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Human Embryogenesis

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1. Introduction

The building of the human embryo is a biological process of transcendent complexity. It fails at least three times as often as it succeeds. It takes about six weeks for a 'normal' version of the process to construct a fetus containing all of the differentiated cell types necessary, in the correct numbers and locations, to form all of the tissue and organ systems necessary to become a living, breathing human baby at birth. At the developmental horizon between embryogenesis and the fetal period, the majority of the cellular and molecular work of the developmental biology is done. The rest – the subject matter of obstetrics – is mostly about growing larger.

The business of becoming human thus begun is never complete. You are neither the same today as yesterday nor the same as the person who may awaken wearing your face tomorrow. Most of the time – over two-thirds of the time in optimal conditions for pregnancy – embryogenesis fails and its products become detritus before anyone knows that anything has happened. When the time for full-term birth arrives, fewer than one in four will remain alive and growing. That is, among highly privileged pregnancies: young, healthy mothers, in couples of proven fertility, under research-level medical attention. In the less favorable conditions of most pregnancies in most of the world we may be reasonably certain that the prospects are not that good.

I am here to discuss what we know about how the human embryo builds itself. If you want, you can step aside and waste as much of your own time as you want arguing about what "know" means. However that turns out for you, we actually do know a great deal about the formation of the human embryo, sound inference from sound observational evidence, repeated and reviewed by multiple knowledgeable and competent scientists. In one form of summary, human embryogenesis has a great deal in common with every other kind of embryogenesis we understand at all, and our observations to date also show it is not exactly like any other kind.

First: No part of human embryogenesis is the "beginning of human life." Every human life today is a continuation of something that began a very long time ago. If the sperm and the egg cell are not already very much alive, nothing is going to happen. If the egg and sperm are not both human (the biological definition of which changes with every generation), nothing is going to happen. Even if everything is as it should be when egg and sperm meet, even so, very often nothing very interesting is going to happen. Much more often than not, some part of the awesome complexity of the process does not work.

The sperm and the egg cell bring life forward from the parents, whose lives came from their parents, whose lives came from their parents, etc., etc., etc., all the way back to the very beginning of any form of life on Earth or wherever else it might have begun before coming to Earth. You may, of course, choose not to know that, but that **is** the way **all** living things work, including the human species.

Much of what we know about human embryogenesis we have learned from embryogenesis of other organisms, but there is a wealth of knowledge, specific to the human process, available from the traces left by variations in embryogenesis among living humans with developmental anomalies, and twins and chimeras. By learning how some people have done their embryogenesis differently, we can learn much about the more usual process.

The formation of the human embryo is a complex system of processes of dividing and differentiating cells, very much like every other kind of embryogenesis, but not exactly like any other kind we know anything about. One same nuclear and cytoplasmic genome must be functionally subdivided and sequentially reprogrammed so that each of many thousands of differentiated types of cells may be functionally defined by the expression of a different subset from each of the multiple layers of coded information in that genome.

2. Zygosis and the asymmetric foundations and outcomes of cleavage

That original zygote genome must first be assembled from parts brought forward in the oöcyte, together with parts arriving with the sperm to be reorganized by functions in the egg cell cytoplasm, directed in part by coded settings in and on the hyper-condensed chromatin of the sperm. The cell division machinery necessary to orchestrate the mechanical onset of the differentiating cell divisions of the cleavage stage must be assembled under the direction of components of the centrosome brought by the sperm. [In some other placental mammals, the oöcyte retains its centrosomes through the meiotic cell divisions, but the human oöcyte does not and the structures of the cell's division mechanisms must be brought back from the sperm.] Also arriving with the sperm is a system of *imprints* on the DNA. The *epigenetic* system of imprints on both sperm and oöcyte DNA will play major roles in development – these are relatively new understandings, still unfolding, and there will need to be more said here about that later.

Asymmetry is fundamental. From head to tail, back to belly and left to right, cells form tissues, organs and organ systems specific to their appropriate three-dimensional positions in the organism and specific to the current time in development, from fertilization on to and through senescence.

For decades, my lab and several others have studied embryogenesis from a variety of viewpoints and approaches, hoping to understand the origins of (left, right, etc.) asymmetry and the mechanisms by which it is originated, elaborated and enforced. My original question when I began these studies a few decades ago was *"where do left and right come from?"* That question was triggered by puzzlement over excess nonrighthandedness in twins – more about that later. My conclusions must – like every other piece of science always – be considered preliminary, but it has been a long time building and it has become unlikely to change much further short of a major new influx of observational data. The origin of the developmental asymmetries of living things is enmeshed with the origin of Life itself. Life, as and in the functions of living things, IS – of its chemical and physical essence –

asymmetrical. DNA is physically and chemically asymmetric. Cell structure is fundamentally asymmetric. Cell division in embryogenesis is fundamentally asymmetric, and in general each embryogenic cell division is an event of differentiation for at least one of the daughter cells of that division. Asymmetry is its own mechanism for generating and maintaining asymmetry, all the way forward from the origin of all Life. Asymmetry maintains and propagates itself, and it is a major component of all Life and all the mechanisms of differentiation.

When we see ciliary motion, or heart looping, for example, put forward as the foundation of embryonic asymmetries (each IS one of the earliest *microscopically visible* gross structural asymmetries of the embryonic body) and we determine that certain gene products are essential for that movement to go in the proper direction, and that at least one of those gene product molecules must be in position on the right side of the embryonic midline to make it happen that way … then we may know with certainty that ciliary motion or heart looping clearly is not the beginning of embryogenic asymmetry. How? How?! did that molecule – that initiated this so-called 'beginning' cascade of events – know which side of the midline he was supposed to be on, at that particular time, to begin this beginning, and make the looping of the heart fall to the normal/usual side? And what does 'side' mean, anyway, in terms of cellular or subcellular structure to which a molecule should respond? Clearly the normal embryo at that stage is long since reliably asymmetric, and the origins of embryogenic asymmetry are much earlier in cellular and developmental Time.

Every vertebrate embryo properly questioned to date is *already reliably asymmetric when it has divided into only three or four cells*, with respect at least to consistent differences among those first few cells in the movements and functions of serotonin (Aw & Levin 2009; Buznikov *et al*., 1964; Fukumoto *et al*., 2006; Il'kova *et al*., 2004). Zeskind and Stephens (2004) report neurobehavioral effects on newborns exposed prenatally to the presently very popular serotonin re-uptake inhibitor antidepressants. If we could find a way to refocus the question on variations around the time of early cleavage, we might have an answer that would be much more to the point.

3. Foundations of embryogenic asymmetry; Introducing chromatin and imprinting

The first few cell divisions of human embryogenesis are visibly asymmetric. One may look at an early cleavage stage human embryo, and know quite confidently which of its cells will divide next – always the largest one among those first few. The first cleavage is asymmetric – one of the daughter cells is larger than the other. That larger cell will be next to divide, leaving the smaller of the first two blastomeres as now the largest of three and now the next to divide. After several such divisions, it becomes impossible to follow in the microphotographs published to date. However, there might be means to follow it further as it has been followed through the entire embryogenesis of *C. elegans* (Begasse & Hyman, 2011).

Every organism yet properly questioned has demonstrated the need and the means to recognize and respond to the differences between old (mother, template) and new (daughter, newly replicated) DNA strands, and between leading and lagging strands, for purposes of cell division and for the control of differentiation of cell function (Huh & Sherley, 2011; Klar 1987; Landsdorp 2007; Merok *et al*. 2002; Pierucci & Zuchowski 1973).

The DNA exists and functions at all times in various degrees of chromatin condensation, wrapping and unwrapping the DNA strands in RNA, histones and other proteins, covering and uncovering the base sequences for varying access to enzyme complexes of replication and transcription. This is the level of control where the effects of imprinting and other epigenetic controls are exerted.

We first learned from mice in the mid-1980s that embryogenesis will fail if the zygote does not contain both maternal and paternal pronuclear half-genomes. If the paternal pronucleus is removed from a zygote and replaced with a maternal pronucleus from another zygote, most of the time the resulting rearranged zygote will die from the effects of the manipulations. When development can continue, the embryo-proper will appear to be well-made, but the extra-embryonic support structures will not. And vice-versa: when development proceeds with two paternal pronuclei and no maternal pronucleus, the extra-embryonic support structures may look wonderful, but the embryo itself will not be at all well put together. The two half-genomes are prepared differently in oögenesis and spermatogenesis for different functions in embryogenesis. DNA base sequences are not changed, but they are marked by chemical modifications in ways that will cause the same DNA sequence in the two half-genomes to be differently expressed (Surani *et al.*, 1984).

We do not know exactly which or how many human genes are involved in the protocols of genomic imprinting. We do know that 'imprinting' in gametogenesis is only a part, and probably a small part, of the whole of epigenetic control of development. Our best guess at the function of imprinting itself has to do with the tug-of-war between the evolutionary long-term best interests of the respective parents. At face value, it seems clearly to be in the male's long-term evolutionary best interest to maximize the number of his offspring. This is not in the long-term best interests of the female, who is better off to husband her reproductive resources, to optimize the strength of her surviving offspring, at the expense of numbers if necessary. That is what we make of the original findings in the mouse … the paternal imprint in the absence of the maternal imprint maximizes the extra-embryonic support tissues, the better for more of his embryos to maximize their harvesting of uterine resources. The maternal imprint works to moderate all of that, to shepherd her reproductive resources – to save some of her self – for the sake of future as well as the present conceptus. This is a good plausible story, but it does not help us much with the fact that, in addition to placental mammals, at least some plants have found imprinting to the evolutionary advantage of their species. Differential expression of the blocks of 'imprinted' genes is heritable through many cell divisions, until reset in the next generation of gametogenesis (which happens in oögenesis in the first few months of female embryonic and fetal development). Most of the rest of epigenetic control is acted out in the resetting of combinatorial expression codes in each of the asymmetric cell divisions of the rest of embryogenesis.

The hardest thing about understanding human embryogenesis is that we cannot see it experimentally. We must *infer* from what we can see in *'experiments of nature'* and interpolate a testable picture of what is happening when and where we cannot see. Statistically useful samples of the normal real thing are unobtainable. We can stimulate human ovaries to produce large numbers of oöcytes, and we can fertilize them *in vitro*, after which we then may briefly watch their development. We cannot, however, safely assume that what we see in those circumstances is, or even very closely resembles, the normal, natural processes.

Such oöcytes and embryos as those are not entirely normal, as plainly demonstrated by the excesses of anomalous results among the progeny from every form of human reproduction that depends upon artificial ovarian stimulation. Papers in the literature following closely upon the invention of human *in-vitro* fertilization consistently reported "no statistically significant excess" of abnormalities. The sample numbers were too small for statistical significance in the demonstration of sizable increases in those small probabilities. Later studies, when available numbers of ART births are larger, make the risks more clear (examples: Buckett *et al*., 2007; Green, 2004; Pinborg *et al*., 2004). Oöcytes from artificially induced ovulation undergo embryogeneses that are less stable, less reliable, less likely to yield a fully 'normal' product. The mechanism most likely as an explanation is disturbance of the integrity of genomic imprinting.

Embryogenesis is all about the differentiation of dividing the single zygote cell and its progeny cells into hundreds of billions of specialized cells in the proper relative positions and growing to form a functional adult body. To the extent that we have come to understand it, human embryogenesis is very much like that of all other placental mammals and not exactly like any of them. The basic elements of this system of processes have much in common with the basic components of embryogenesis in every animal life form since before the radiation of the cnidarians (Morris, 1994). We have learned a great deal from fruit flies, from worms whose adult bodies include 959 cells all of whose paths through embryogenesis have been mapped, and from sea urchins and starfish, and we have learned important things from mice and cattle and sheep and birds and fish – as a far-from-exhaustive list of prominent examples.

In every case, the progeny of successive divisions from the original zygote must be differentiated to use different combinations of the thousands of genes in the one same diploid genome, to take the forms of and assume the functions of thousands of different cell types. The head–tail axis must be defined, and back vs belly, and left vs right; all three mechanical dimensions, and we must not neglect the fact that all of that changes with time. The many different tissues required for proper functioning of a complex body must be built of the right kinds of cells and put in the proper relative positions within the framework thus defined. Otherwise, it fails. In fact, 'otherwise' and 'failure' are the most common results for the human embryo in particular. It is difficult to know quite accurately, but it appears that human embryogenesis must be among the least efficient kinds of embryogenesis in terms of normal live births per fertilization.

From other chapters here, you should be able to form a good vision of the generalized story of embryonic development. This chapter will focus on commonly observed departures of human development from what we understand the "normal" process of human embryogenesis to be. Malformations, aneuploidies, 'birth defects' in general, twinning and chimerism, taken together, comprise a substantial fraction of the outcomes of human prenatal development – even of the small fraction that survives to delivery. From understanding these frequent anomalous outcomes, we can project a vision of the normal process in the light of which we might better claim to understand human embryogenesis.

4. Anomalies and failures of development

'Unusual' or 'anomalous' is of course a matter of perspective. We may prefer to think that the only relevant result of gestation of a human conceptus is a healthy live birth, and to

think of anything short of that or other than that as an outcome sufficiently rare and peripheral to ignore. We rarely see what we do not expect to see. We generally believe that we know what we should look for and that we see all of what there is to see.

If, as usual among ordinary folks and obstetricians, we think of pregnancy as beginning with maternal awareness and clinical recognition, then miscarriages and stillbirths qualify as unusual. Only perhaps fifteen to twenty per cent of recognized pregnancies end before live term birth. That IS a minority, but it is a substantial minor fraction. Here today, our concern is for all that happens before they are recognized, the fact that over two-thirds of them typically fail before recognition, and what they were or should have been doing when they failed, how they come to fail.

About half of those spontaneous abortions have a recognizable (cyto)genetic problem in the form of chromosomal anomalies that are big enough to see in a microscope with proper preparation and staining. The other half of them have had no readily diagnosable problem. Recent advances in DNA microarray technology now allow us to see sub-microscopic anomalies in the DNA of some of them, and even to find some of the single-base-pair mutations when we have a reasonable idea of what to look for.

The probability of miscarriage is not uniformly distributed over the population. If a couple has one miscarriage, they are rather more likely to have another one than the couple in the house across the street is to have their first one. Spontaneous abortions are sufficiently common that we seldom investigate before a couple has their second or third one. When we do investigate repeated abortion, we find that the causes tend to repeat, in those broad classes with vs without chromosome anomaly. With very rare exception, the developmental problems that cause failure of recognized pregnancies [spontaneous abortions] are put in place during embryogenesis, before the maternal or clinical recognition of pregnancy.

In the research that led to self-administered pregnancy tests, it became clear that pregnancy can be recognized by biochemical signals (immunoassay of chorionic gonadotropin) from the differentiating trophoblast (in the process of building the chorion and the fetal portion of the placenta) a few weeks ahead of usual maternal awareness or clinical recognition. A much larger fraction of pregnancies discovered this way will disappear than the fraction that will miscarry after more conventional recognition of pregnancy. The majority of failures occur before recognition, during embryogenesis. More than twice as many instances of human embryogenesis end in failure as result in a living fetus carrying a recognized pregnancy forward (Boklage, 1990, 1995).

From the completion of embryogenesis at the recognition threshold [usually about eight weeks since the last normal menses, about six weeks after fertilization] and on through the fetal period [the remaining 30-32 weeks to normal time of birth], the loss rate is much slower than it was during embryogenesis. By the time miscarriages and stillbirths are over, fewer than one in four products of successful syngamy and zygosis remain to be born alive.

5. Secondary and primary sex ratio, imprinting and sex differences in speed and efficiency of embryogenesis

Sex ratio at birth is one of the outcomes from which we can learn some of the facts of embryogenesis. With rare and poorly understood exceptions, the number of males among

human live births exceeds the number of females. The 'secondary' (at birth) sex ratio exceeds one (fraction male exceeds 50%) in most samples ever observed. All endings of recognized pregnancies short of live birth (miscarriages and stillbirths) also, with a very few reported exceptions, include an excess of males. If males comprise more than 50% of live births in spite of excess males among all the losses of recognized pregnancies throughout gestation, then … it has been supposed that the 'primary' sex ratio (at fertilization) must be much higher to supply an excess of males for all prenatal losses and still have an excess of males at birth. This hypotheses has been subjected to many competent tests, and the answer is always no; there is no excess of Y-bearing sperm in the normal ejaculate, nor among the products of fertilization. There is no excess of Y-bearing sperm in the ejaculate after chemotherapy or after any of several efforts at changing the fractions of X-bearing and Y-bearing sperm for purposes of helping a couple influence the likely sex of their next offspring.

There is no significant departure from 50:50::X:Y-bearing sperm at fertilization, but there is a very real excess of males throughout pregnancy from recognition through delivery. What happens in the interval between fertilization and recognition of pregnancy? Embryogenesis – that's what happens between fertilization and recognition of pregnancy. Embryogenesis is approximately complete, with all organs and organ systems in place and needing (almost) only to grow, at about the most usual time of maternal recognition of pregnancy – about eight weeks since the last normal menses, about six weeks since fertilization – when the second consecutive menses goes missing.

Can there be anything about embryogenesis that routinely generates enough of an excess of male conceptuses to last for the remainder of pregnancy? Yes. In a word: speed. Male conceptuses generally do embryogenesis faster. In mouse, human and a few other kinds of embryo so far studied, the presence of a paternally-imprinted X-chromosome slows embryogenesis. Since only female embryos have a paternally-imprinted X-chromosome to slow them down, male embryos (who get not X but Y chromosomes from their fathers) do embryogenesis faster. Because many of the most important cellular achievements of embryogenesis are time-critical chemical signals, to other cells in the embryo or to the placenta, or through the placenta to the maternal physiology, then getting through embryogenesis less quickly very likely means doing it with less success. Since some of the products of every stage of development are signals from cell to cell within the embryo, or from the embryo to the maternal physiology, signals necessary for continuation of the pregnancy, then the establishment and maintenance of viable pregnancy is more efficient in general for male embryos. The extra losses of females because of their slower and less successful embryogenesis can set up an excess of males sufficient to show an excess of males in all losses of recognized pregnancies and still have an excess of males among live births.

6. Imprinting, the rest of epigenetics and major continental subpopulation variations in the epidemiology of embryogenesis

Significantly consistent differences in secondary sex ratio among human major continental subpopulations turn out to match corresponding gradients in several major parameters of the physiology of embryogenesis. The list includes at least: frequency of twinning, same-sex vs opposite-sex fractions of delivered twins, chorionicity fractions of twins, age of females

at menarche, age at first birth, age at last birth, and the fraction of births that are premature or of low birth weight. All of these may be seen as arising from differences in relative speed and efficiency of embryogenesis between male and female embryos and among these subpopulations in the strength of the male-female differences. These are reviewed and discussed in Boklage (2005).

Surveys of the genome for gene sequences subject to imprinting have not in general shown a great deal of activity on the X-chromosome in comparison to levels indicated on several of the other chromosomes. It remains likely that the molecular survey criteria used to identify imprinted DNA sequences are imperfect and that there may be any number of different groups or classes of loci subject to imprinting. At least as likely, the 'rest of epigenetics', changes in gene expression mediated by genome markings or modifications other than imprinting, may greatly exceed imprinting in scope.

The parent-specific genome modifications during gametogenesis that we know as 'imprinting' set up only one 'kind' of epigenetic control, wherein the effective developmental difference is not a matter of DNA sequence itself, but a matter of relative expression of the same sequence, differing according to the parent of origin of that particular copy. Epigenetic controls are a means, perhaps the primary means, by which 'environmental' variations can affect all of development, from embryogenesis on through life. The mother's nutrition, the mother's emotional state, the mother's medications, nutrition of the maternal grandmother during the mother's fetal development… all of these have been shown to have effects on prenatal development that are not governed by variations in DNA sequences. Throughout life, physiology can – indeed, must – change to adapt to environmental variation. There are physiological reasons, for example, why losing excess weight or leaving behind any other sort of addiction is so hard. A number of drugs, particularly psychoactive drugs of abuse, and various foods, have been shown to cause changes in physiology mediated by changes in multiple gene functions that may persist long after the drug is withdrawn. Variations in imprinting and other elements of epigenetic control are major functional contributors to variations in the course and outcomes of embryogenesis. Our understanding of those controls is increasing rapidly, but has a long way to go.

7. Twinning

Among the most obvious of the more-or-less 'unusual' outcomes of human embryogenesis is twinning. According to the inferences about prenatal mortality and survival discussed above (Boklage 1990, 1995), twins born as members of live pairs represent no more than about one-in-fifty of all products of twin embryogenesis. Like singletons, over three-fourths of twin conceptions disappear completely (with loss of both conceptuses) before term birth. Most of the remaining one-fourth of twinned embryos arrive at term alone, as sole survivors, outnumbering live-born twins apparently at least ten- to twelve-fold. The strongest indications are that roughly one live birth in eight is a product of a twin embryogenesis, with the great majority of them showing no easily recognized evidence of their origins in twinned embryos. These sole survivors have been totally ignored in all the various literatures about the epidemiology of twinning. Developmental consequences of twin embryogenesis are not terribly hard to find in twins born in pairs (Boklage, 2009) and are therefore to be expected in the lives of those sole-survivor individuals. We do not yet

know any simple or inexpensive way to identify all of the sole survivors for an accurate count, but clearly a substantial fraction of all human embryogeneses are twinned and a similar fraction of all live births arise from twinned embryos. I remain satisfied with the estimate of one in eight, with the realization that it may vary considerably up or down with variations in overall efficiency of pregnancies in general.

Since the mid-1960s, from deep within the old orthodoxy [in place since before Galton, 1875], that '*identical*' twins arise from 'splitting' embryos and '*fraternal*' twins arise from double ovulations unfolding into parallel and independent embryogeneses, it seemed obvious that we should be able to learn a great deal about embryogenesis from the ways in which the two 'kinds' of twins differ from each other and/or from singletons in their development.

Just suppose … that the "common knowledge" is the truth. *Just suppose* that all the unusual things about the development of twins really are due to consequences of 'splitting' the embryos of the 'identical' twins (only) and thereby disturbing the establishment of their embryogenic asymmetries. *Suppose also*, as per the "common knowledge", that 'fraternal' twins arise from separate and independent egg cells, and that their development is the same as that of singletons – except perhaps for any effects specific to living through development – beginning in the cleavage stage or at least no later than the blastula – as twins. If all of that were true, if the common knowledge were the simple truth that it has been assumed and reported to be, we should be able to compare the development of 'identical' twins with singletons and learn a great deal about how embryogenesis generates the doubled three-dimensional body symmetry to make two embryos out of one. Dizygotic twins, from that perspective, would be the obvious 'controls' against any effects of simply being twins. That describes the climate in which these studies of human embryogenesis began, and that has been the outline of the plan of my research for the last few decades.

8. Probing twin embryogenesis

The answers have been surprising and consistent and clear. All "kinds" of twins as groups are about equally different from singleton development, in the same multidimensional directions, at about the same multidimensional distances. Things just are not like the common knowledge would have it. The evidence is clear and ample. *We have no reason to imagine that the cellular processes of embryogenesis in dizygotic twin pairs are any different from those for monozygotic twins.* A single contiguous mass of cells within a single *zona pellucida* confining the mass and substance of a single secondary oöcyte becomes organized by processes of cell differentiation into two complex asymmetric plans to become bodies for two fetuses. The cells may all contain copies of one zygote nucleus (the monozygotic twins), or – if syngamy yields two genetically distinct zygote nuclei, there may be two genetically distinct sibling cell lines (for dizygotic twins).

Like fertilization, or zygosis, or any other proposed definition of conception, the onset of twin embryogenesis, the 'conception' of twins if you will, is not an *event* that can be considered to occur in an instant, but is instead a complex *system process* that occupies space and time. It has no instantaneous beginning or end, but constitutes a developmental horizon, perhaps crisp and clear from a distance, but not subject to clear definition from anywhere within conceptual or temporal proximity.

Whether the twins thus initiated are monozygotic or dizygotic is a genetic distinction, not a cellular one. Two zygotes never had to mean two cells. Cells within an embryogenic cell mass that will generate monozygotic twins all have copies of one nuclear genome. [Keep in mind the occasional occurrence of post-zygotic mutations that may establish a second genetically distinct cell line – even a second embryo. That is the common knowledge explanation of "mosaic" embryos – who may have cytogenetically different cell lines, and are not usually tested for other genetic differences.] Dizygotic twins are built from cells with two different nuclear genomes, most likely different in every chromosome. Syngamy and zygosis have assembled *two zygote nuclei instead of one within the confines of the single secondary oocyte and its zona pellucida*. We must, of course, discuss how that can happen. Very briefly, the frequency of triploidy (the most common of all chromosome anomalies) tells us that tripronuclear zygotes are quite common, more or less equally possessing two paternal contributions or two maternal contributions. Those events are sufficiently frequent that neither can be considered to limit the frequency of their joint occurrence with both two paternal and two maternal pronuclei, to form two zygote nuclei after syngamy (*cf.* Boklage 2009, 2010).

Every trace of embryogenesis we have properly examined, with several independent samples and methods, shows that dizygotic twins differ developmentally from singletons at least as much as the monozygotic twins do, in very similar multidimensional directions and at very similar multidimensional distances (Boklage, 2006, 2009). There are enough differences to significantly distinguish monozygotic from dizygotic twins – as groups of individuals, without any consideration of within-pair similarities or differences. Those differences, however, are very small compared to the common differences of both 'kinds' of twins from singletons.

9. Handedness in twins and their families, and "mirror"-twinning

The minority version of brain function asymmetry (nonrighthanders = lefthanders + 'ambidexters') is more frequent in twins than in the general population. The lore has it that the excess belongs primarily to the 'identical' twins by virtue of 'splitting' their embryos, disrupting the proper asymmetries of ongoing embryogenesis. Actual data, on the other hand, show that the excess occurs *equally* in *both* 'kinds' of twins *and* in the singleton siblings of the twins. The parents of twins are more often nonrighthanded than *their* same-sex siblings – the maternal aunts and paternal uncles of the twins (calculated separately because of the consistent sex difference in frequency of nonrighthandedness in the general population). Each nonrighthanded parent increases probability of nonrighthandedness in the children – regardless of multiplicity – by a factor of about 1.5 (Boklage 1976, 1977a,b, 1981, 1987a).

In all of this, there is no effect of zygosity or chorionicity. Monochorionicity has been thought to indicate exclusively monozygotic twinning events occurring later in embryogenesis than those of dichorionic twins. The 'later splitting' has been imagined to be more likely to disrupt the ongoing establishment of embryogenic asymmetries. In fact chorionicity is not associated with any difference in the distribution of handedness. The excess of nonrighthandedness in twins is not specific to 'identical' twinning. Nor is the excess of nonrighthandedness in twins any greater among monochorionic pairs as proxy for 'late splitting' (Carlier *et al.*, 1996; Derom *et al.*, 1996).

The idea of "*mirror-imaging*" in twins, near and dear to the hearts of twins and their parents though it may be, does indeed mean something special about twin embryogenesis, but what it means is much more complex and fundamental than what they have been thinking (*cf* Boklage 2010), and nowhere near as much fun. In short, twins of both "kinds" are substantially more symmetrical in their craniofacial development than singletons. Dental diameter measurements from left sides and right sides from singletons are quite significantly consistently different – discriminant function calculations can identify the side of a singleton's head from which a set of dental diameter measurements were taken with over 95% accuracy. The same is not true of measurements from twins of either "kind". Discriminant function calculations report probabilities of over 90% that the left- vs right-half-jaw sets of measurements, within statistical error, might as well have been drawn from a single sample.

The excess of nonrighthandedness in twins arises from an inherited tendency to nonstandard brain function asymmetry that is concentrated in families which also have an inherited tendency to deliver live twin pairs. Neither differs as a function of zygosity or chorionicity (Boklage, 1981, 1985, 1987a; Carlier *et al.*, 1996; Derom et al. 1996) Something about establishing an unusual version of motor brain function asymmetry during embryogenesis and something about becoming twins at about that same time in embryogenesis is the same or closely related.

You will find it written in many places that dizygotic twinning and only dizygotic twinning is at all hereditary, and then only in the maternal line, by way of an inherited tendency to double ovulation. The entire literature of the biology of twinning is predicated on variation in the births of live twin pairs being considered a perfect proxy for variation in conceptions of twins – as if every twin conception must generate a live twin birth and that variation in twin deliveries directly reflect variation in double ovulation. Since older mothers for example deliver more twins, so the story goes, it must be because they have more (double ovulations and therefore more) twin conceptions. There is no significant attention paid to the facts 1) that those born alive as members of live twin pairs are a tiny fraction of those conceived as twins and 2) that we know very little about the complexity of the processes that make the differences between those conceived as twins and those born alive as twins. Because the live-born fraction of twin conceptions is so small, very small differences in the determinants of prenatal survival can make large fractional changes in the numbers born alive. For this reason above several others, the use of the Weinberg Difference Method in general is of dubious value, and its application to any population of twins with any significant anomaly is absolute nonsense.

10. Malformations in twins

Malformations, particularly the most common, midline/fusion malformations, are more frequent in twins. Neural tube defects, congenital heart defects, and orofacial clefts are the most common, therefore best known. These all involve structures formed in embryogenesis from the fusion in the midline of bilaterally-approximately-symmetrical half-structures. Following fusion of the asymmetric half-structures, the resulting midline structures are remodeled with and by mesenchyme cells descended from neural crest cells. Like nonrighthandedness, the malformations that are more frequent among twins than among singletons are also more frequent among the sibs and offspring of twins, without zygosity

differences except in a few situations where the correlation is in fact stronger among the dizygotics and their families (Boklage 1985, 1987a,b, 2010).

Again like nonrighthandedness, throughout the history of studies of the biology of twinning, it has been reported that the malformations that are excessively frequent in twins are due predominantly to the 'identical' twins because of the 'splitting' required to generate monozygotic twin embryos causing disruptions of embryogenic symmetry operations.

Schinzel *et al*. 1979 provided a reasonably thorough review which is often offered as the standard reference on the relationship between monozygotic twinning and malformations. It included nothing new, being instead a good summary of prevailing prejudices and presumptions as if they were the available "common knowledge" facts of the matter. There are deep and wide problems with the sorting in every one of the sources they gathered to put their review together. With rare exception, none of the twin pairs included in those calculations were actually diagnosed for zygosity, let alone soundly diagnosed. The MZ excesses of the malformations considered there have in general been decided by sorting into same-sex vs opposite-sex twin pairs, under the assumption that the boy-girl pairs are in all ways developmentally representative of all dizygotic twins, and that the apparent concentration of difficulties in the like-sex pairs is due entirely to their concentration among the monozygotic members of the like-sex pairs. I have discussed the severe faults of that idea at length in Boklage 2010. OS-DZ pairs are unique. They are not developmentally representative of any other group. The members of OS-DZ pairs are not representative even of their own respective gender groups, twin or single. Risks of developmental anomaly or pregnancy wastage are in fact at least as great for same-sex DZ twins as for MZ twins (Boklage 1984, 1985, 1987c,d).

11. Blastulation, gastrulation, neurulation, the neural crest and asymmetries of human embryogenesis

The midline/fusion embryonic asymmetry malformations are sufficiently frequent in twins that they can readily be imagined to occur only in twins – if 'twins' properly includes the sole survivors. The numbers would allow it. They all intimately involve the neural crest. The structures in question are built by fusion in the midline, from left- and right-half structures, followed by remodeling with and by mesenchymal cells from the neural crest.

Until about the fourth and fifth days after fertilization, the embryo-in-progress is a solid ball of cells called the *morula* (*L., mul*berry). The outermost layer of cells becomes a membrane and then an epithelium (now the *trophoblast*, the future *chorion*) by forming *tight junctions* between the cells of its outermost layer to replace the *gap junctions* through which the cells of the morula communicated while held together inside the zona pellucida from zygosis through cleavage. Zona-breaker cells of the trophoblast now attack the zona pellucida with enzymes that leave the zona softened and weakened. The outer (trophoblast epithelium) layer of cells pump fluid from outside to inside, and the morula swells (for the first time growing beyond the mass and size of the 'egg cell' secondary oöcyte at ovulation) and sheds the softened zona. This is *hatching*.

The *inner cell mass* remains attached to a patch of the outer layer cells that we will now call the *polar trophoblast*, separated from the *mural trophoblast* (the *wall* of the embryo, exclusive of

the *polar* patch) by the fluid pumped in through the trophoblast epithelium. The polar trophoblast will now attach to and penetrate the endometrium, catalyzing the formation of the placenta from interacting fetal and maternal tissues. By separating the *inner cell mass*, attached to the *polar trophoblast*, from the *mural trophoblast* and filling the intervening space with fluid and sloughing the dissolving zona pellucida, the *morula* now becomes the *blastula*, bounded by the trophoblast, containing the inner cell mass in the fluid-filled *blastocyst* cavity – a lump inside a ball.

The inner cell mass will now form and separate a double layer of cells facing the blastocyst cavity, forming another smaller cavity between that new *bilaminar disk* stage of the embryo and the polar trophoblast. The bilaminar disk has one microscopically visible patch of distinctly 'taller' cells in each of its layers. Cells of the *prochordal (sometimes called prechordal) plate,* near the anterior end of the embryo, are longer than their neighbors in the direction of the blastocyst cavity. That direction thereby becomes recognizable as the *ventral* direction. The cells of the *primitive streak,* near the posterior edge of the disk, are taller in the *dorsal* direction, into the newly formed amnionic cavity. The anterior-posterior (head-tail), and dorsal-ventral (back-belly) asymmetries are thereby made visible and the left vs right dimension is also therefore constrained with no dimensional degrees-of-freedom remaining.

The attachment of the inner cell mass to the polar trophoblast has previously identified the dorsal aspect of the embryo. So, the definition of the anterior-posterior axis by the appearance of the prochordal plate and the primitive streak actually constrains the whole system of three axes. It must be remembered that the cells in question began their various biochemical differentiations before those differentiations became microscopically visible in spatial coordinates.

The human blastula does not form a *gastrula* stage exactly like the structures in other embryos to which we usually give that name, but what comes next, the formation of the trilaminar disk stage, is the human homologue of *gastrulation*. From the primitive streak, cells multiply and spread to the anterior and laterally between the layers of the bilaminar disk, forming the third embryonic layer in the middle (the *mesoderm*). The dorsal layer is now the *ectoderm* (which will soon be divided into the neural plate and the non-neural ectoderm that will form the skin), the ventral *endoderm* which will form the gut and associated structures, and the new middle layer will be the *mesoderm*, primarily to build muscle and bone.

When the embryo becomes the trilaminar disk, the process of *neurulation* begins. Most diagrams of this period show the cells of the new middle layer multiplying rapidly from somewhere in or near the anterior end of the primitive streak, diving under the ectoderm just anterior to it, between the ectodermal and endodermal layers, and spreading anteriolaterally, toward the prochordal plate and to the sides. These cells appear to correspond closely in function to the cells of the amphibian *Organizer*, the avian *Hensen's node*, and the zebrafish *shield*.

These cells will induce the formation of the neural plate, the *neurectoderm*, from the portion of the ectoderm lying dorsal to them. The edges of the neural plate– at the border between neural and non-neural ectoderm – begin to roll up and toward the midline in a wavelike structure, led by differentiating cells at the boundary between the neuroectoderm and the non-neural ectoderm. The peaks of those rolls will meet in the

midline and fuse to form the neural tube. The cells of the neural crests (of the 'waves') meet in the dorsal midline to differentiate and begin migrating laterally and ventrolaterally to an enormous number of destinations to perform a dazzling array of embryogenic functions on each side of the midline.

12. Formation of the neural crest

Formation of the neural crest is a major watershed moment in embryogenesis. It may well be that no other group of cells comparable in number has more functions to perform in embryogenesis, especially in the realm of the determination and elaboration of embryogenic asymmetries and the formation of midline structures by fusion of bilateral halves.

The autonomic nervous system, the enteric nervous system, the pigment cells, most of the bones of the head and face, the jaws and the teeth, the bones of the ears, the inner structures of the heart, the adrenal medulla … an incredible variety of specialized cell types will be formed either from, or under influences of, mesenchymal derivatives of the neural crest cells (Chang *et al.,* 2008; Kirby *et al*., 1983, for examples from a large and varied literature).

The radiation and development of the neural crest cells is driven by a gene regulatory network the complexity of which we have just begun to appreciate. This is a system of interacting signals, transcription factors, and cascades of downstream effector genes that will guide the final migrations and differentiations of the neural crest cells (Betancur *et al*., 2010; Huang & Saint-Jeanett, 2004); Abnormalities in neural crest development cause '*neurocristopathies*', which include conditions such as frontonasal dysplasia, Waardenburg-Shah syndrome, and DiGeorge syndrome, along with all of the individual non-syndromic midline/fusion malformations.

13. Craniofacial development in twins vs singletons

The developmental anomalies which are more frequent in twins than in singletons are of these kinds: anomalies in the development of midline structures formed from bilaterally-almost–symmetrical half-structures by fusion in the midline, followed by remodeling under influence of neural crest mesenchyme. The excess of nonrighthandedness in twins mentioned above, for example, long assumed and reported to be a certain consequence of monozygotic twinning, but shown here to be equally frequent in dizygotic twins and in the close relatives of all "kinds" of twins, seems to be a clear example. We do not to this day, however, know the cellular bases for the normal asymmetries of the motor functions of the brain, so it is not obvious in molecular terms how they might be disturbed by the cellular events of twinning.

We know a good bit more about the cellular and molecular bases of craniofacial development, as represented in the development of the teeth (Boklage, 1984, 1987d, 2010). Multidimensional structural relationships in craniofacial development, represented in a sub-system model by the covariance matrices of 56 buccolingual and mesiodistal dental diameter measurements, clearly discriminate between twins and singletons, as groups of individuals, with over 95% accuracy. A small fraction of cases entering these analyses identified as singletons are scored as twins by some of the discriminant functions, but no one identified as a twin is ever misclassified as a singleton. This could have been predicted.

Any large enough sample of singletons is highly likely to include some 'sole survivors' of twin embryogenesis. The presently best available estimate is that about one live birth in eight arises from twin embryogenesis (Boklage 1990, 1995). At that rate, the probability that a sample of ten single births will include at least one sole surviving twin is almost 75%.

These results further make it clear that girl-boy pairs are absolutely not developmentally representative of all dizygotic twins. They are different from all other groups. Of course, normal boy-girl pairs are dizygotic. Their developmental patterns are different from singletons and from same-sex twins of either sex. To assume that they are entirely representative of all dizygotic twins, and therefore that every difference between opposite-sex and same-sex twin groups arises entirely from the monozygotic members of the same-sex group, is shortsighted, lazy, baseless and untenable.

If embryogenesis is to be double, to build twin bodies from a single contiguous mass of embryonic cells, then differentiation as twins must begin with a doubling of the definition of the embryonic axes, just as in the simplest of embryos. In the human embryo, the dorsal-ventral direction is the first axis grossly visible, as the bilaminar disc separates from the rest of the inner cell mass attached to the polar trophoblast, before the prochordal plate and the primitive streak appear, to make the anterior-posterior direction apparent. There will need to be two of everything required to induce the formation of primitive streaks, neural tubes and neural crests. It has been proposed that the formation of the primitive streak defines the onset of human individuality because it marks the end of the possibility of twinning and of one conceptus (with one sacred immortal personal human soul) becoming more than one person.

14. Pyloric stenosis, Hirschsprung disease, enteric nervous system, and neural crest: Twins! Yes! But … No sign of monozygotics!

Pyloric stenosis affects about one in 600 children. It is a disorder of the development of the enteric nervous system, which includes more neurons than the spinal cord, all apparently derived from cells of the neural crest (Farlie et al., 2004, Barlow et al. 2008). Pyloric stenosis is over 30% more frequent in twins than in singletons, four times as frequent in males as in females, rarely concordant in twins, and we really can find no reason to believe that any of the affected twin pairs are monozygotic. The greatest repeat risk is among twin brothers of affected females. This is an intriguing prospect contrary to all of the old background … a highly heritable multifactorial midline malformation – a neurocristopathy – exclusive to the embryogenesis of dizygotic twins (including sole survivors)?!. If, as it seems, this particular developmental deviation does not in fact occur in liveborn monozygotic twins, then it might be lethal in MZ embryos OR it might require the presence of two different genomes or epigenomes, and singleton cases must all be sole survivors of twin embryogenesis. These results are not yet published – we're thinking of running a contest with a prize for a testable mechanism – that it should also be plausible presently seems too much to ask.

Hirschsprung disease is a less common disorder of the enteric nervous system, affecting the colon in about 1/1500 children, with epidemiology very similar to pyloric stenosis: >80% males, excessive in frequency among twins, even more highly heritable than pyloric stenosis *and* even more rarely concordant in twins (Bolande, 1997; Jones, 1990; Kenny et al. 2010; Martucciello, 1977; Moore, 2006; Shahar & Shinawi, 2003; Tam, 1986; Tam & Garcia-Barceló, 2009; Templeton, 1977).

15. Chimerism and chimeras

Arguably the most intriguing variation on the themes of human embryogenesis is spontaneous embryonic chimerism, and it provides essential insight here. Spontaneous embryogenic chimerism is a branch of the twinning process. A spontaneous embryogenic human chimera is an individual whose body is composed of two embryonic cell lines with different genotypes. For present purposes, this does not include chimerism installed by way of a blood transfusion or other tissue transplant, nor does it include colonization of women by cells transferred into their bodies from a conceptus. It is herein meant to be understood as the embryogenesis of dizygotic twins occurring within a single contiguous mass of cells (Boklage, 2006). Assortment of the cells of two different genotypes into the separate twin body symmetries from a single mass of cells is unlikely to be perfect. Either or both of the cotwins thus derived may incorporate some cells of the other cotwin's genotype (Abuelo, 2009; Boklage, 2006).

According to very nearly everything you will read, human embryogenic chimeras are exquisitely rare. This is quite compatible with chimerism being thought to arise from the fusion of placental circulations of independent dichorionic dizygotic twin fetuses. It does not happen that way. Anastomosis of placental circulation has been found to have happened only a handful of times in examination of several thousand fused dichorionic dizygotic placentas (Foschini et al., 2003).

When directly tested for, chimerism has been found in over eight per cent of a sample of dizygotic twins and 21 per cent of dizygotic triplets (van Dijk et al., 1996), using an exquisitely sensitive test with fluorescent antibodies against five red blood cell antigens. Given that chimerism, when present, need not be present in blood, and the considerable possibility of sib-sib matching for alleles at five loci (so that cells of co-twins would not be detectably different - a negative test for chimerism), these numbers clearly represent a minimal estimate of the chimerism that was there. Remembering that the two cell lines of human embryonic chimeras are by definition the genetic equivalent of dizygotic twins, and that the majority of products of twin conceptions are born single, chimerism found in twins born alive as twin pairs represents a minor fraction of the chimerism that might be found if all cases could be identified.

In another direct study, female cadavers were tested for chimerism in multiple tissues, indicated by fluorescent hybridization histochemistry for Y-chromosome DNA sequences, scored as positive only when labeled in tissue-specific cells to exclude possibility of having captured blood cells 'just passing through'. In about one third of the women tested, chimerism was found in one or more of the tested tissues. History of having borne one or more sons (exposing the woman to the possibility of fetomaternal cell transfer), or of having had one or more transfusions, did not increase the frequency. Since only male 'foreign' cells were visualized, the true frequency of chimerism might have been twice what was observed, closer to two-thirds of the women sampled (Koopmans *et al*., 2005). The idea that chimerism could be as frequent as two-thirds of live births is hard to believe. Ideally, that study should be extended to similar numbers of virgin females, to control for the possibility of transfers from unrecognized transient conceptions of sons or the transfer of any other types of Y-bearing cells through vaginal mucosa by insemination.

It is abundantly clear that chimerism is not rare. Because of the much greater numbers of twins born alone, chimerism may well be several times as frequent as births of live twins in

pairs. Chimerism has no macroscopic phenotype of its own. It has been, and generally still is, called "extremely rare" because it is *discovered* rarely and only by accident. The first reported instance was in 1953, when a unit of blood from a normal, healthy English woman was found to have about a 70:30 mixture of red cells of two different serotypes. Since then, a number of other cases have been found by way of such mixed-field agglutination, but admixtures of less than 15 to 20 per cent are not usually discoverable in standard serological blood typing. Molecular genotyping, as in forensic identification, is – more-or-less as a matter of policy – no better: when a genotyping scan shows an extra allele peak with less than 30 per cent of the strength of the main peak value, it is marked as noise and ignored. When an extra allele peak appears at 30 per cent or more of the main peak value, especially if multiple loci are involved, the sample is declared contaminated and discarded (but see Erlich, 2011). Because "everybody knows" chimerism is exquisitely rare, we do not in general look for it, and we hardly ever find what we do not believe will be there.

The majority of living humans are built of normal cells, and there is every reason to suppose that the great majority of chimeras must have two normal cell lines. A couple of sensational cases were covered in the popular press. Karen Keegan needed a new kidney. Her husband and three sons were tested first. The probability of a match was small, but keeping it in the family has advantages. Her husband proved to be an excellent prospective donor. Almost overshadowing that good news was that the DNA results said two of her three sons [she conceived, carried, delivered and raised them] are not her sons, but cannot be hospital label-switching accidents. The DNA results say they are sons of her husband and another woman. Examination of frozen samples from previous surgeries showed that the "other woman" exists genetically in the form of some cells in her body from her unborn dizygotic twin sister. This woman is a chimera, with no sign in her phenotype, discovered entirely by the accident of carefully genotyping her whole family for purposes unrelated to her chimerism (Yu *et al*., 2002).

Another case in progress shortly thereafter concerned a young woman who needed public assistance to start over after separating from the father of her two children and her fetus. DNA said the two children were children of her partner and another woman. There were questions of welfare fraud, that she might be seeking public assistance for children who were not her own, and questions as to what she had done with the real mother. Were there crimes rather worse than fraud involved? A representative of the court was in the delivery room to gather samples for DNA on the spot. The newborn is full sibling to the other two – same father, same mother who still is genetically not the woman from whose belly the baby had just been seen to emerge. No old surgical samples were available this time, but samples from various more-or-less accessible parts of her body yielded some cells of "the other woman", the twin sister who was never born and is a perfect genetic candidate for being the mother of all three children. This woman is a chimera, with no sign in her phenotype, discovered entirely by the accident of carefully genotyping her whole family for purposes unrelated to her chimerism. Lydia Fairchild's case never to my knowledge made it into the scientific literature, but can be found in many popular press items on the web.

Some human chimeras are discovered when they are observed to be hermaphrodites and investigation reveals a mixture of XX and XY cells. Experimentally constructed mixed-sex chimeras of mice almost always have a normal male phenotype at delivery. A paternally-imprinted X-chromosome [present only in female embryos], retards the growth of

embryonic cells in human as well as in mouse. Faster growth of the XY cells in mixed-sex embryos might reasonably be expected to minimize the fraction of mixed-sex human chimeras that are detectably hermaphroditic. Some chimeras are discovered when twins are observed to be dizygotic (different sex is the easiest, but not the only, way to tell) and monochorionic (*cf* Erlich, 2011; Parva *et al*., 2009; Walker *et al*., 2007).

Mixed-sex twins are less often found as chimeras but they are found at sufficient frequency to know they have their place. Because experimental mixed-sex mouse chimeras almost always show up as normal males, I find it plausible that the lower-than-binomially-expected frequency of mixed-sex human chimeras is probably caused by the large growth-rate advantage of XY embryonic cells – reviewed in Boklage, 2005.

16. Monochorionic dizygotic twins are chimeras, and they are common

For most of the history of any analyses of twins, in fact apparently at least since Galton in 1875, monochorionicity has been considered to be absolutely diagnostic of monozygosity. Samples recorded as opposite-sex and monochorionic have been summarily deleted from data bases as obvious errors. It could not be otherwise, since it is clear [so says the common knowledge] that dizygotic twins must come from separate and independent egg cells and could not possibly be from a single embryo as monochorionic twin pairs must be. [Here is one part of the problem: 'monoembryonic' has never been the same as 'monozygotic.' Just as a zygote may become more than one 'embryo', one embryonic cell mass may contain more than one zygote (nucleus).] Over the decades, a few monochorionic dizygotic pairs had been discovered and ignored as meaningless anomalies. A (then-)young physician from Glasgow was publicly declared laboratory-incompetent and shouted down at the Fifth International Congress on Twin Studies in Amsterdam for telling us he had found a few dizygotic pairs among the monochorionic pairs in his practice ["…everyone knows… after all," and the pillars of the Society came down upon his head and shoulders]. I did not know then what I know now and could not defend him.

Recently, the number of reported cases has more than doubled, and the reality of monochorionic dizygotic twins has begun to sink in. Monochorionic dizygotic twins are necessarily chimeric, since they share at least some embryonic cells of one another's genotype in the form of the shared chorion. Monochorionic twins of the same sex were unanimously for decades declared monozygotic without 'wasting the reagents' to genotype them for zygosity – of course it is "well known" that such twin pairs are "always" monozygotic. That 1986 study from Glasgow (Mortimer 1987) and a later and larger one from Taiwan (Yang *et al.,* 2006) agree – about a quarter to a third of consecutive, unselected monochorionic twins are dizygotic, and therefore necessarily chimeric.

A recent paper on the risk of monozygotic twinning in deliveries from artificially assisted pregnancies counted very nearly all of the "monozygotic" pairs included in their analysis merely from ultrasound indications of monochorionicity (Vitthala *et al*., 2009). The rest were estimated by Weinberg Method calculations, as the excess of same-sex pairs over the number of opposite-sex pairs, with the required unacknowledged assumptions that the pairings were independent at fertilization and stable throughout gestation, in spite of often-reported excess losses of males and of members of same-sex pairs.

The future chorion differentiates, first as the trophoblast, from the outer layer of cells of the morula stage of the 'embryo' before 'hatching' – that is, while still inside the one same *zona pellucida* that came out of the follicle surrounding the egg cell. [Until just before 'hatching' by shedding the zona, as the morula is changing to become the blastocyst, the cell divisions of the cleavage stage of embryogenesis have partitioned the original substance and volume of the oöcyte, increasing in cell number and decreasing in cell size.] Because they are often born as chimeras, and because they are sometimes born monochorionic, we are left with no reason to doubt that naturally-conceived human dizygotic twins can and usually do arise from a single contiguous mass of cells divided from the contents of a single secondary oöcyte. The rest of the background cited above includes no evidence that any naturally conceived dizygotic twins ever develop without the embryogenic differences between singletons and monozygotic twins (Boklage 2009).

Every publication that ever said that dizygotic twins come from double ovulation either just says it as if it-is-a-fact-and-everyone-knows-it, OR provides a reference to another writing as authority for the statement. That reference in its turn either just says it as if it-is-a-fact-and-everyone-knows-it, OR provides a reference to another writing as authority for the statement. Follow each and every such chain as far as you can, you will find no one offering any evidence. There is no evidence anywhere that any spontaneous human twin pair ever came from two egg cells. Once upon a long time ago, someone said it was so, and someone else heard that and thought it made sense and he wrote it down. Someone else read it and then wrote 'it has been written that it is so'. And someone else read that and wrote "it is well and widely known that it is so" and someone else read that and wrote "it is common knowledge that it is so" and so it ever since has been.

17. How can embryogenesis generate two embryos from a single 'egg cell'?

We are left with the question of HOW a single secondary oöcyte can serve as substrate for the embryogenesis of twins with two sibling genomes in the same embryo, differing in both the maternal and paternal contributions.

The paternal part of the story is the simpler part: the orthodox story of 'normal' human embryogenesis includes a very rapid calcium-mediated change in the zona pellucida and the egg-cell cortex after penetration by a sperm cell. This change in the boundaries of the oöcyte is called the '*polyspermy block*'. It is supposed to prevent the entry of a second sperm. One thing we know for certain about it is that it does not work perfectly, maybe not even very well. We know that dispermy is common, because diandric triploidy is common. A triploid embryo has three copies of all chromosomes instead of the normal two; there are three parental half-genomes grown from three pronuclei entering zygosis. The great majority of triploid embryos fail to complete embryogenesis. Most triploids ever seen are seen as spontaneous abortions, of which they constitute one of the largest fractions. Very few are born alive, only to die within at most a few days.

There is disagreement, more apparent than real, in the literature as to the relative frequency of diandric (with two paternal pronuclear contributions) and digynic (with two maternal pronuclear contributions) triploids. There is, however, at least as much variability among the samplings reported as there is in the results. The available data must be reconsidered, the sooner the better, with larger samples to make it possible to fractionate the results by

time to failure. Normal diploid embryos grow more slowly with a paternally-imprinted X chromosome (reviewed in Boklage, 2005). There are at least these five possible configurations [xxX xxY xXX xXY xYY] where x represents a maternal X-chromosome, X a paternal X, and Y is Y. There are three configurations with paternally imprinted X-chromosomes, one of which is digynic (xxX) and two diandric (xXX and xXY). The xYY is by far the rarest among triploid spontaneous abortuses, and presumably therefore the least likely to complete embryogenesis. xxX and xXY have one paternally imprinted X; xXX has two of them. We have no reasonable way to examine the progress of embryogenesis to learn about the relative longevity of these various triploid configurations, except by extrapolation and inference from a more thorough study of a larger sample of triploid abortuses.

At any rate, it is clear that dispermy is not rare, nor is the presence of two maternal pronuclei rare, among failures of embryogenesis. Most discussions of possibilities like these have been based on believing that the most likely source of two maternal pronuclei is the failure to sequester the second polar body after the second meiotic division of the oöcyte, which is believed normally to be triggered by, and occur after, penetration of the secondary oöcyte by the sperm. However, several of the papers on the subject suggest that errors of the first oöcyte meiosis are the major source. This may result from the error of believing that the output of Meiosis II is a pair of 'identical' sister chromatids, and that only Meiosis I errors would bring different maternal contributions into a tripronuclear zygote. We all believed that once upon a time, and we thought wrong and did things wrong because of it for a long time. That thinking ignores the effects of recombination … we still expect the centromeres segregating in Meiosis II to be sister centromeres, but the arms of those chromosomes have undergone at least one obligate recombination per arm, and the changes from those rearrangements segregate in the second meiotic division.

18. Conclusions

In every way that we have been able to sample the effects of twin embryogenesis on development, we find monozygotic and dizygotic twins to be equivalent in their clear differences from singletons. The embryogenesis of dizygotic twins is subject to each and all of the anomalies long attributed entirely to monozygotic twin embryogenesis. The twinning mechanism in the embryogenesis of dizygotic twins is the same as that of monozygotic twins, and subtly different from two simultaneous occurrences of singleton embryogenesis (even anencephaly does not represent a very large deviation from the normal developmental protocols – if the product of embryogenesis is not round, at least the basics of the plan has been executed). This is not compatible with the idea of dizygotic twins arising from independent double ovulation. The developmental histories of dizygotic co-twins are not independent and they are not like those of singletons. Whatever happens in embryogenesis to generate dizygotic twins is the same thing that happens to generate monozygotic twins. One embryogenesis becomes two; two body symmetry plans, two sets of axes, are differentiated from a single contiguous mass of cells.

Here it is customary and plausible to fall back on our understanding of these processes drawn from more accessible embryos. Gene products involved in generating the necessary changes in cell shapes and functions are known to serve the same or closely related functions in embryogeneses ranging from flies and worms and tadpoles to fish and birds and mammals. There are genes whose products are synthesized only in anterior cells where

head and brain will soon be formed. There are genes whose products are synthesized only in posterior cells that will become germ-line, sperm or egg, cells. There are genes whose products are synthesized to form gradients from anterior to posterior and from dorsal to ventral and vice-versa. For many of those genes, the amino acid sequence of that gene's product, and the DNA sequence encoding it, have been highly conserved across millions to hundreds of millions of years. A favorite of mine is a gene discovered in the fruit fly *Drosophila melanogaster* where it was named *eyeless* because those flies have what looks like burn scars the size of eyes where their eyes should be. The homologous gene from mouse, Pax6, can be patched into transgenic flies whose gametes can then give rise to flies with perfectly good eyes – proper compound insect eyes; not single-chambered ('simple') mouse eyes. Several disorders of eye development in humans are known to be caused by mutations in the homologous human gene, PAX6. Of course, it takes more than one gene product to build an eye, whether the compound insect eye or the single-chambered eyes of mammals and mollusks. But every animal eye the development of which has been properly examined has a close homolog of *eyeless* that it needs to generate a normal functional outcome.

There are many other genes and multigene families involved in embryogenic differentiation, under multiple layers of control. Developmental variations in gene expression to define the myriad cell types of the embryonic and fetal body are combinatorial, rising in number exponentially with the number of genes involved. Potential variation is in fact effectively infinite, because those innumerable combinations of genes are not just binary, on-or-off, but tunable to generate varying amounts of each of their products.

As another layer of control, a great many of the products of developmental genes can be made in multiple forms. At least many, and probably most, of those DNA sequences include multiple *exons* – DNA sequences that can be expressed as amino acid sequences in protein products, alternating with always-one-less-number of *introns* – intervening sequences that will be removed by splicing and will not become part of the messenger RNA to code for the protein product of that gene. The *primary transcript* of RNA copied directly off the DNA sequence of the gene needs to splice out the introns and join the exons to become the mature messenger RNA to be transcribed into protein. In many, maybe most, cases, alternative splicing can make a messenger RNA from any combination of those exons. Ten is not at all an unusually high number of exons for a given gene, and ten exons can yield over 1,000 different versions of that gene product [ten independent binary choices, $(\text{in-or-out})^{10} = 1{,}024$] depending on which exons are included in a given messenger RNA. Instances are also known in which messenger RNAs include exons from more than one gene's primary transcripts.

Remember, furthermore, that all of these developmental variations must occur in the proper place in the embryo and at the proper time. Most of the coding / gene expression changes are most likely to occur in the process of cell divisions, when the chromatin packing of the chromosomes must change with condensation and re-expansion. The DNA is asymmetric in the chemical differences between its strands and in the chromatin changes associated with each round of DNA duplication. The leading and lagging strands for replication are different in their sequence composition and alignment. Each comes through a replication event with an old copy that just served as template, and a new copy. Modifications, such as methylation and demethylation, of DNA bases are realigned in each replication.

DNA in the cell is never naked, and seldom even available for the base-sequence-specific replication or transcription of any given sequence. The DNA is covered in RNA molecules

and histones and non-histone proteins, in a constantly moving and changing multidimensional structure. Genes that need to be transcribed in any given cell at any given time must be made available for the RNA polymerase complexes, to reach their promoters, under the influences of their enhancers, silencers, insulators and perhaps other elements of transcriptional control remaining to be discovered.

When replication is due, the entire sequence will become available to multiple replication complexes, no more than a few hundred bases at a time. The five major types of histone molecules that form the bulk of the chromatin structure are subject to modification at multiple amino acid side chains, each with a different effect on the degree to which that region of chromatin sequesters its DNA. Some of the lysines can be acetylated, some of the lysines and some of the arginines can be methylated, some serines and threonines can be phosphorylated, and some lysines can be ubiquitinylated or sumoylated. Some of the modifications occur in the core regions of the histone complexes and some in the amino-terminal tails of histone molecules. Some change the strength of the electrostatic binding between the basic histone proteins and the nucleic acid. Some change the available density of binding sites on the chromatin for non-histone proteins that participate in other ways in the regulation and variation of histone binding. This is the heart of *epigenetic developmental regulation*, where environmental effects ranging from air pollution to mother's moods can reach into embryogenesis and fetal development, where cells can be reprogrammed to reflect an acquired addiction, where proper regulatory control of cell division and differentiation can be lost to cancer.

The structure of every cell's chromatin in fact **must** change over **the whole genome** with every replication of the DNA. Every cell division requires the entire chromatin package to be rearranged, to move aside at least enough to allow DNA replication. Every cell division requires the entire chromatin package to be rebuilt to accommodate the two new DNA strands, each made of one old template strand and one newly replicated strand – the old leading strand is now partnered with a new lagging strand and vice-versa. For all these reasons, by all these means, every cell division is asymmetric in several dimensions and is therefore a perfect place to execute programmatic changes to the combinatorial genetic and epigenetic switches that constitute cellular differentiation.

Caenorhabditis elegans is a nematode worm that is usually about one millimeter long in its adult form. It has 671 cells in its newly hatched larva at the completion of embryogenesis, some of which die, some of which continue to divide, to reach 959 cells in its adult body. Each one is in the exact place it is in by virtue of an invariant developmental history of asymmetric cell divisions – up here, right next, then tailward, then left, now ventral, etc., as the case may be, most of which involve an asymmetric change of epigenetic program. Each adult cell's specialized definition is specified by its history of sequential asymmetric divisions. Many of the genes involved in those differentiations still have homologs with crucial functions in the embryogeneses of placental mammals, including the human. Sidney Brenner, Robert Horvitz and John Sulston won the 2002 Nobel Prize in Physiology or Medicine for explaining this (*cf* Sulston *et al.,* 1983; Sulston, 2003). It took about a hundred years from the start of that project until it reached its present approximation of being finished.

There are millions of cells in the muscles and nerves, skin and bone of my left hand that have to perform exactly the same functions as corresponding millions of cells in my right

hand, only in a different direction. Many of them can trace their developmental programs back to neural crest cells that came from opposite sides of the dorsal midline to the periphery to do their jobs, that came to their places in the dorsal midline from exactly opposite positions at the boundary of the neural and non-neural ectoderm, that came to those places at that boundary from a single cell proliferated from the tip of the primitive streak as daughter cells moving in opposite directions. It is reasonable to suppose that their fates have been mirrored throughout the trip.

Embryogenesis of the human is not as strict and precise as that of *C. elegans*. *C. elegans* is more toward the 'mosaic' embryogenesis end of the spectrum and the human more 'regulative'; the cells of *C. elegans* appear to be more, and human embryonic cells less, *determinate*. However, recent work shows more regulative embryonic behavior in *C. elegans* embryogenesis than we have been accustomed to believing. The human body with billions of times more cells than *C. elegans* has, is vastly more complex, but seeing the overlaps of functions to be served and gene products to serve those functions, we are left with little room to doubt that the systems of processes are homologous.

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[1] "The reader will easily understand that the word "twins" is a vague expression, which covers two very dissimilar events - the one corresponding to the progeny of animals that usually bear more than one at a birth, each of the progeny being derived from a separate ovum, while the other event is due to the development of two germinal spots in the same ovum. In the latter case they are enveloped in the same membrane, and all such twins are found invariably to be of the same sex."

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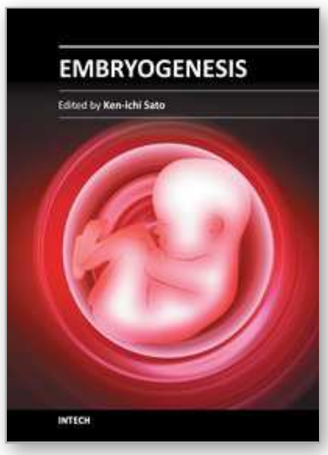
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