally separated cells might form a twin is limited to the time prior to this.

A human, described by Hertig and Rock[18] and estimated to be 12 days old, has Hensen's node already. Hence the time-table seems to be:[19]

Day	Stage
1	Fertilization
2-3	2-16 cells
4-5	Free blastocyst
5-6	Attached blastocyst
7-12	Implantation, Hensen's node
13-15	Chorionic villi
15-17	Notochord
17-21	Heart, somites, neural folds

C. TWINNING

Twinning is a relatively rare phenomenon. The classic figures, derived as an approximation before the era of the fertility pill, are that twinning occurs in 1 out of 80 births, triplets in 1 out of 80^2, and 1 out of 80^3 for quadruplets.[20] There are two kinds of twinning: dizygotic (DZ) and monozygotic (MZ).

(1) Two embryos, two genotypes

These are dizygotic: that is, they result from the union of two ova with two sperm. The zygotes have different genotypes and they resemble each other no more than any two siblings, although they share the same uterus at the same time. About three-fourths of all twins are of this type.[21]

There is a genetic basis for this type of twinning. If the first pregnancy results in DZ twins, the second has five times greater chance over the normal of also resulting in DZ twins.[22] Also, there is a strong racial correlation: blacks have a DZ rate of about 16/1000 births, while Caucasians have a rate of 8/1000, and Orientals a rate of 4/1000. These are only average figures. Bantus, for example, have a rate of 20/1000, while in large parts of Asia the rate is as low as 2.5/1000.[23]

The environment, however, plays a strong role. For example, the tendency to produce DZ twins increases with age and with parity; also it is higher among women who conceive during the first three months of marriage.[24] There is a decreasing frequency of DZ twinning taking place both in the United States and Moscow.[25, 26] The rate varies with the nutrition of the parent as well as with geography: France has a DZ rate of 7.6/1000, while Spain has a rate of 5.9/1000 and East Germany 9.1/1000.[27]

Since DZ twins are separate embryos from the beginning, they are dichorial and diamniotic. It is rare that the walls between adjacent chorions will break down and result in DZ twins that are monochorial. In six studies, only two monochorial DZ twins were reported in 763 DZ twin pairs, and there is some question that the two cases may have been incorrectly reported. In 90% of monochorial twins, the fetal circulation undergoes anastomosis within the placenta, making possible the exchange of cells between the twins. This can also occur between dichorial twins, but much less often.[28] If this takes place early enough, stem cells can be exchanged and a cell lineage with its own genotype can be established in an individual of a different genotype. This explains the existence of the so-called "blood chimeras" such as the one reported by Hosoi et al. They found a pair of twins in which the male had 89%A_1 and 11% A_1B red blood cells; 32% of the male's

lymphocytes were diagnosed as XX. The female had 88% A_1 and 12% A_1B red blood cells, and 40% of her lymphocytes were XY. [29]

Although embryologists [30] believe that it never occurs, it is theoretically possible for the intervening amniotic walls to break down, resulting in mono-amniotic DZ twins. In this case it would be possible that DZ twins could fuse secondarily to form a chimera. However, this has not been demonstrated in humans, and seems unlikely.

(2) Two embryos, one genotype

These are monozygotic twins. One-quarter of all twin pairs are the result of a single egg fertilized by one sperm.[31] The two individuals have the same genetic characteristics (unless a somatic mutation takes place). This type of twinning is probably not genetically determined and does not seem to be as environmentally labile as DZ twinning. Although the incidence increases with age, it does not increase with parity, nor does it change with race or geography. Worldwide, there is a relatively stable rate of 3.5/1000 births.[32] However, there is an increase of MZ twinning with strained parental relationships and with an increase of physical labor on the part of the mother. [33]

The data thus suggests that hormonal variations in the mother can influence MZ twinning. This would fit with the theory that MZ twinning is the result of developmental arrest due to a temporary oxygen deficiency. We know, for example, that the permeability of the zona pellucida is controlled by maternal hormones as well as the rate of implantation. Temporary changes in either of these could cause an oxygen deficiency. Two pieces of collateral evidence seem congruent: in tubal pregnancies the MZ/DZ ratio is five times the normal, and the chances for malformations are many times greater in MZ twins than DZ. This theory is also supported by some evidence from lower animals. [34]

MZ twinning can occur at different stages in development.

If the blastomeres separate at a very early stage, for example at the two-cell stage (or at least before the fifth day), and each moiety includes the primary organizing substance, both blastomeres or groups of blastomeres can re-regulate to form two entire blastocysts. The result would be MZ twins that are dichorial as well as diamniotic. In 10 investigations, 354 MZ twins were dichorial and 763 monochorial. [35]

Once the trophoblast covers the ICM, at the 16-32 blastomere stage in humans, separated groups of blastomeres would no longer reorganize into complete embryos. The trophoblast cells are determined and can no longer re-regulate. Now, only in those cases where the trophoblast remains intact, but the ICM cells accidentally separate into two groups will MZ twins be produced. These will be diamniotic but monochorial. As noted above, this is the most common of the MZ twins, occurring in about two-thirds of the cases.

It is possible that in the monochorial MZ twins, the amniotic barrier could break and secondary fusions between the embryos take place; however, there is not much evidence that this takes place in man. Most authors believe this unlikely because monochorial diamniotic twins never show fusion of the intervening amnions, and the monoamniotic twins have the umbilical cords arising close together, suggesting a separation within the same amniotic sac. [36]

The rarest of MZ twins are those that form during the production of Hensen's node. The separation of the embryo could take place after the formation of the embryonic disc, just before, or during Hensen's node formation. These would be monoamniotic as well as monochorial. One would expect that the later the separation would take place, the less complete it would be; thus, most authors feel that this is the cause of Siamese twinning. If this is true, and since the division would begin dorsally in the embryo, one would expect a series of such twins from the Janus twins, completely fused along the venter, through superficial ventral fusion to sharing all or part of the umbilical cord. Monoamniotic twins do not fare too well since there is a chance of entangling the umbilical cords.[37] About 2/100,000 births are monoamniotic. Re-fusion to form a chimera of previously separated twins is certainly possible and may have taken place in the cases of Siamese twinning where the fusion is dorsal. The evidence, however, is not clear. [38]

(3) One embryo, two genotypes

There are two ways in which this type of individual can occur.

First is the *mosaic*. This is a condition where a genetic event results in colonies of cells with different genotypes living in the same individual. This may take place in several ways. For example, a non-disjunction of chromosomes could take place at mitosis in any somatic cell at any stage of life. If this took place early enough, it would result in patches of cells in the body, of different genotype. Again, there may be a mis-function of the spindle, so that one chromosome is left behind and lost in cell division. If this took place, for example, in the sex chromosome, then colonies of XO cells would result in an XX or XY individual. [39] Another possibility is a mutation in the DNA of an early blastomere. Translocation in a blastomere is another.

There is evidence that women are always mosaics.[40] Since the mammalian male has only one sex-linked set of genes and the female has two, it is obvious that only one set is necessary for normal body development and function. The female, with two sets, would have too great a dose of chromatin in the X chromosome.

In order to redress this excess, it appears that, after the genital primordia are laid down in the early embryo, one X chromosome is inactivated in each cell of the female. There seems to be no favoritism

between the two; thus, on the average, one-half of her cells have one X chromosome functional and the other half have the other. Any sex-linked factors that are heterozygous would be expressed differently in one-half of her cells.

The two kinds of cell populations, being of different genotypes and producing different antigenic substances, can be detected by the use of selective antibodies. The body does not normally immunize against one or the other of these cell populations because of the constant presence of both antigens. However, it is conceivable that, given the predominance of one genotype in the reticulo-endothelial system, there might be a gradual weakening of this tolerance in some cases. Indeed, there is a condition in chimeras of New Zealand black mice, where just such a progressive weakening of tolerance takes place and the body slowly immunizes against one cell population, resulting in a hemolytic anemia.[41] This may be the etiology of auto-immune diseases in humans and would account for the fact that such auto-immune diseases are found more frequently in women.

The second way in which two genotypes can be present in the same individual is by the formation of a *chimera*. Here, cells from distinct embryos intermingle to set up colonies in an individual. Some authors distinguish between primary chimeras, which are formed early in development, and secondary chimeras which would form after organogenesis. This second category would include such phenomena as parabiosis and transplants.

The above-cited case of Hosai[42] is explained as a blood chimera. These are caused by the migration of stem cells from one DZ twin to the other during development when anastomosis occurs between fetal circulations. There are many examples of this kind. For example, Booth,[43] and his co-workers found a pair of DZ twins in which the male had 86% A red blood cells and 14% O; the female had 1% A and 99% O.

There may even be migration of cells from mother to child in the rare cases when there is anastomosis between maternal and fetal circulation. This is one theory as to the etiology of Hodgkin's disease.[44]

Primary chimeras are embryonic fusions early in development. They have been experimentally produced in lower animals, inluding mice and rabbits, as a tool in developmental and genetic research.[45] The zona pellucida is removed by various techniques and the dividing blastomeres of each embryo are held together. They re-organize into a single individual. As many as eight embryos have been aggregated, with normal sized offspring resulting. This has been done in the lower animals, up to the time of trophoblast differentiation. Once the trophoblast is formed, the embryos will no longer aggregate; although one can still pipette blastomeres from the ICM of one embryo and inject them into the other to form "injection chimeras."[46] Some valuable genetic information has been derived from these experiments,

but naturally occurring primary chimeras that result from the aggregation of separate embryos have never been certainly demonstrated in any mammal. The presence of the zona pellucida around the embryo until well after trophoblast formation is a barrier against such an event. Whether it could occur experimentally in humans is a moot point. A laudable reluctance to experiment with humans has thus far inhibited this study. Generally, we can explain most cases of humans with two genetic cell populations as either blood chimeras or mosaics.

There are, however, some cases whose explanation might include the aggregation of two distinct cell strains into one embryo.

One type of such cases is the most frequently mentioned in the literature,[47] that is, the case of the XX/XY individual. About 70% of all such persons are hermaphroditic. If these are not just blood chimeras, then early aggregation of two cell lines is possible. Ford, for example, reports a situation where the stroma cells of the gonad in a mouse were XX and the germ cells XY.[48] This would suggest an event that took place very early in development. The most common explanation, however, is that non-disjunction of an XXY individual took place early in cleavage.[49]

If a number of loci are involved, then such an explanation becomes difficult to defend. For example, Moores found a woman with two red blood cell populations differing in ABO, MN and RH factors, and who also had mottled skin.[50] This could be explained by a translocation to the X chromosome of a portion of an autosome that subsequently becomes inactivated with the X chromosome in half of her cells.

A clearer case is that reported by Gartler.[51] This was a girl with one hazel eye and one brown eye. Her lymphocytes were half XX and half XY. There were two populations of red blood cells, and analysis showed that her father had to contribute to both cell populations, but her mother made only a single contribution. The usual explanation is that this is a case of dispermy, where one sperm nucleus fertilized the ovum and the other the second polar body.[52] McLaren considers this the most reasonable explanation biologically, pointing out that relatively large polar bodies occur with some frequency in lower animals and that this is the cause of natural chimeras in mice. However, it is equally possible to account for this on the basis of an anomaly that is present in about 5% of all human sperm cells: that is, sperm heads with two nuclei.[53] These are the result of incomplete separation after second meiotic division in spermiogenesis and each haploid nucleus is encased in its own membrane, but the two are molded into a single sperm head. If such a sperm entered an ovum, separation of the two nuclei would be slow — perhaps slow enough to delay fusion with the female pronucleus until after mitotic division of first cleavage. Then, if each sperm nucleus fertilized one of the daughter nuclei of the ovum, just such an individual as described here would result.

A case reported by Lejeune is interesting.[54] It was an XX/XXY individual that had two red blood cell populations and sectoring in the iris. Although genetic analysis is not complete, it would seem to require fertilization of the ovum and the first polar body by two sperm. The trouble with this kind of explanation is that polyploidy usually renders the human so abnormal as to inhibit development; there is a question whether an individual, in whom triploidy occurred so early in development that half of the cells would be triploid, could survive.[55] It is also possible, however, that this is a case of non-disjunction of an XXY individual in a later cleavage division with a subsequent somatic mutation accounting for the other differences.

Perhaps the clearest case of aggregation of the results of two separate fertilizations is that of De la Chapelle.[56] This was an XX/XY hermaphrodite who had two distinct red blood cell populations. Analysis of the parents showed that there were two genetic contributions from the father and two from the mother. This probably was a case of two sperm nuclei, one fertilizing the ovum, and the other the first polar body.

To summarize, there are a number of ways in which primary chimeras could arise naturally in mammals.

(i) Precocious mitosis in first cleavage and either dispermy or a double-nucleated sperm (Ford, type 4).[57]

(ii) Dispermy, where one sperm nucleus would fertilize the ovum and the other the second polar body with later aggregations of the two cells lines (Ford, type 3).

(iii) Normal fertilization, but incomplete separation of the second polar body from the vitellus; some or all of the chromosomes of the polar body would then mingle with one of the blastomeres in cleavage, yielding triploid or trisomic cells along with the normal diploid cells (Ford, type 5).

The other possibilities have never been found in natural development.

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