Session 1: Early Embryonic Development: An Up-to-Date Account

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DR. OPITZ: Thank you, Dr. Kass, ladies and gentlemen, Members of the Council, members of the audience. I feel very privileged and happy to be here and to share with you some data, facts, experiences and insights that I have gathered over a half a century of working in the field of development. And if you think that I don't quite look as old as all that, I'd like to just recall from a personal perspective that when I was a 15-year-old immigrant, newly arrived in Iowa City, the music department where my uncle was a Professor of Cello and Chamber Music was right next to the Zoology Department and my uncle's next door neighbor had been Professor Witschi, Emil Witschi, one of the great founding fathers of modern developmental biology, particularly endocrine development. So my second day in Iowa City, my uncle took me over to Dr. Witschi's department at the age of 15. I became immediately an animal caretaker, laboratory assistant and as of Day 1, I was introduced to human embryology.

The very first question that Professor Witschi asked me is well, John, and what is the biogenetic fundamental law? And I said well, I don't know, sir. And right then and there he told me about Haeckel's famous statement that ontogeny recapitulates phylogeny and not only that, he was a historian and his intellectual ancestry, in fact, went back to Johannes Mueller to the very beginning of the 19th century and he had many primary documents from all of those great men from Johannes Mueller to Haeckel to Virchow (?) and of course, to his teachers.

So I come from a tradition primarily of European, specifically German, morphology which accomplished a huge amount of work during the 19th century in just establishing the facts of embryogenesis, of thousands of different forms of life and today, I'll focus primarily on human development, but remember that I do so from an evolutionary perspective. And I will not hesitate anywhere along the line to use animal homologies if they serve to illustrate the point I am making.

I appreciate also the help of staff who were most kind this morning to help me come to grips with the technology of this presentation.

Shortly before I left Salt Lake City, this was one of the cartoons in the paper, so I represent the chap on the left, namely that I'll try to represent the facts as they are or I think they are.

Dr. Kass mentioned the word "mysterious" and while the process of development has lost much of its mystery, I never on a daily basis have lost my awe about the very process of development and the coming into being of living individuals from the moment of fertilization to the time of sexual maturity.

I'm guided by a few sentiments, namely the old one of Hippocrates, I think is still correct, namely that description is infinite and easy; an explanation is still limited and difficult, especially in the field of developmental biology.

Goethe's statement that "we see only what we know" applied to me most quintessentially when it was discovered recently that one of the syndromes that I described over 30 years ago, the so-called RSH or Smith-Lemli-Opitz syndrome, turned out to be a simple inborn error of metabolism involving the synthesis of cholesterol from its immediate proceedings, namely 7-dehydrocholesterol and for decades we had known that these babies, these fetuses have a low cholesterol level, but since cholesterol was demonized as something bad, we never gave the matter any thought that there might be a cause and effect relationship between the low cholesterol level and the child's developmental abnormalities and stunted growth and mental retardation. And then it turned out, in fact, this was a simple inborn error of metabolism, a defect in the synthesis of cholesterol. We again learned Goethe's aphorism there and we learned something extremely important about the

earliest stages of human development, namely, that cholesterol is not only desirable, but is absolutely necessary for normal development.

The last statement of Goethe there, it's difficult to translate, but what he implied when he formulated the concept of the signs of morphology in 1796 and in the early 19th century is that the study of form, both of embryos and of adults is at the same time an intent to understand its coming into being. Now we shouldn't put more into that statement than is actually there. Even though this was set at a time of Lamarck and Transformism, descent and of evolution were already very widespread and widely debated. All right, so much for philosophy.

Let me again try to begin by defining life and I think there exists a reasonable consensus amongst biologists on this definition, namely that life consists of all of the self-contained units of nature considered primarily of organic matter, autonomously and I stress the autonomously capable of undergoing development, reproduction and evolution. Note that this definition excludes the viruses because they're not autonomously capable of undergoing development and reproduction.

Now like all scholars, I suppose you've all got a stack of books for Christmas and the one that I got was by Christian De Duve, "Life Evolving," in which he gives the definition of life which I find highly tautological, but which he defends vigorously as being nontautological, namely that life is what is common to all living beings. However, on the same page, there is a sentiment there that I can subscribe to, namely, that there is only one life and it's certainly probably axiomatic that all living beings descend from a single ancestral form. And I will come back to this point again and again during this presentation.

Now then, we need a perhaps not an axiomatic, but an operational definition of development, namely that it is the biologic process that is the attainment of the mature form characteristic of a species. Or to paraphrase from one of my earlier definitions, the process which generates a sexually mature organism from a fertilized ovum. And the reason I put the first line in brackets is that in Goethe's original definition of morphology, he included Das Tierreich, and the mineral kingdom as well, and indeed, mineralogists and gemologists and crystallographers speak of the development of crystals, etcetera, etcetera, but strict to the term development refers only to these biological processes.

Now in all sexually reproducing organisms, reproduction occurs as part of a life cycle. And there is a continuum of life cycles that takes us back to the very first organism here on earth. Here, out of Scott Gilbert's wonderful textbook, the sixth or seventh edition which will be off the press in just a few days, that is the seventh edition, is the life cycle of a frog. Notice the adult on the extreme left, generating germ cells which lead them to fertilization. And in purple, the purple spot at the bottom of the egg in the two-cell stage, and in the eight-cell stage and so on, that is the germ plasm which was already identified by August Weismann, almost 110 years or so ago, which provides a continuity of germinal information and the separation of the germ line and the somatic cell line during development. Then at the very bottom at 6 o'clock, there is "birth" in the frog which is namely the hatching out of the gelatinous ovum, the formation of the tadpole and then the metamorphosis is the coming on land of the animal after it has sprouted legs and lost its gills, before it loses its tail and then becomes a sexually mature adult in the life cycle of one year.

Now let us be sure we understand the fundamental distinction between reproduction and sex. Reproduction refers to the propagation of the species, whether this is by fission or by budding or by runners as in plants whereas the term sex refers to propagation by mating and genetic recombination. That is the exchange of genetic material in a process called meiosis, an enormously complex later edition of evolution to our ways of reproducing and maintaining life during which the chromosome number, the genetic constitution is doubled before it is reduced to a haploid chromosome number.

Asexual reproduction results in clones and confers potential somatic immortality. So the clone of amoebas that you have in your petri dish on your laboratory bench goes back to the very beginnings of the common ancestor of the amoeba and of us. And it, of course, does not exclude extinction, but so long as these organisms are capable of continuing to reproduce, they have immortality. It was sex

which introduced death into multicellular life. At least to somatic death, but potential continuity of life and I say potential because it isn't guaranteed through the germ plasm.

Chris Wiley once put it very nicely in 1999 that the germ cells are the stem cells of the species.

Let me perhaps illustrate what I mean in an example which was also in Scott Gilbert's textbook. Let me go back to this slide here.

There's a wonderful organism that many of you studied also in high school biology called volvox. And volvox is one of the most instructive early forms of multicellular life. Basically what it is is it's a colony of chlamydomonas or dinoflagellates. They're banded together into this wonderful hollow sphere which actually acts together as a unit. It ordinarily has a half number of chromosomes. In other words, it is haploid and reproduces asexually. However, when towards the end of the season, things get dry in the pond and pond starts to freeze over, then suddenly very powerful sexual induction protein is produced, half of the organisms become male; half of them become female. The females undergo oogenesis. The males spermatogenesis. Sperm packets are released which float toward the female, release the sperm and then fertilization occurs to create a diploid organism. And at 3 o'clock on the right hand side of the slide, you see the zygotes, these diploid organisms which have a very, very tough shell and can survive the winter and then when the spring rains come, meiosis occurs, germination and reduction to the haploid state and a repetition of the asexual cycle. And once that happens, the former adult dies. So there's continuity through the germ line and death of the somatic cell line.

Now when we speak of the relationship between sex and death, we're referring to two phenomena here, namely the death of the parents which are mostly somatic events, namely all of those thing which will ultimately kill us through cancer or stroke or hypertension or diabetes and so on, and the death of embryos which are overwhelmingly germinal events. There's an occasional child or adult that dies over a germinal event like a teratocarcinoma or a gonadal blastoma or something like it, whereas most embryos, as I'll explain in a second, die due to germinal events.

There are now wonderful websites available to anybody who has a interest in human embryology and in fact, many of the patients that I see in the clinic have already consulted those.

Let me particularly point to the last one which is available right here in town, just not very far from here at the Armed Forces Institutes of Pathology. There's a National Museum of Health and Medicine, which now has the Carnegie Collection of human embryos and it was based on the Carnegie Collection that the staging of human embryos was based. And this staging is now universally used throughout the world and that website is available for free.

The first one, no, the second one, "From Conception to Birth, a Life Unfolds," by Alexander Tsiaras who incidentally is an artist and he has beautiful 3-dimensional, multi-colored images. The program is available from amazon.com for $30 as a CD ROM.

Four Stages of Human Development

All right, conventionally then, human development is subsumed under four stages. 1) **Pregenesis**, which are the events that occur in the parents and basically represent a consummation of events that occurred in the grandparents. Then there are two stages of embryogenesis called 2) **blastogenesis** and 3) **organogenesis** and then finally 4) **phenogenesis** are the events that occur during fetal life and post-natal life.

1) Pregenesis

Pregenesis, also called progenesis or proontogenesis or the German morphological term is [Vorentwicklung] is a complex process which subsumes the establishment of the germinal tract during parental ontogeny. The migration of the original primordial germ cells which do not arise in the gonads, to the gonadal ridges, ridge differentiation into ovaries and testes, and only then can the production begin of egg cells and sperm cells through the process called meiosis, recombination and germ cell formation. And it ends with fertilization, syngamy and karyogamy.

Here are diagrams which are directly readapted from my teacher/professor who actually did the work here in the Carnegie Institution on human embryos on the migration

of primordial germ cells. This is an illustration from Scott Gilbert's book, showing the events in the mouse, whereby you can see at the very caudal end of the embryo, on the right hand side of the embryo to your left where it says "alimentary", the red spots in the alimentary yolk sac, hindgut rudiments are the primordial germ cells. And they then undergo a very circuitous, complex route of migration from the hindgut into the gonadal ridges where they will then induce the development of gonads and ovaries. Very early, as these germ cells, as they migrate are alkaline phosphatase positive and you can see on the bottom left hand slide there, three germ cells alkaline phosphatase positive, that's the dark stain in the wall of the hindgut and on the right then you can see as they migrate into the gonadal ridge. And that phenomenon of germ cell formation and of migration into the gonads is an exceedingly ancient phenomenon and is present already is Drosophila.

Now, here's an important point. The specification of germ cells identity and the capability of germ cells to perpetuate themselves and to be totipotent, not just pluripotent, but totipotent, is conferred through a very complex cascade of molecular transcription factors, the most important of which up until recently was OCT-4, O-C-T-4, which is a nuclear transcription factor, stained here on the top left hand side, a bright orange-red. So here you can see the inner cell mass of the mouse embryo and that there are a few cells already in the inner cell mass that are probably destined to become primordial germ cells.

In the middle top panel, on the tail end of the embryo which is on the bottom of the slide here, with LacZ reporter gene construct, these cells which will become the primordial germ cells are stained a very dark brown. And then on the top right hand embryo, you can see the germ cells migrating, actively like amoebas from posterior to anterior into the gonadal ridges.

And on the bottom right hand side then is what the original oogonia looked like after they first established a varying differentiation and the nuclear still shows some OCT-4 staining and the spermatogonia on the left are beginning to lose it, but the oogonia will continue to show OCT-4 expression. And it is one of the reasons and causes of the many failures of stem cell transformation in vitro that if OCT-4 is not re-expressed, the construct will not have the potential to begin development from the beginning.

2) Blastogenesis

All right, let's rapidly go through blastogenesis. It is the process from the first cell division to the end of gastrulation. In humans (or in the human system) stem, that's day 1 through day 28. That is the first four weeks of development or the first half of embryogenesis, ages 1 through 13.

During the first week then there's stages 1 through 4; stage 1, fertilization; stage 2, the first cleavage division; stage 3, the free blastocyst in uterus and I'll illustrate this in a second; stage 4, the blastocyst's actions and begins implantation.

Now during stage 3, as the free blastocyst is in the uterus, still in its vitelline membrane, during increasing cell division, the volume of the zygote doesn't increase. And the reason why this is is because of a process of compaction, had been initially a very loose ball of cells. They then grow very tightly together and I'll illustrate this to you with scanning electron micrographic pictures, so that gap junctions can develop between these blastomeres and the cell's cell communication process so necessary for development can begin.

Now on the top picture here is the process of fertilization. In the middle picture you see a scanning electronmicrograph, the single sperm having just entered a mammalian ovum, but the most important item is on the bottom panel. There are those four images there show you the formation of the male pronucleus and the female pronucleus and their fusion in the middle or the middle left hand panel in what is the essence of fertilization is not the fusion of the germ cells because that need not necessarily lead to development, but the process of karyogamy. That is the fusion of the male and the female pronuclei so that the diploid number of chromosomes is reestablished, each pronucleus having a half number of chromosomes and only then can the spindle be set up for the first cell division in the beginning of development.

On the top then you see stages 1 through 5 and on the bottom, 10 cell embryo, human embryo with a zona pellucida removed on the

left before compaction and on the right, 10 cells beginning compaction. And you notice a tight, tight gap function between these two arrows on the right hand side and then in – as you can see, the two little images within the uterus, the blastocyst hatches. And it has to hatch before it can implant on the uterine wall.

Stage 5, the embryo is fully implanted. Stage 6, the primary villi appear and the primitive streak appears. Then here's some beautiful images from Bill Larson's third edition of his textbook on human embryology. Here, you can see the little blastocysts implanting. And in blue is the hypoblast and in yellow is the epiblast. So during the second week of development, the human embryo then develops two layers. During the first week, unilaminar; during the second week, bilaminar; during the third week, it is trilaminar.

There's further progress in implantation. And then on the bottom you can see the fully implanted human embryo with the amniotic cavity being formed on the left and the primary yolk sac being formed to the right. In blue is the epiblast on top and the hypoblast in yellow on the bottom.

Here's the formation of the primary yolk sac and of the extra embryonic mesoderm. Notice now the big, jelly-like space around the embryo. This is the extra embryonic mesoderm and as it cavitates on the bottom right hand panel there, that cavity will form the chorion cavity.

The primary yolk sac is then shed and a secondary yolk sac is formed. Again, from the hypoblast, so this is basically then an endodermal structure.

During the third week then we see the formation of the trilaminar embryo, the beginning of gastrulation and notochord formation. At stage 8, the primitive pit, the neural plate, neural groove forms; stage 9, formation of the caudal eminence, the first somites, the neuromeres and the primitive heart, too.

Now a recent human embryological work has shown that the dating of cardiogenesis, the formation of the heart which is in every embryology textbook is probably too late, that there is a beating heart tube present day 17 already. That's an important to remember. It's not completely formed heart yet, but there certainly is a beating heart to present. And here you can see the human embryo no more than 1.5 or 2 millimeters in size with the amnion cut open. You're looking upon the embryo from top. You can see the primitive pit, the primitive node, the primitive groove. In other words, the primitive streak. And already, two structures are evident, one in front and one behind, namely the buccopharyngeal membrane and the cloacal membrane which are two regions of the embryo where mesoderm never intercalates itself, between the future ectoderm and endoderm.

Notice also that this thing has a polarity and indeed the polarity, the future polarity of the embryo is probably established during the very earliest stages of cell division. There's a front end. There's a rear end, in other words an AP axis which automatically defines a right-left axis and there obviously is a dorsal side and a ventral side that is a backside, a topside and a belly side or an underside.

Now the quintessence of gastrulation is the establishment of the three germ layers and all metazomes that undergo this complex process of development undergo gastrulation. It's a sine qua non of mitosome development. And what you see here, on the top, left hand panel is at the primitive streak, subduction occurring of the epiblast into the primitive streak, down and underneath and initially replacing the hypoblast to become the definitive endoderm and in red forming the mesoderm which is between the former epiblast now, the ectoderm and the definite endoderm.

And on the bottom hand panel, the red arrows then show the migration of the mesoderm between the ectoderm and the endoderm and again, notice the two areas in front and in back where the mesoderm intercolates. The red arrow that points straight from the primitive node makes the prechordal plate.

Now it has been shown that even before or right at the very beginning of primitive streak formation, you can label the surface epiblast very carefully the four stratas with peroxidase or with dyes, or with oil droplets and so on and you can follow the fate of the various regions of the epiblast as it is subducted into the primitive streak. And you can identify the future head process, the notochord, the

endoderm, the mesoderm and the surface epiderm that will form the neural tube later on.

Now during the four then, the end of blastogenesis, the neural folds fuse and here you see an image of the neural folds fusing from the top on down. They fuse initially in the middle, in the middle of your back and then they sort of zipper towards the end, and towards the tail end and the bulges, the symmetrical bulges on either side of the neural tube are the so-called somites which are mesodermal condensations which later on help to give rise to the vertebrae.

At stage 11, the primordial germ cells migration in humans, the cranial neural pore closes, the buccopharyngeal membrane ruptures, the optic vesicles and pit are forming. At stage 12, the caudal, that is the tail end, pores close. There's a cystic diverticulum that is a bladder diverticulum, pancreatic bud. The urorectal septum is forming that will separate the anterior bladder from the posterial cloaca. The upper limb buds begin to appear and pharyngeal arches 3 and 4 and then this on page 99 are the final stages of blastogenesis at the end of which the embryo looks as it does on the right hand side.

You have the head and notice the change in shape of the embryo. Initially, early during the formation it's straight and then it becomes to curve into the C-shape curve. There are something like 28 somites present, four branchial arches, the heart is pumping, the nasal pit is obvious. The eyes you can begin to see the eyes, the otic vesicle and so in essence you have a reasonably fully formed embryo.

Now this image is better seen on the slide projector, if you may please. It did not copy very well into the computer. It's the frontispiece of my teacher's textbook on vertebrate embryology. This is a Carnegie embryo – it's not much better – which he serially sectioned and reconstructed. Now this is not 28 days, but rather 30 days. So it's a little bit later than the canonical end of blastogenesis and there you can see in detail all of the structures, including the primordial germ cells settled in the gonadal ridges in red. That red streak is the dorsal aorta. There's the heart. You can see the eyes. You can see the otic vesicle. So this is what the human embryo looks like shortly at the end of blastogenesis.

DR. ROWLEY: And the size?

DR. OPITZ: The size is no more than about 3 to 4 millimeters. It's minute still, but nevertheless, it is exceedingly complex already at that stage.

3) Organogenesys

Now if you could go back, please, to the – thank you. All right. Now the second of embryogenesis, I'm going to just summarize in this one slide. They are stages 14 through 23. The length then from about anywhere between 4, 5, 6 millimeters to 3 centimeters, that is 31 millimeters. It's the middle to the end of embryogenesis proper and the end of organogenesis, that is the end of the eighth week is what used to be called in classical morphology and I think it is still appropriate to do so, metamorphosis, namely the transition of life then from the embryo to the fetus.

And there is a good reason for doing so because this is the time when all marsupials are born, all kangaroos, opossums, etcetera, etcetera, at the end of embryogenesis and they make their way into the pouch and then continue their development on the outside.

And the organogenesis is then characterized by two important processes, namely the formation of organs and of histogenesis, that is the formation of cells and tissues. And it was recognized early during the 19th century already as Meiko once said in 1822, that's the das die form vor der Strunktur ensteht, namely, the form arises before structure, that is the growth form before the cellular specification. He said that even before the cell theory and before he had a microscope available. And the embryology textbooks towards the end of the 19th century made this canonical point.

Now on the right hand panel then, it's a double panel out of Carlson's textbook. On the top right hand, the small embryo there then is – what's is say there, 4 weeks? No, 8 weeks. So the small embryo in the top middle, 8 weeks. This is what the embryo looks like at the end of embryogenesis and thereafter you see the progressive changes during fetal life.

4) Phenogenesis

The term phenogenesis has a double use. It refers to the events during fetal life, namely from the 9th to the 38th week of gestation which is equivalent to the 40th week of pregnancy, right? And from the time the embryo is 3 centimeters to when the fetus is 50

centimeters and that growth in length, that 47 centimeter growth in length occurs mostly during the second trimester from a weight of 8 grams at 8 weeks to an average weight of 3,400 grams at birth and this weight is gained mostly during the third trimester.

So therefore, the period of phenogenesis is one of tremendous growth, progressive maturation towards post-natal adaptation and the attainment of all of those quantitative traits which constitute family resemblance and ethnic resemblance. So that's a very important difference.

Embryogenesis is about the attainment of qualitative differences, eyeballs, liver, kidneys, limbs and so on. And fetuses grow and the anthropometric characteristics change on a daily basis. They increase in length and weight and head circumference, etcetera, etcetera, etcetera.

And post-natal adaptation then involves not just the cardiovascular system, the closure of the ductus and of the endoventriculum and endoatrium communications, but continued growth and maturation to change body proportion from little toddler with a huge head and a small body to a more adult proportion as portrayed by Leonardo da Vinci and his Vitruvius cartoon and then finally pubertal changes and adulthood, pregnancy, gestation, parenthood, senescence and death which are normal stages of homogenesis. Remember that even in old age, the nose and the ears and other parts of our body continue to grow.

Developmental Defects

1) Defects of Pregenesis

Now let me summarize briefly with perhaps some oversimplification, the development defects of each of these stages of human development. By far, the most common defects of human development are defects of pregenesis and they mostly lead to the lethal chromosomal imbalances, mostly trisomies, also monosomies, monosomy X. In other words, Down's Syndrome, trisomy-21, trisomy-18, trisomy-13 and so on. These are defects of meiosis.

It is estimated by multiple sources and authors and has been for decades that at the very beginning of life, of human development, of conception, about 50 percent of all potential human beings have a chromosome abnormality, mostly a lethal chromosome abnormality. Chromosome abnormalities are the commonest cause of death in humans. They kill at the very minimum two-thirds of potential humans, more likely 80 to 90 percent and they mostly do so through these lethal aneuploidies.

Now during fertilization, some triploids arise, that is, individuals with a triple set of chromosome numbers and during the first cell division, some cases of tetraploidy with a quadruple number of chromosomes, 99 percent lethal disorders. And the only reason that a few 18-trisomy syndrome and 13-trisomy syndrome babies survive to birth is because of the phenomenon of confined placental mosaicism whereby the placenta, which is a smart organ, chunks out the extra chromosome, establishes a normal cell line and it is the normal cell line that supports the severely defected babies until birth so that they can be born. Down's Syndrome doesn't do that. Down's Syndrome is the only trisomy that does not involve confined placental mosaicism.

2) Defects of Blastogenesis

The defects of blastogenesis then, put parentheses around that, are the gross, mostly lethal, not necessarily, but mostly lethal malformations. And the multiple gross, lethal malformations, there was an entity that used to be called "associations." And I'll spell it in lower case letters, rather than with capital A.

3) Defects of Organogenesis

The defects of organogenesis then are the later, milder, usually single malformations such as a cleft palate, a cleft lip, an extra finger, a hypospadia.

4) Defects of Phenogenesis

The defects of phenogenesis are intrauterine growth retardation or for that matter overgrowth which is far less common than growth retardation and minor anomalies, namely those – to some extent objective, but also quantitative differences in multiple subtle facial structures which take away family resemblance and which make the parents wonder when they look at their baby, where did he come from? He doesn't look any one of us and that is a red flag. This is one of the most sensitive signs we have for the presence of a chromosomal abnormality because chromosome abnormalities produce multiple, multiple minor anomalies that take away family

resemblance so that the parents will then say well, he doesn't look like any one of us. And so on.

The ***defects of histogenesis***, I guess is important to mention. They cause dysplasias. All the moles and birthmarks and so on, but also some developmental tumors, teratomas and embryonal cancers.

Now could we see the next series of slides, please? They show up much better than – here then is a little Japanese Down's Syndrome – oh here. I can probably advance this. A little Japanese Down's Syndrome child. It used to be thought that the reason for the gravity of this condition is because they had such terrible series major malformations. As a matter of fact, most of the anomalies in Down's Syndrome are minor anomalies. And you can still recognize the ethnic origin of this child and you do sort of a minute point by point comparison between say Caucasian and black and Mongolian children with Down's Syndrome, they look more like each other than they do to their brothers and sisters.

In a bad year, this occurs 1 out of 750 deliveries. In a good year, 1 out of a 1,000 deliveries. It's a very common condition. It used to be the commonest cause of developmental disability that we saw in our university clinics. Now we don't see these patients any more because the pediatricians take care of these kids themselves.

Now here is a girl, one of the very first ones that David Smith and I studied with Turner's Syndrome. This is an aneuploidy in which the individual instead of having 46 XX chromosome constitutions, got a 45 X chromosome constitution. In other words, a sex chromosome is missing, neither an X nor a Y. This is one monozygotic twin girls and when I first arrived in Madison Irene Uchita and Walter Nams were able to demonstrate that in Turner Syndrome there is an increased incidence of monozygotic twinning. What they were not able to answer was the question which is the chicken and which is the egg here? Does the aneuploidy cause the twinning or does the twinning cause the aneuploidy and to this day I don't know the answer, but the association is unquestioned and I'll show you another striking example of that, namely that in these aneuploidy syndrome including Down's Syndrome and Kleinfelter's Syndrome, there's an increase incidence of monozygotic twinning.

It's temperamental. Thanks, Chuck.

So let me illustrate just a little bit more. Also to illustrate what the concept of Turner Syndrome means. These two little girls, you can see they're Mennonite girls out of the Lancaster County area, they were born at the same time. The one on the right is obviously a bit of a runt and was brought into the clinic because she is so short and I'll show you the growth curve of these two girls in a second. And the little one showed some signs of Turner Syndrome and when her chromosomes, she turned out to be a mosaic of two cell lines; one, a normal one, 46XX and a Turner Syndrome cell line 45X.

And they thought hm, are these two girls identical or are they not identical? They took blood from the normal, bigger sister also and she also had exactly the same mosaicism. She was 46XX, 45X. So are they mosaics or are they chimeras? Then a skin biopsy was done on the little girl. She was pure XO. And a skin biopsy from the big girl, she was pure XX.

So what happens is that at the moment of monozygotic twinning at the first cell division when these two blastomeres fell apart and formed – one formed one twin and one formed the other twin, a chromosome, an X chromosome or Y chromosome was probably an X chromosome, was lost out of the cell that made the small girl and the other cell line was normal. And due to placental vasculature connections in the single placenta in these individuals, they exchanged blood cell lines and they became chimeras. So they're not mosaics. They're chimeras. They're grafted, the XX, the XO girl grafted her XO cells into the XX girl and vice versa.

Next one, please, Chuck.

Here's the growth curve. You see the little one was way below the third percentile before she started to be treated with growth hormone and the growth curve of the normal girl was between 3rd and 50th percentile.

Next, please. And when the DNA analysis was done, ignore the two left hand lanes, those are control lanes, but the right, the third and the fourth lane where they used probes for chromosome 2, chromosome 17, chromosome 15 and chromosome 16, you can see these are

identical twins. There are several very important points to be made here about this case.

2) Defects of Blastogenesis

Now the defects of blastogenesis which is a common one, Dr. Rowley will recognize this as the handiwork of Dr. Edith Potter at the Chicago Line, the so-called Potter Syndrome or Potter Sequence due to absence of the kidneys. Absence of the kidneys means absence of amniotic fluid, hence these are cramped, contracted and this is the so-called Potter Sequence. So it's a very early defect of blastogenesis.

Otocephaly, already well-known by the early French teratologists of the 19th century where there's a defect of the mandibular arch, early defect of blastogenesis, an inviable defect.

Next, please. Sirenomeli, named after the mermaid, where there's a single, apparently fused or undivided lower limb, usually with severe genital-anal-renal abnormalities. As you can see in the two upper panels also, radius abnormalities. So multiple defects of blastogenesis or an association, a lethal disorder. Next, please.

Here's a defect that Dr. Oscar Borin, a German co-worker of mine and I have studied intensively, so called lumbosacral agenesis where portions of the spinal cord and the vertebral column are missing. In the one baby on the right, there were only four cervical vertebrae missing and most spinal cord below, and interestingly enough, even without a spinal cord, there was normal late development here. And if these kids don't die of pulmonary abnormalities because they lack pulmonary chest power, they have normal intelligence. Severe defect of blastogenesis. Next, please.

Anencephaly. Sometimes even with complete absence of the brain and the spinal cord, nevertheless, normal hand and upper limb and lower limb development, so no central nervous system is required for limb development.

Next please.

This fetus that Dr. Gilbert and I studied that came from St. Vincent's Hospital in Green Bay, sort of the epiphysis of everything that can go wrong during blastogenesis. The only normal parts of this baby are the upper limbs. And you can see that there is an anencephaly the entire face is cleft. You can see the right half and the left half of the nose. There was rudiment of an eye on the left. There were no normal vertebrae at all. There was a single umbilical vessel, renal abnormalities, diaphragmatic abnormalities and so on.

Nevertheless, this baby lived to 23 weeks of gestation. And when the parents came in for counseling and that picture on the chart and I've since then adopted this as a practice, on my desk, the mother picked up the picture of this baby and asked me is this my baby? And I said yes, that was your baby and she clutched the picture to her chest and mourned for three or four hours, tears running down, regardless of how malformed and sometimes unrecognizable these products of conception may be. They may be mourned as much as if a normal baby had been struck by a car or died of leukemia, sometime later.

Is that the last one, Chuck? The next one looks – yes, let's go on to this one here.

Now again, there's a very similar story. This is a quintessential twinning defect of monozygotic twinning again, but here the umbilical cords of the two twins are connected and so one becomes a parasite on the other one and the other one that is with the reverse perfusion, then begins to sort of rot away because of loss of blood supply and so called acephalus acardia anomaly which has been very well known since the beginning of the 19th century.

And so as they lose their head and they lose their heart because of the perfusion in the upper part of the body is lost, finally then they lose their toes and their limbs and so on and then ultimately they may just be left as a shapeless, formless, lump of tissue which nevertheless still at times at birth shows some signs of movement and again, the parents may mourn this as the loss of my baby. And so the question arises then, given that this baby and in this particular case this baby was born, still moving at 23 weeks of gestation, the parents preferred that even this inviable remnant of a fetus be baptized and be given a name and that some meaning be bestowed on its existence and its passing.

Next. Mine you, the co-twin was perfectly normal.

This is a photo that I took in the Virchow Museum in Berlin. This is conjoined twinning. When the events of twinning occur relatively late, notice their tubular columns here. And the co-twin, sitting on the shoulder of the normal twin was anencephalic. This is one of the few specimens that survived a direct hit on that museum during the end of the second World War. Virchow at one time had over 50,000 specimens and there are only a few hundred of them left.

Next, please. I would like you to see also the Hensel twins. Many of you may have seen – those are those fixed slides, Chuck. If we can't see the previous one, let's just stick with this one.

Did any of you see – there they are – the documentary about the Hensel twins on public television the other day? It was fairly recently. If I were you, I would go. This is a most dramatic kind of a story, that these two girls with a single body and two heads and dramatically different personalities, so different in fact, when they came home one day from school and the dad was sitting there absentmindedly reading the newspaper, one of the girls said, "Dad, we learned to swim today." And the dad said, "Well, which one of you jumped into the swimming pool first?" So you know, absentminded dad. Wonderful, wonderful little girls. Now the question is one soul, two souls. The Catholic Church in this hemisphere began to regulate or to address this question already during the 16th century by ordering that both be baptized. And in this particular case, one is Abigail, the other is Britney and their anatomical arrangement is as you can see here. They've got two heads.

Next one, please, Chuck. Thank you. There was an arm between their shoulders which was removed. You can see two sets of lungs, two hearts, a single liver, two guts down to the ileocecal valve and a single pelvis, single anus, single external genitalia, two legs and two arms. How these girls manage with two different will powers and minds to coordinate, let me say swimming or bicycle riding and so on is really a miracle for the neuroanatomists and neurophysiologists.

Next, please. All right. Why don't you put a little piece of paper over that and we'll come back to you.

If you could return to these – let me see. I need to go forward because I wanted to show you the scheme of twinning which I took out of Ronald O'Reilly's textbook. There's a wonderful scheme which summarizes twinning in – I can't see why I showed you these slides, these Kodachromes because they really did not import very well on this disk, this program at all. Sorry about that. But they take up a lot of memory, so it takes some time to advance slides.

The dizygotic twinning in this connection is relatively uninteresting. Monozygotic twinning has been very well studied for a long, long time. And one can make a correlation between the time when the twinning event occurred and the outcome, namely, whether there are two individuals, two amnions, two chorions and two placentas. This is an event most likely had occurred during the first or the second cell divisions when the blastocysts then parted and set up independent housekeeping.

The later – during blastogenesis that the twinning event occurs with the midline being developmentally highly unstable kind of a landmark. The greater is the likelihood that you'll end up with a set of conjoined twins. Now the greater is the likelihood that you'll end up then with a single chorion, finally with a single amnion and finally with a single placenta.

And the events can occur according to O'Reilly, not let me show you this figure, a similar kind of an experience. This is a fetus that I was privileged to study with colleagues from San Jose, Costa Rica.

It's gone again. In any event, the baby that I was trying to show you – yes – very good – was a hemibaby. It was a half a baby. It was the right half of a baby which I suppose in order to survive up until 23 weeks and a weight of 500 grams, formed into a donut like shape that you saw there, so this diagram which incidentally will be in your handouts, shows a hemibaby consisting of – why don't we just leave it at that, consisting of a half – there it is, of a half right baby.

And now the question there is half a soul or a whole soul? In any event, the mother went ahead and named the baby anyhow and the baby was buried. This is an exceeding rare anomaly of blastogenesis. I presume that this is

the defect of the earliest stages of development, but I really don't know because there is virtually no published precedent about this in the human literature.

All right, here's the diagram from Ronald O'Reilly and if you look at the bottom below the dotted line, those are the dizygotic twins. They're basically uninteresting. And starting in the most right hand column from top on down, that has been the twinning event occurs as late as 14 days. There's a time scale on top of the illustration there. And you end up with conjoined twins, as presumably happened in the Hensel twins.

And in the extreme left hand side then, if the two blastomeres fall apart and set up independent housekeeping as in one, two, three – the first panel on the right, then you can see, there are two independent – separate intercellular masses, separate amnion and separate chorion and separate placenta. If the fission occurs in the inner cell mass, let me say at Day 5, then within a single blastocyst cavity, you've got two embryos. You've got two amnions, but a single chorion and a single placenta. If it occurs as late as Day 8 or Day 9, then you've got – you end up with a set of monozygotic twins with a single amnion, a single chorion and a single placenta. So the membrane and the placentation situation at the time of birth is – it gives us a good idea as to what occurred and when it occurred. So these then are genetically identical individuals and yet every mother can tell their monozygotic twins apart on the basis of small physical differences, personality quirks and differences, differences in voice and so on and so forth, showing that even though they are genetically identical all development is an epigenetic process that is continuously modified by an interplay between environment and genetic constitution.

All right, you've seen that baby. You've seen the Hensel twins. Now my time is over, Dr. Kass, right? And I don't know I should go on any further.

The reason I put in this – maybe a few more minutes. This ART business here because of a very interesting and I think rather dramatic new development which I again, by chance, picked up the mail as I was going to the airport, the last issue of the American Journal of Human Genetics had an astounding report in it by ART, the referred to assisted reproduction techniques, which is widely practiced, not just in the United States, but worldwide, affecting as it does 15 to 30 percent of all couples being infertile; 37 to 70 million worldwide, Now these are very gross estimates. In the United States in 1999, 1 out of 150 children were born, were conceived by ART and since Louise Brown in 1978, about one million kids worldwide have been born in some form of ART or another. And about 40 percent of all infertility, we deal with the male factor in fertility. And the practice in ART then involves procedures for the collection of eggs and sperm fertilization in vitro. That is in a petri dish in the laboratory and then the embryo transfer and then the question arises, do you transfer a single one or several? The probability of implantation being relatively low, so that people try to increase the probability of implantation by putting in three or four. Do you put in very early cleavage stages or do you put in a blastocyst and if you put in blastocyst, there is a substantially increased risk of monozygotic twinning thereafter.

Now the ART forms then and pardon this dreadful pun, but that's how the specialists themselves refer to it, is still the commonest is artificial insemination by donor. In vitro fertilization first practiced Bob Edwards and Patrick Steptoe in 1969 leading to the birth of Louise Brown in 1978. Then GIFT, that is gamete intra fallopian transfer, this is mostly the injection of sperm into the fallopian tube, allowing normal fertilization occur. Let's say if the tubes are closed or if there is a problem of sperm concentration. Then ZIFT refers to the zygote, interfallopian transfer, I'm sorry, that's a misspell – it should be IVF, in vitro fertilization, interfallopian transfer of a fertilized embryo and then this dreadful acronym ICSI, pronounced "icksy" refers to intracytoplasmic sperm injection and embryo transfer, a technique which arose in 1992 and already the need for it and the technology of it has way stripped our ability to understand the biology that's behind it. And the reason I mention this and then I'll sit down and shut up is because last year I saw a paper and I think it was in the *American Journal of Medical Genetics* that a child with the Angelman Syndrome had been born after intracytoplasmic sperm injection and that immediately aroused an alarm bell in my mind

because if there is a cause and effect relationship between this procedure and the child's condition, then you might make the prediction that not so much Angelman's Syndrome but the Wiedemann-Beckwith Syndrome would occur with increased frequency in children conceived in this manner.

Now let me try and explain what these conditions are. The Angelman Syndrome and the Wiedemann-Beckwith Syndrome are two clinically radically different looking conditions. The Angelman Syndrome involving acquired microcephaly, severe mental retardation, seizures, usually no speech development and a very characteristic kind of behavior. And what was discovered in these children is that there's either a deletion of the short arm of chromosome 15 or else an imprinting defect whereby in an attacked chromosome, the gene expression on a short arm of chromosome 15 was altered through abnormal imprinting, depending on from which parent the gamete came, whether it was paternal or maternal. And in fact, we now know that there are several regions in the human genome which are differentially imprinted, mostly turned off, hypermethylated or hypomethylated, depending on whether they come from the mother or through the father.

So the process of myosis then may confer an epigenetic modification of the genome by regulating gene dosage, especially during earlier stages of embryo development, whether this is the trophectoderm development or the innercell mass development by differential imprinting of genes.

The Angelman Syndrome and the Wiedemann-Beckwith Syndrome are complementary syndromes due to imprinting defects of exactly the same genes on the short arm of chromosome 15. And what people at the National Cancer Institute here across town and at the University of Washington-Seattle have found is that 5 percent of all babies conceived in this way have Veidemann-Baechler Syndrome. And so the LOS Syndrome, the large offspring syndrome, that's being described so many times in infants conceived in this manner, now finally has an explanation.

These are large because they have Wiedemann-Beckwith Syndrome. Wiedemann-Beckwith Syndrome babies are large and they have infantile embryonal carcinomas. So there's an increased incidence of all kinds of carcinomas and Dr. Rowley knows this a whole lot better than I do that hepatoblastomas, rhabdomyosarcomas, adrenal-cortical carcinomas, what else do you know, Janet? Those are some.

So in other words, it may – in spite of all the best intentions here, this may contribute then to childhood morbidity and mortality and to cancer, morbidity also. So our technology got a little bit ahead of our understanding exactly what goes on because during sperm injection the neural events that occur during fertilization in the sperm capacitation and the dissolving of the acrosome, the shedding of the midpiece, none of that occurs and the process then of forming a male pronucleus is dramatically radically altered and different than if you do it. Now I don't know about the other 95 percent of the kids since I don't see those very commonly, but at our university, for example, this is practiced and I will be at pains to call this article to the attention of colleagues and I'm not so sure how this can be prevented. The need, in any event, is enormous for this technology and many, many clinics who practice this throughout the world as a matter of fact, without really being fully aware of the consequences that this might engender.

Let me perhaps stop here so that you have time for discussion and for questions and then we would perhaps carry on later on.

Thank you very much.

Question and Answer Session

CHAIRMAN KASS: Thank you very, very much, Dr. Opitz for a wonderful presentation. We do have at least 15 minutes at this point to run a little over, but Dr. Gómez-Lobo, please.

DR. GÓMEZ-LOBO: This is a question out of ignorance, of course. When does the developing human organism acquire its genetic material? What I'm trying to get at is this, is there any genetic material coming into the embryo after syngamy.

DR. OPITZ: After karyogamy.

DR. GÓMEZ-LOBO: After karyogamy. My second question, if I may has to do with karyogamy. Do you have something like in fanciful, numerical estimate of the possible combinations in karyogamy?

DR. OPITZ: The answer to your first question is no. Although the maternal genetic

contribution which comes through the cytoplasm of the ovum is variable because of the mitochondrial DNA. So the DNA content is not the same in every zygote. It can vary dramatically and considerably depending on what the mother contributes by way of mitochondrial DNA in its cytoplasm.

With respect to your second question, you can combinatorily account for variations in every one of the 23 pairs of chromosomes. So the number of combinatorial permutations that you can get out of a fertilization is astronomical, especially if there has been exchange of genetic material, but in homologues, so that in fact, except for monozygotic twins, the probability of having two identical human beings, same parents, is much easier.

Did that answer your question?

CHAIRMAN KASS: Robby George.

PROF. GEORGE: Doctor, thank you for your wonderful presentation. Is an embryo of any mammalian species something distinct in kind or nature from developed members of the species in question or is the embryonic stage a stage in the development of a determinant member of the species?

DR. OPITZ: It's a stage. So there is, in other words, increasing potentiality, increasing valuation towards birth, towards full maturity, but in humans, remember that's a relatively arbitrary cut off point because 200 grams, 300, 400 gram babies may survive, born prematurely.

PROF. GEORGE: Is there any biological sense now, any biological sense in which an embryonic or fetal cow, let's say, is prebovine rather than bovine in nature?

DR. OPITZ: No, it's always bovine in nature from the conception. That was the point already emphasized by Von Baer in the 1820s, that even though the early embryonic stages may look remarkably similar, if you look closely enough from the very beginning we have the unique and distinctive development path whereby increasingly you can tell the development from one species to the next species and so on.

PROF. GEORGE: I was wondering how early in embryonic development in humans can we detect the production of an immunosuppressant that would prevent the rejection of the embryo by the mother?

DR. OPITZ: That question I don't know. I am not a biologist, but I do know that human gene expression occurs very early already in first or second cell division. So unique gene expressions occur very early during embryogenesis.

Now mind you, many of these gene expressions are generic because the generic body plan that may say of mammals and vertebrates, all is built exactly the same by using exactly the same molecular machinery so that you have initially the molecular expression patterns in very early zygotes and embryos is more phylum-like, you know, Class, Order, Family, you know genos-like and then later on as development proceeds, it becomes more, more and more specific to the species and then finally to the individual.

PROF. GEORGE: Thank you, Doctor.

CHAIRMAN KASS: Michael Sandel and then Janet Rowley.

PROF. SANDEL: Thank you. I have two questions about the rate of natural embryo loss in human beings. The first is what percent of fertilized eggs fail to implant or are otherwise lost? And the second question is is it the case that all of these lost embryos contain genetic defects that would have prevented their normal development and birth?

DR. OPITZ: The answer to your first question is that it is enormous. Estimates range all the way from 60 percent to 80 percent of the very earliest stages, cleavage stages, for example, that are lost.

PROF. SANDEL: Sixty to 80 percent?

DR. OPITZ: Sixty to 80 percent. And one of the objective ways of establishing the loss at least as of the moment of implantation, well, even earlier, let's say as of five days because the blastocyst begins to make a chorionic gonadotrophin and with extremely sensitive assay methods, you can detect the presence of gonadotrophins, let me say, first around Day 7. That's the beta of human chorionic gonadotrophin. And if you follow prospectively the cycles that has been done on quite a few occasions in the Permanente study in Hawaii and so on, a group of women, of nonfertility, who want to conceive and you detect the first sign of pregnancy there of

human chorionic gonadotrophin, about 60 percent of those pregnancies are lost.

It is independently corroborated by the fact that the monozygotic twin conception rate at the very beginning is much, much higher than the birth rate and then if you follow with amniocentesis, the presence of the two sacs in about 80 percent of cases,the second sac disappears, one of the sacs disappears.

CHAIRMAN KASS: The 60 percent then would be of those that have at least reached the 7 days so that you could trace the – so there might be even greater loss at the early cleavage stage, is that correct?

DR. OPITZ: That's correct. And the earlier the stage of loss, the greater the rate of aneuploidy. There exists sort of a standard, textbook formula whereby 60 percent of spontaneous abortions have a chromosome abnormality. Six percent of all stillbirths and 6/10ths percent of all live born children. Now the latter figure is probably closer to 1 percent if you include some growth variants. So that's sort of a rule of thumb.

In my own lab in Helena where I did all of the autopsies on all pregnancy losses for 18 years, the rate of chromosome abnormalities was a little bit higher.

PROF. SANDEL: So if we take the 7-day stage, it's 60 percent. The 80 percent is if you go back to the moment of fertilization. But if you take just starting at the 7 days, there's 60 percent rate of natural loss. And of those 60 percent that are lost from the 7-day stage, what percentage of those have abnormalities or defects such that they wouldn't otherwise be able to be born?

DR. OPITZ: I would say somewhere around 50 to 60 percent and mind you, many of these are empty sacs, tiny, tiny stunted little embryos, but when you culture the sacs you find a chromosome abnormality, even though the embryo has vanished already.

PROF. SANDEL: So of the 60 percent that are lost at the 7-day stage, 40 to 50 percent did not contain defects or abnormalities, could have been born?

DR. OPITZ: Right.

PROF. SANDEL: And become babies.

DR. OPITZ: Your point is well taken, which doesn't mean that the chromosome abnormality isn't there. There's a wonderful lady, Dagmar Kalousek at the University of British Columbia, who has studied this question very intensively and published on it and incidentally the question that you addressed is a reference to that in the bibliography which is in your handout. Of course, this presentation will be a handout in which I tediously enumerated all of those data that are being published until recently.

And Dagmar Kalousek has shown that even the low chromosomes are apparently normal for XX on structural abnormalities, they may be abnormal. The commonest chromosome abnormality in humans is chromosome trisomy-16 which you may detect at chlorionic villi sampling and then at amniocentesis, it's gone.

And what the embryo has done is it has chopped out the extra chromosome out of the somatic cells, but in the process it has a two-thirds probability of forming an isodisomic pair whereby both homologues either from a mother or from the father – they look perfectly normal, but there's the defect. And so it is even recommended that you do imprinting studies on every pair of chromosomes, even in those that are apparently normal and nowadays, with subtelomeric probes, we can even discover additional things because if the embryo is grossly abnormal, let's say it's a 10 millimeter embryo under the dissecting microscope, the changes are that it is a chromosome abnormality.

So the selection against chromosome abnormalities in humans before birth is enormous, it's over 90 percent. I would say probably even higher than that.

CHAIRMAN KASS: Janet Rowley?

DR. ROWLEY: Well, I think just to follow on with this before I ask my own questions, what has been learned by these kinds of studies is that nature is remarkably effective in identifying its mistakes and in disposing of those mistakes before they develop, they can't develop into a normal fetus, so that this is really the – I think, one of the lessons that we've learned from this.

PROF. SANDEL: Janet, could I just interrupt just to ask a point of clarification on this. Of the 60 percent that are lost from the 7-day stage, they're not all mistakes, are they? Some of them

are, but you were saying they're not all mistakes.

DR. OPITZ: They may appear normal, but almost by definition they can't be normal because they died. There must be some reason for it.

Now it could be placental. It need not be intrinsic, but remember, that a major portion of the placenta also is of fetal origin.

PROF. SANDEL: Sorry, Janet, go ahead.

DR. ROWLEY: No, I think that's – one of the things that you didn't touch on that's important, I think, is the relationship of aneuploidy with age and we've often focused on maternal age, but I think there's evidence in some of the chromosome abnormalities that paternal meiotic errors are also involved in this so that was my first question to ask you to amplify on that.

And the other issue that I wanted to bring out is the importance of environment and environmental influences on embryology and I realize in one sense you can say this is a lecture in itself, but we know, for example, that neural tube defects are very high. Women in poor areas that don't get folic acid, then a simple way to not take care of all of these, but to diminish the frequency of spina bifida and other things is just to make sure that the mothers get adequate nutrition.

In what we're learning about many of the defects that you illustrated here, I wonder how many of those might also be apparently of some consequence of environmental exposure.

DR. OPITZ: You raise three important points. I guess one of the major impetus for the development of prenatal diagnosis is this relationship that Dr. Rowley alluded to between maternal age and nondisjunction or the presence of chromosome abnormalities with women reproducing at the age of 45, having almost a 1 out of 7 chance of having a chromosomally abnormal baby. So there's a direct linear relationship between maternal age and the presence or a chromosome abnormality. It is also true as Dr. Rowley has pointed out, that there is some relationship with paternal age, mostly in the occurrence of new mutation, that is of gene changes, rather than of chromosome changes, but some of the Robertsonian, I think, translocations may also arise with increased paternal age.

The influence of the environment, it cannot be underestimated. Even though amnio, chorion, placenta, you know, buffer the fetus to some extent, nevertheless there's a very active circulation from the mother to the baby and it is very important that where we can prevent birth defects due to environmental causes, we do so and one of the most rational and most effective defects, not just for neural tube defects, but for other major defects of blastogenesis, including congenital heart defects, has been the introduction of the recommendation that every woman wanting to conceive take at least 4 milligram of folic acid per day in order to prevent these common defects.

Now in the state in which I worked for 18 years, Montana, by far the commonest environmental defect that we saw was the fetal alcohol syndrome which is 100 percent preventable. And on certain reservations and I will footnote that statement, we estimated that 60 to 70 percent of all kids born on the reservation had fetal alcohol syndrome, Rosebud was a particular example, but the Crowe Indian Reservation, the Northern Cheyenne Reservation, the Blackfeet Reservation were similar, but in our overall patient cohort that we were examining in Montana before my wife and I left, my wife being a particular expert in the fetal alcohol syndrome, 30 percent of them were non-native, you know, Caucasian individuals, smoking. And particularly the combination of smoking and drinking and it has a dramatic effect on the placenta. So interuterine growth stunting occurs very, very commonly and then there's a whole raft of medications that are known to cause birth defects and environmental abnormalities of the baby.

CHAIRMAN KASS: Could I ask – these are – they're partly biological questions, but with a certain quasi-philosophical edge to them, if you wouldn't mind. One has to do with the question of individuation which is one of the issues that comes up here and I ask how the phenomenon of twinning, how that enters into what one regards as the thing which gives rise to twins, whether one sees this really as something whose individuation is yet undetermined and only can be somehow guaranteed after the time of twinning is past, or whether one sees twinning as some kind of a response to some abnormal event in an

individual to which this is then somehow a reaction.

And second, I'm interested also in the question of wholeness and both as a biological fact, but also for what – for its bearing on things like blastomere biopsy when one removes, let's say, as many as two out of an eight cell stage and what the implications are for the residual organism. In Dreisch's famous experiments, as you know, one took half and yet one was still able to produce a whole and I'm puzzled about that here and if I might just add, I was very interested in the difference between the stage of the early embryo when the cells seemed to be in there, lacking the compaction and lacking the intracellular connections and wondered whether from an embryologist's point of view, that's somehow crucial as part of the answer to the question about whether you have a whole yet or whether you've got something other? I'm not sure the questions were stated as well as I would like, but I think you get the drift. I'm interested in the question of wholeness and the question of individuation as a biologist sees these.

DR. OPITZ: Well, I think every fertilized zygote has the potential of becoming monozygotic twin.

CHAIRMAN KASS: Everyone?

DR. OPITZ: Everyone. Just simply because of the phenomenon that the midline which is established very early, even has the phenomena of polarity before you can even see a midline at the primitive streak and so on, the midline is morphogenetically highly unstable and I once enumerated all of the biological attributes, you know, of the midline which would support this kind of a statement, with most of the products of monozygotic twinning, then dying. That is the co-twins.

And the surviving co-twin, having a very high incidence of additional midline anomalies, heart defects, vertebral abnormalities, etcetera, etcetera. So this would seem to be an intrinsic attribute of the midline.

Now your question then of wholeness is also well taken because the – it's beginning to be shown in mammalian embryogenesis that already at the time of the first cell division the axes for the polarity of the embryo are being set up. And that removing, let me say 2 out of 8 or 2 out of 10 blastomeres may perhaps disturb this, but interestingly enough, as in the Roux versus Dreisch experiment, some individuals have regulative development whereby they can heal and repair and start over again as if nothing had happened and then sort of redetermine the remaining eight blastomeres as if they were whole and others like in the sea urchin, for example, when you take those two away, then in fact, that part of the body is missing. And so which is regulative kind of development.

My preference is to look at instead of wholeness as integrity, developmental integrity and the individuation issue then developing an impact after the risk of twinning has past, after 14 days. But even if it did occur and you've got the Hensel twins, you have two individuals in one body. And even the half baby, you know, was a living human organism up until about 23 weeks and 500 grams and it even had a beating heart.

CHAIRMAN KASS: Thank you. Frank Fukuyama and then Bill and I think we will break.

PROF. FUKUYAMA: Are there any chromosomal abnormalities that don't show up until a much later stage of development, when the individual is an adult or by the time they get to an adult, do you pretty much know? I'm thinking of this, for example, just with IVF and some of the ART procedures where the – Louise Brown is still in her 20s. Is it possible that things will show up at later stages that we simply don't know about or is once you get past a certain age, you're pretty much home free?

DR. OPITZ: It's possible, Dr. Fukuyama, but unlikely. There have been some adults who started to reproduce and all of a sudden miscarriage after miscarriage after miscarriage and then you begin to investigate and you find that you've got a chromosome abnormality, usually in a mosaic form.

And usually if development is abnormal on account of a chromosomal imbalance of abnormality, you will see it at birth or shortly thereafter. Or let me say during infancy or childhood, if the individual then is a little bit slow, is not doing well in school and is then brought into the clinic for evaluation, so I think it is unlikely, but the point is well taken.

Let me maybe, if you don't mind rephrase the question, what chromosomal abnormality should we be alert to as a concept of these ART forms, and one of them is imprinting. It's an imprinting defect and where the chromosomes look perfectly normal, there's no deletion there, there's no X chromosome or missing chromosome, but the genes are not expressed properly at the right time and the right place because of faulty imprinting and that can persist into adult life, into later childhood and embryonal carcinoma.

CHAIRMAN KASS: Bill Hurlbut and then we'll take a break.

DR. HURLBUT: I want –

DR. ROWLEY: Can I just interrupt just because I think it's important to clarify that when, in the instance that Dr. Opitz gave of a woman having multiple miscarriages due a chromosome abnormality, there are things such as gonadal mosaicism where some of the oocytes developed in that woman as he's already shown, the gonads in her are developing during the – well, before the 24th week of gestation. Those abnormalities will only show up in the oocytes that she produces as an adult. Then there are other meiotic errors where a gamete is chromosomally abnormal, but that error in formation in that abnormal gamete probably occurred just in the cycle or just before the release of the oocyte. At least I think that's correct.

CHAIRMAN KASS: Bill Hurlbut, please.

DR. HURLBUT: I want to follow up on Leon's questions and try to make some sense of the moral meaning of these matters and if it's okay, I'd like to ask Professor Opitz' opinions on the moral matters. Is that within the –

CHAIRMAN KASS: I will reserve the right to cut you off, if it seems to break the rules.

DR. HURLBUT: Okay, I ask this in the spirit of really wanting to understand these issues myself and I guess what's in my mind is why don't all the cells at the various stages up through the blastocyst even until the formation of the primitive streak, why don't each of the individual cells go on to form a trajectory of distinct development? In other words, there must be something binding them as an integrated unit in the drive of the direction of the individual maturity. Let's start with that, is that –

DR. OPITZ: So what you're addressing is the question of determination, progressive determination during progressive differentiation. For the on-going developments, the more determined is the developmental fate of these tissues.

Now we used to say once you've got a brain made, those brain cells, you know, are terminally differentiated. When they die, they can't ever be replaced and there's no – etcetera. Now, of course, through the work of Irv Weissman and many others, we know that there are, in fact, even in the brain there are stem cells left over which can rejuvenate and can give rise to various kinds of cell lines in the brain, including the supporting cells, the astrocytes, the oligodendra sites and even neurons.

Now during the course of normal development, these matters are constrained, phylogenetically constrained into only very, very few specific outcomes. Now you can probably start over again taking certain pieces or certain cells as has been done, certainly successfully in a mouse and in other mammals and so on, but the further on in your development, the more determined is the outcome of the specific developmental process.

DR. HURLBUT: But you did say there is polarity, even as early as cell division.

DR. OPITZ: And it is potential polarity in the sense that if you don't disturb the system, you can recognize the meridians and the anterior, posterior, right and left and dorsal and ventral sides already during the earlier stages of cell division. If you disturb the system and take out, for example, those two blastocyst cells, the system can re-equilibrate itself and can reestablish communication between each other so that the remaining cells will say, all right, we'll start over again or we'll re-equilibrate the system.

And this phenomenon of developmental, how shall I say, equilibration or homeostasis was recognized very early during the 20th century and Waddington called this canalization or buffering and the earlier the stage of development, usually in mammals, the greater is this buffering capacity to repair, to heal and to reconstitute, also at the same time the greatest vulnerability then towards major disasters happening like twinning, for example.

DR. HURLBUT: So this restitution of the integrated process can take place sometimes in two trajectories of development, if the disturbance is great enough, but there is from the beginning a drive in the direction of a single maturity and it's only when it's disrupted that it becomes two? What I'm getting at here is you said that there are early cell divisions. There seems to be gene expression. Is there even differential gene expression fairly early like four to eight-cell stage and could we see that as the development of a single individual in which case some events may disturb it which become then two individuals, but – see what I'm getting at?

DR. OPITZ: Yes. I wouldn't say the development of a single individual. I would just say, gene expression pattern appropriate to the cell at that stage of development. And then after the establishment of the basic body plan, that is when you begin to see the establishment of the specification of cell lineages, specific cell lineages and one of the last few issues of *Science* had a wonderful article in it on the specification of the germ line, for example. And the interesting thing is that these – you're starting off with totipotent cells in the inner cell mass or a few of the inner cell mass cells. And they then become part of the somatic component of the posterior, the rear end of the embryo, the allantois, the yolk sac, the hind body and so on. And then they are respecified through the developmental context, environment in which they happened to be developing into germ cells.

And you can, in fact, take nowadays single cells and do microarray genetic analysis of the gene expression patterns of these single cells and this is how it was discovered that germ cells in mammals, that is in mouse, certainly are respecified as totipotent cells from the development of milieu in which they were and were influenced. And it's mostly interferon which does it and then suddenly the cells that have been respecified begin to express two proteins which are unique primordial germ cells.

DR. HURLBUT: One final question and Leon may veto this question. We've had to struggle here with this question of when there is intrinsic moral value in this developing entity and the criteria that had been used in various deliberations on this worldwide relate to primitive streak and so forth. Usually, the principles being some kind of differentiation which you seem to indicate is already taking place in its primordial forms very early, the issue of twinning, which you say is a fairly ambiguous issue, and then third, implantation. I didn't ask about implantation, but my assumption from my scientific understanding is that it's a difference of quantity, if you will, not quality. There's already growth factors influencing the developing embryo in the fallopian tube.

But I want to ask you your feeling about the moral meaning of this, is there some sense in which before say 14 days there is something of different moral meaning at 14 days?

DR. OPITZ: Bill, let me be – I don't mean to be a moral coward here, sidestep that issue, by not addressing my – or expressing my moral feelings about the subject which I think is slightly besides the point because I have a strong suspicion that everybody in this room has got their own moral feelings and opinions on the subject, but I do – the point I want to make very strongly is this, that there's a continuum in developmental potential to the very moment of conception.

As a matter of fact, there's a continuum even into the germ cells which ought to be treated with exactly the same respect as the fertilized ovum, as the implanting ovum, as the developing embryo, simply because germ cells, for example, are extraordinarily vulnerable to teratogens, viruses, x-radiation, chemicals, etcetera, etcetera, etcetera which in the long run, being damaged in any one of these wanton and random kind of race may harm humanity infinitely more than the loss of a trisomic baby.

CHAIRMAN KASS: Dr. Opitz, thank you very much for a lucid, illuminating and forthcoming presentation and response to the questions.

We've run over to take advantage of Dr. Opitz' presence and generosity. We're running probably 15 minutes behind.

Why don't we start at 5 after the hour. We'll steal 15 minutes from our long lunch. Let's take a break and then go into the next session.