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Of microbes, mice and man

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ABSTRACT This chapter reviews my 18 years of research in Anne's Unit including studies on temporal and spatial aspects of X-chromosome inactivation and imprinting and the role of methylation in X-inactivation in these processes during female mouse embryo development. To enable molecular studies of embryos, we developed a plethora of single cell assays for specific enzyme activity, gene mutation and methylation, and RNA transcription. While in Anne's Unit, I used these same single cell assays to pioneer the procedures for preimplantation diagnosis of genetic disease, now an established clinical approach to prevention of the birth of children with severe genetic disease. At the Institute of Child Health in London, we continue to develop new highly sensitive molecular procedures - currently for the creation of cDNA libraries from human preimplantation embryos, primordial germ cells and embryonal stem cells. We are using these cDNA preparations to isolate human developmental genes and embryo/cancer genes. One of the more fascinating aspects arising from my time in Anne's Unit is the way in which my research findings challenged a number of accepted dogmas in development concerned with the origin and totipotency of the germ line and the possibility of transgenerational genetic inheritance by epigenetic modification of the germ line.

Introduction

On the occasion of this book in honour of Anne McLaren, we are invited to make informal contributions of the "self-review" type, with reminiscences and speculations, past and future. So, as my title might indicate, I will use this opportunity, to take a nostalgic look at my progress through science during the18 years that I spent in Anne's Unit - the Medical Research Council Mammalian Development Unit at University College in London. I am eternally grateful to Anne for her support, guidance and inspiration over the years in so many different ways. Uppermost for me was her constant enthusiasm and attention (I don't think I ever saw Anne sleepy at a seminar or conference) and the encouragement I experienced from her unfailing positive energy. All of us in the Unit were also encouraged in our inventiveness skills. Anne ran the Unit on a "shoestring" (we were renowned for "value for money") and there was very little in the way of custom-made equipment. For a long time there was only one CO_2 incubator in the whole Unit and all of us were involved in embryo culture. I will never forget "doing the slow motion droplet walk" through three sets of heavy wooden swing doors with precious embryos in tiny wobbly droplets under oil. Others had to slow motion their droplets up and down the stairs! But Anne taught me that, by honing my own skills of dexterity and visual sense, I could observe more accurately than the most elaborate expensive machines and equipment. I learnt to work at the lowest magnification possible to 'leave room' for later more exacting work in the microenvironment of the embryo. My biochemical and molecular research was always carried out starting from the basics – no kits and short cuts – experimenting first on new ways of "micro-measurement", and then on the optimal conditions - magnesium concentration, pH, temperature and so on – with reconstruction experiments to know as well as one could know the validity of the experimental design. My work in Anne's Unit, and speculation arising from it, has challenged a few accepted paradigms, such as dogmas relating to the continuity and the origin of the germ line in mammals, the generally held belief that the beginning of development is the '*tabula-rasa*" state of the gametes, and the strongly held view that adaptive directed (Lamarckian) changes in transgenerational genetic inheritance do not occur.

Of microbes

Prior to joining Anne's Unit my research was on microbial genetics and on slime moulds. It was this early period of research, starting in 1959, that shaped and defined my later approach to the mouse embryo at the cellular and molecular level. One advantage of working with bacteria is that there is generally only one cell type (with exceptions such as spores), although that one cell type may behave in different ways in different situations. In slime moulds

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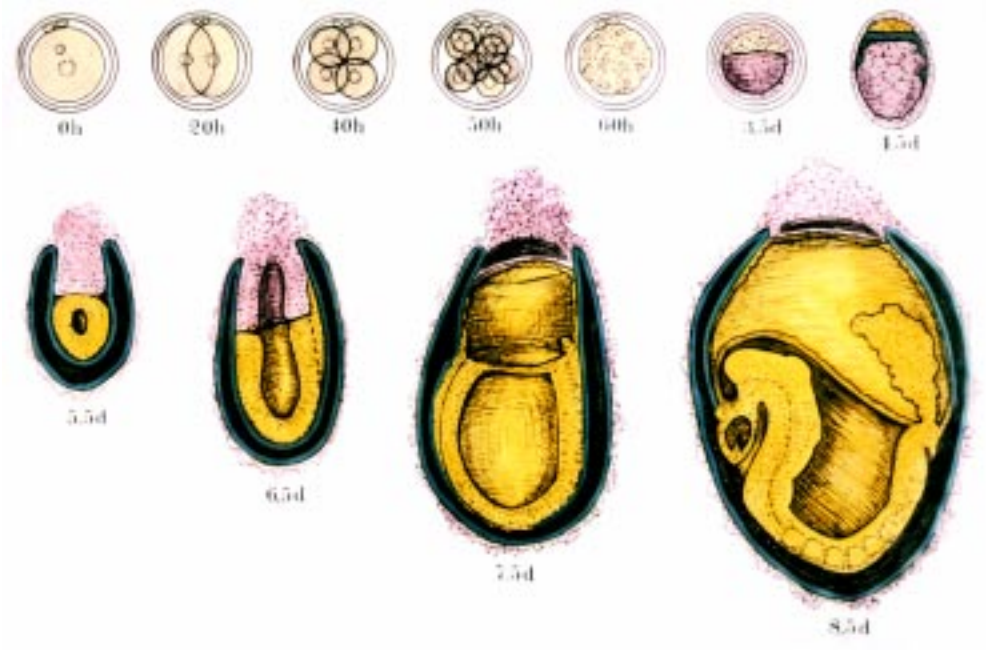


Fig. 1. Preimplantation and early postimplantation stages of mouse embryo development *(figure kindly given to me by Rosa Beddington).*

there are just three cell types and we can ask fairly simple developmental questions about how these three cell types are formed. Mammals are much more complicated with a body made up of about one hundred different cell types, all with the same complement of genes but with different sub-populations of genes active in each different cell type according to the requirements for its function. My research with simple microbial systems for over 15 years before I moved to Anne's Unit had already prepared me for the single cell approach to mouse embryo development. Slime mould aggregation is a beautiful system in which to study cell communication in space and time and cellular differentiation in development. My research in the early 70's had opened up new ways of analyzing the dynamics of the cells' response to cyclic AMP and establishing the parameters of aggregation. We were making exciting progress (Alcantara and Monk, 1974). However, as fate would have it, the Medical Research Council (MRC) decided to close the Microbial Genetics Unit in Edinburgh.

Of mice

I needed to find another unit that would have me and I knew that I wanted to continue with developmental studies. About this time, in the early 70's, I met Richard Gardner in Oxford. He was talking about his interspecies chimeras formed by aggregating rat and mouse embryos and transplanting the composite embryo into the uterus of a foster mother mouse, where it developed into a fetus composed of both rat and mouse cells. I was enormously impressed and very enthusiastic to know more about mouse embryology. In fact, it occurred to me much later that I had always wanted to be an embryologist. I remembered that as a child playing at "herding the chooks" (and other animals) in Australia, the idea that a complete fully formed individual could arise from a single fertilised egg was the most wondrous thing in the world. Harry Harris at the Galton Laboratory, University College London told me that Anne McLaren was just starting up a new unit there- the MRC Mammalian Development Unit. I remember phoning Anne in Edinburgh in 1974 to ask her if she would take on a microbial geneticist/slime mouldologist in her new unit in London. She didn't know me, nor much about my fields of research, but as typical of her open-mindedness and supportive attitude, she said yes to this seemingly unpromising new postdoc. So it was that I made this enormous leap from microbes to mice. I am indebted to Anne McLaren for taking me on in my hour of need and for her patience and faith in me in the early days when I was unable to kill a mouse and could not tell the males from females when it was my turn to probe for copulation plugs on the weekend rota. Anne seemed to know that I would eventually "make it", even if I didn't know it for quite some time.

My task in moving from microbes to mice was to "go molecular". Previous studies in mammalian embryology were concerned with the morphology of development, and the culture, metabolism and manipulation of embryos. My future work was to extend the molecular and cellular approaches I had used with the microbes to studies on gene mutation, expression and modification in the cells of the early mouse embryo. The aim was to attempt to understand the molecular basis of the unfolding genetic programme of development.

When I moved into the field of mouse embryology in Anne McLaren's Unit in early 1975, there was very little information on the molecular aspects of early development. This was due to the fact that the mouse embryo consists of so few cells. The fertilised egg is just a single cell, about 100 microns in diameter, which at the start of development cleaves into 2 cells, 4 cells and so on, to reach about 100 to 200 cells in the blastocyst at the time of implantation. These preimplantation stages of pregnancy are free-floating as the embryo passes down the oviduct and into the uterus. Then the embryo implants in the uterus and starts to form the different cell lineages and to shape up into the fetus (Fig. 1, kindly given to me by Rosa Beddington).

Back in the 70's, most biochemical or molecular studies required millions of cells. We used to call it "bucket biochemistry". Working with bacteria or slime moulds, it was possible to obtain millions of cells in just a few hours. So in moving from molecular studies with bacteria or amoebae to early mouse embryos, I was immediately faced with this overwhelming problem. So few cells! Instead of giving up, I made one of those critical turning point decisions in life. I decided not to work with the embryos in the first instance but to direct all my attention towards the molecular techniques - to make the biochemical and DNA techniques far more sensitive. In fact, they needed to be at least a million times more sensitive! I set about inventing new ways to measure chemical reactions in tiny volumes, assaying small samples in microcuvettes balanced on corks in the spectrophotometer, scaling gels down to a few microlitres in a 2ul microcap (e.g., Monk and Petzoldt, 1977), using very hot radioactive reagents, and refining methods of analysis of DNA modification and gene expression to

the sensitivity of a single cell. From this time on, the refinement of different specific molecular micro-techniques sensitive to the single cell, and the application of these techniques to research into early development, became the hallmarks of my career. The aim always was to make the techniques so sensitive that I could obtain information from just one single embryo or even one single cell from an embryo. By inventing these procedures, the number of mice needed for molecular research was hugely reduced and, to my mind, now justifiable.

My specific studies in Anne's Unit, and now in my own Molecular Embryology Unit at the Institute of Child Health, have been concerned with the study of gene expression and its regulation in early embryonic development. We have used as powerful model systems the phenomena of X-chromosome inactivation, and imprinting, in development, and the role of DNA methylation in controlling these processes. I used the same single cell procedures, perfected over the years to study these phenomena, to pioneer the procedures of preimplantation diagnosis of genetic disease in the 80's. These areas of my work in Anne's Unit are described in more detail below.

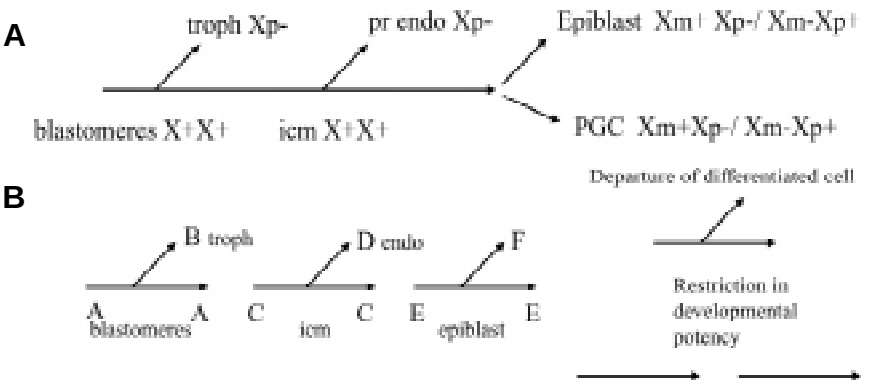


Fig. 2. Stem line models of X-inactivation in development. (A) *A stem line model of X-inactivation in development.* **(B)** *A stem line model of development showing two types of differentiation event.*

X-chromosome inactivation in development

Having decided to work on the microtechniques to a sensitivity of a single cell, I began to look for sensitive techniques already in the literature, even for something that went down to a 1000 cells in the first instance. I found an assay for an enzyme, hypoxanthine phosphoribosyl transferase (HPRT), that had been used by Eileen Adamson, that was sensitive to about ten thousand cells. Then, a happy accident (which could be called incompetence) led to a single-cell-sensitive assay for HPRT. The assay uses a radioactive substrate and due to my forgetting that adjustment of specific activities of label required addition of unlabelled substrate, I used neat tritiated hypoxanthine at the required substrate concentration. The Packard scintillation counter produced masses of counts for a single preimplantation embryo.

It was a memorable occasion when Anne and I looked at my first set of results in 1976. I remember exactly my small laboratory in the UC Galton laboratory, the weather and the view out the window, and the excitement and gratitude one feels with the realization of a gift of a breakthrough that one had not even dreamt about. The assay was so sensitive I could easily monitor this enzyme in early embryos and even in a single cell from an embryo and my research in the field of molecular embryology had begun. Again, fortuitously, it happened that this gene was on the X chromosome and the X chromosome had been of great interest to Anne for some time. I extended the highly sensitive single assay for HPRT to a neat double microassay system (similar to one I had used to isolate DNA replication deficient mutants in the bacteria) in which a related autosomal enzyme (adenine phosphoribosyl transferase, APRT) could be measured at the same time to serve as an internal control and to standardize the results. This assay could be used to determine X-linked gene dosage and thus the number of X-chromosomes active within an embryo, or a single cell of an embryo. We also used this assay to carry out the first experiments to show that it was possible to conditionally regulate specific gene expression in mouse preimplantation embryos by induced transcription of antisense DNA (Ao *et al.,* 1988). In the subsequent few years, I developed microassays for other genes on the X-chromosome, most notably an assay for different forms of an enzyme called phosphoglycerate kinase (PGK) using a home made machine invented by Professor Theodore Bucher from Munich. Now, we could monitor which X chromosome was being expressed in the female in different tissues at different times, and even in a single cell. This PGK assay became one of the foundation assays for the future work in the Mammalian Development Unit on germ cells, sex differentiation and sex chromosome influences on growth and development.

X-chromosome inactivation in mammals is the mechanism of dosage compensation in which one of the X-chromosomes in the female is inactivated. This means that females are equivalent to males with respect to dosage of genes on the X-chromosomes. Random inactivation of either the maternally-inherited or the paternally-inherited X chromosome means that all females are genetic mosaics. This X-inactivation mosaicism is readily visible in females heterozygous for a gene on the two X chromosomes with observable phenotypic effects – for example the tortoiseshell cat shown in Fig. 3A. But whether you can see it or not, all females are X-chromosome-inactivation mosaics.

Using the single-cell-sensitive assays for HPRT/APRT and PGK, we were able to demonstrate the picture of changes in X-chromosome activity shown in Figs. 2 and 3. Two X chromosomes are active during the formation of the egg, whereas in the sperm the single X chromosome is inactive and sequestered away from the events of meiosis in the sex vesicle. After fertilisation, two X chromosomes are active in the blastomeres of the cleaving embryos. Then one X chromosome (the paternal X, Xp) is inactivated in the trophectoderm and then in the primary endoderm as these lineages differentiate from the pluripotent embryonic cells and, finally, random X-inactivation occurs in the fetal precursor cells (what Mike Snow in Anne's Unit termed the epiblast) of the egg cylinder. This indicated a link between X-inactivation and differentiation (Monk & Harper, 1979; see also Monk, 1981; Takagi *et al.,* 1982; Tam *et al.,* 1994). At this time I depicted development in terms of a stem line model (Fig. 2 A,B, Monk, 1981). The stem line model distinguishes two types of differentiation event in development (Fig. 2B), the departure of differentiated cells type (e.g., cells B depart from cells A), and a restriction in developmental totipotency occurring in all the cells of the stem line (e.g., cells A to cells C), so that they now produce a new differentiated lineage, as

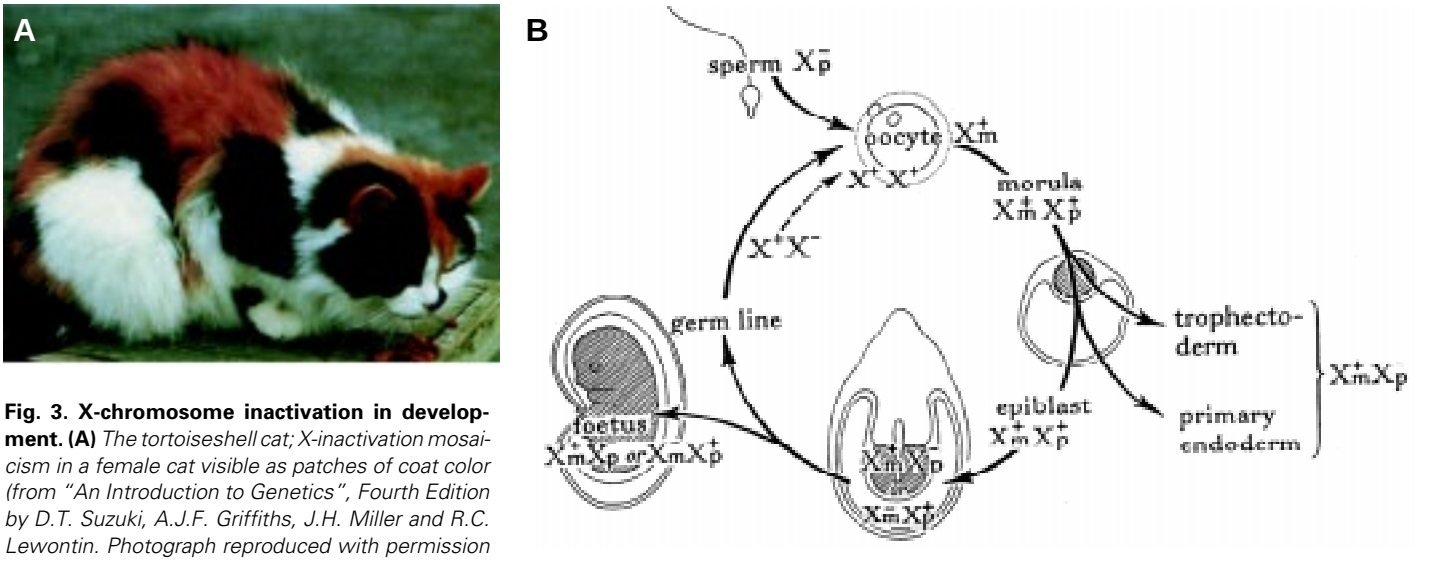


Fig. 3. X-chromosome inactivation in development. (A) *The tortoiseshell cat; X-inactivation mosaicism in a female cat visible as patches of coat color (from "An Introduction to Genetics", Fourth Edition by D.T. Suzuki, A.J.F. Griffiths, J.H. Miller and R.C. Lewontin. Photograph reproduced with permission W. H. Freeman and Company.* **(B)** *Cycle of changes in X chromosome activity in female mouse embryonic development (reproduced from Monk (1992) with kind permission from Kluwer Academic Publishers).*

development progresses.

What about the germ line? Since there was some expectation that the germ line would be continuously totipotent, it was rather a surprise when Anne and I showed that X-inactivation did occur in the female germ line. The inactive X chromosome was re-activated again at around the time of onset of meiosis (Monk and McLaren, 1981), although the process of meiosis itself was not required for this re-activation (McLaren and Monk, 1981; 1982). Clearly, the female germ line has lost its developmental totipotency when one whole chromosome is inactive. Since Mary Harper and I had found that X-inactivation was linked to cell differentiation (Monk and Harper, 1979), it seemed reasonable to argue that, conversely, X-reactivation may be linked to a de-differentiation event occurring in the germ line (Monk, 1981; see later for mechanisms). This prompted me to join the circle and change my stem line diagram into the circle of development and X-inactivation (Monk, 1981; Fig. 3B). It also started me on the line of thinking that the changes in X-chromosome activity were telling me something much more important and significant about the underlying developmental events, e.g., it is the time of the onset of meiosis when the germ line must be at its totipotent ground state (see later).

At this time I wanted to know whether there might be a gradation of non-random paternal X-chromosome inactivation as different tissues delineated from the "stem line" of development (Fig. 2A). For example, would the mesoderm show a greater proportion of cells with the paternal X chromosome inactivated than the primary endoderm, and the endoderm with more cells with paternal X inactivated than the neural ectoderm? To answer this question, Andy McMahon, Mandy Fosten and I set out to observe the X-inactivation mosaicism in these different tissues in a 10.5-day mouse fetus. We also decided to include the germ cells, as I thought at this time that if the germ line were to remain totipotent, it should be delineated very early (and then necessarily from very few cells) and therefore might inactivate the paternal X chromosome too. The result of this study showed that the heart mesoderm, liver endoderm and brain ectoderm showed correlated mosaicism (the same proportions of cells with the paternal X active and with the maternal X active). This meant that random X-inactivation occurs in a common pool of cells, which must undergo considerable mixing, before delineation of these tissues (McMahon *et al.,* 1983).

However, a very surprising result came out of this work. The mosaicism in the germ line was also correlated with that in the somatic tissues. Statistical analysis showed that this meant that the germ line must normally arise from a sizeable pool of cells and therefore relatively late in development (McMahon *et al.,* 1983). This confirmed and extended earlier experiments of Gardner (1977) which showed that single inner cell mass cells could give rise to both somatic and germ line cells in chimaeras (although their fate may have been changed by the manipulations in these experiments). A late origin of the germ line was contrary to the situation in flies and frogs and to the current dogmas of continuity and early origin of the germ line in mammals. Most people believed that the germ line was set aside very early in development, from one or two cells, and before any restrictions in developmental potency could occur. Indeed, Soriano and Jaenisch (1986) subsequently published a paper claiming to show that our results on the origin of the germ line from about 50 cells must be wrong. We worried about this disagreement with our work but could not see where we had made mistakes in our research plan nor in our conclusions. Subsequently, the elegant experiments of Lawson and Hague (1994) have confirmed our results that the mammalian germ line does indeed arise from around 50 cells after implantation. These germ cells can be seen at around 7 days' gestation as a cluster of alkaline phosphatase positive cells (Ginsberg *et al.,* 1990).

Imprinting and transgenerational change in epigenetic inheritance

Inactivation of the paternally-inherited X chromosome from the sperm in the extra-embryonic trophectoderm (precursor lineage of the placenta and membranes) and primary endoderm (which forms part of the yolk sac) was one of the first examples of imprinting in the mouse. Paternal X-chromosome inactivation was first demonstrated by Takagi and Sasaki (1975), confirmed by West et al (1977) and then we showed paternal X-inactivation to be primary

non-random inactivation (and not just cell selection) (Harper *et al.,* 1981). Preferential paternal X-inactivation means of course that the X from the sperm and the X from the egg must be distinguishable in some way even after the 4 or 5 cell divisions (and DNA replications) that have occurred before the blastocyst stage. The distinguishing mark is some form of epigenetic modification of the DNA. A heritable modification of the information encoded in the base sequence of the DNA is called epigenetic inheritance (Waddington, 1953). Epigenetic modification of the regulatory sequences of a gene affects the *potential* of that gene to be expressed.

Imprinting is an example of epigenetic inheritance passing through the germ line, e.g., a paternally expressed gene (expressed in progeny when it is inherited from sperm) will be silent in a generation where the gene has passed through the mother, and reactivated in the progeny of her sons and so on. Imprinting has been observed in a wide variety of systems – plants and insects, as well as mammals, and has many important biological consequences - evolution, sex determination, development, genetic disease and cancer. There are about 40 imprinted genes identified so far in the mouse. Imprinting effects are also commonly observed in progeny of interspecific crosses and tend to be more marked (developmental abnormalities, non-reciprocal lethality, temporal differences and quantitative and qualitative differences in expression of parental alleles of specific genes). The offspring of an interspecific cross between a horse and a donkey, the hinny (donkey mother) and the mule (horse mother), demonstrate the non-reciprocal phenotypes arising from imprinting. There are also effects of uterine environment on the phenotype of the offspring (Allen *et al.,* 1993; a subject of great interest to Anne herself) in addition to the non-reciprocal genetic imprinting.

Imprinting has challenged the central dogma of unidirectional flow of information from DNA to RNA to protein. The Lamarckian idea that environment could exert effects on genetic information in the germ line has not been a popular one for a long time. It has been said that adaptive change in inheritance (transgenerational changes in genetic inheritance according to changes in environment) is not possible for want of a molecular mechanism. Clearly this statement is no longer true; epigenetic modification of genetic information in the germ line does occur and is heritable from generation to generation. In somatic cells, we know that changes in epigenetic information can be induced by the environment; this is the mechanism of activation and silencing of different genes characteristic of a particular tissue and its specific functions. Adaptive changes in inherited information from one generation to the next would only require that such environment-induced epigenetic modifications causing changes in gene expression could occur in the male and female germ line, escape the erasure that occurs in preimplantation development and in the germ line (see later), and thus be inherited by the next generation (Monk, 1990a; 1995). Imprinting, an example of transgenerational inheritance, is easy to observe due to the uniparental inheritance and the "switching" with the sex of the parent. Epigenetic silencing causing random hemizygous expression would not be so easy to detect, yet might occur in response to changes in genetic background or environment (Monk, 1990b). It is noteworthy that a permanent epigenetic silencing of a transgene after its passage through the female germ line has been reported by Hadchouel et al (1987). Epigenetic silencing which is not erased in preimplantation development, nor in the germ line (see below), would create silent heritable genes which might reactivated by unknown mechanisms (the "throwback"). Recently, experimentally-controlled environmental change has been demonstrated with the appearance of previously silent genetic characteristics by heat shock in *Drosophila* (Rutherford and Lindquist, 1998; see also McLaren, 1999).

The role of methylation in the regulation of development, X-inactivation and imprinting

We were next interested in the molecular mechanisms of the inactivation and reactivation of the X chromosome and in the non-random (imprinted) inactivation of the paternal X chromosome in the extra-embryonic membranes. Epigenetic mechanisms that

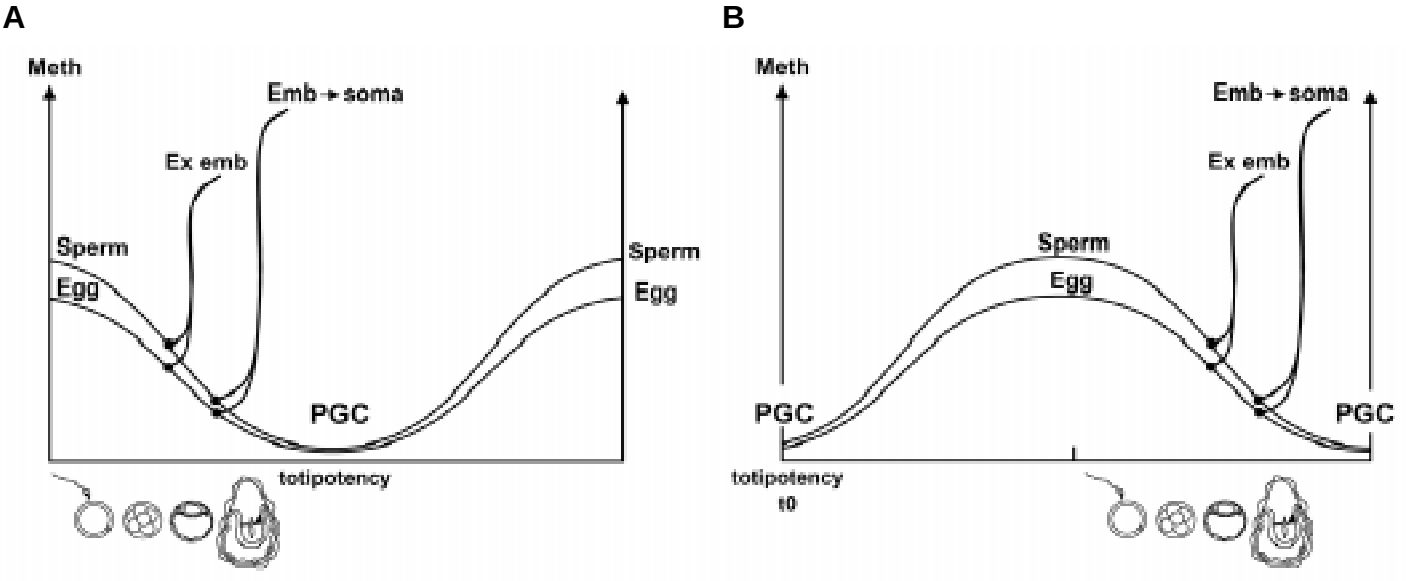


Fig. 4. Changes in methylation in early mouse development. (A) *Viewed with the cycle starting at fertilisation.* **(B)** *Viewed with the cycle starting with the totipotent ground state of the primordial germ cells.*

could modify the DNA of a gene and regulate the expression of that gene include nuclear compartment (e.g., scaffold, nuclear membrane attachment), chromatin conformation, histones, histone acetylation and methylation. Transcription itself is a modification of the DNA in that the transcription apparatus is expected to hold the DNA open in a special way. Much earlier, Holliday and Pugh (1975) and Riggs (1975) published their models which predicted that DNA methylation could affect the potential for gene transcription and play a role in development and differentiation in higher organisms. The addition of methyl groups on the cytosines in the major groove of the DNA affects the conformation of the DNA and protein binding to the DNA. There were no molecular techniques sufficiently sensitive at this time to look at specific gene methylation in embryos, so a new student, Susan Lindsay, and I collaborated with Dick Flavell and Robin Holliday (then at National Institute for Medical Research in London) to determine whether genes on the active and inactive X chromosomes in human cell lines were differently methylated. And they were (Lindsay *et al.,* 1985).

This encouraged me to go back to the embryos and see what changes in methylation might be occurring during early development that would underlie X-chromosome activation, inactivation and reactivation (in the cycle of Fig. 3B). Naively, I expected a demethylation in development (when the paternal X chromosome becomes active), *de novo* methylation at the blastocyst stage when X-inactivation began, and a demethylation, or lack of methylation, in the germ line when reactivation occurs. Working together with Michael Boubelik and Sigrid Lehnert, I devised highly sensitive procedures to look at overall DNA methylation of the embryonic genomes at these different stages, and this is exactly what we found (Fig. 4A; Monk *et al.,* 1987: Monk, 1990a). However, had I thought about this some more in advance, I might have realised that this would not be a popular finding. It was generally believed at the time (and still is by many today) that the gametes represent the "*tabula rasa*", or the "ground state", of developmental potency and that the act of fertilisation is the beginning of development. If this were the case, the gametes should be in the transcriptionally open state, and, in terms of methylation, development would start with the lowest degree of methylation and be associated with an increase in methylation as genes were silenced in different tissues. Others in the field saw the starting state at fertilisation as fully methylated and demethylation occurring as genes became active. Nobody expected the swooping changes in methylation we discovered (Fig. 4A; Monk *et al.,* 1987: Monk, 1990a). Leaders of the field listened with disconcertingly shaking heads during my presentation at a meeting at the Royal Society (Monk, 1990a). But now our results on changes in methylation in development are confirmed and presented by Rudolph Jaenisch at an imprinting meeting in Dublin last year as "textbook stuff".

Our picture of changes in methylation in development immediately explained a number of developmental requirements and observations – erasure of gametic epigenetic programmes with loss of methylation (activity of DNA methyltransferase also decreases, Monk *et al.,* 1991), imprinting as a failure to erase certain gametic methylation differences (imprints) before delineation of extra-embryonic membranes and somatic tissues, *de novo* methylation establishing new genetic programmes occurring during implantation and post implantation (and therefore independently, and potentially differently, in the three primary germ layers) and escape from *de novo* methylation (Grant *et al.,* 1992), and /or continued erasure, in the germ line (delineated after implantation, McMahon *et al.,* 1983).

The task of the next student to join the lab, Mark Grant, and a visiting post-doc, Maurizio Zuccotti, was to study changes in methylation of specific X-linked genes in early embryos. For this we needed single gene sensitive techniques to look at methylation of single oocytes, embryos and isolated ICMs. Judy Singer-Sam in the States was developing refined procedures for the sensitive study of methylation by PCR. Using procedures we had developed in collaboration with Judy (Singer-Sam *et al.,* 1990), Mark, Maurizio and I showed that methylation of the X-linked genes was co-incident (whether immediately before or after we could not say) with X-inactivation at implantation and occurred earlier for a gene closer to the X-inactivation centre (as would be expected if inactivation were to spread along the X chromosome in time) (Grant *et al.,* 1992). This was the earliest time methylation gene silencing in development had been observed. Our results contrasted with an earlier work claiming to show that methylation occurred as a later event to inactivation (Lock *et al.,* 1987) and have re-opened the question as to the role of methylation as cause or effect in gene silencing. It is probable that it can function both as cause and as effect in different situations (see later).

Having shown that the molecular mechanism of imprinting - the epigenetic modification that distinguishes the X in the egg from the X in the sperm – did not reside in the X-linked housekeeping genes themselves, we turned our attention to a newly discovered gene *Xist* (Brown *et al.,* 1991), expressed only from the inactive X chromosome and mapping to the X-inactivation centre. Clearly this was the gene to study for possible methylation mechanisms of imprinting. We asked the question - are the *Xist* genes differently methylated in sperm and egg? Could this be the molecular mechanism of imprinting? Maurizio Zuccotti and I showed this to be the case (Zuccotti and Monk, 1995; see also Ariel *et al.,* 1995). This was the first demonstration of a differential methylation imprint in sperm and egg which governs expression of parental genes in development. However, it should be noted that methylation in the regulatory regions of *Xist* is mosaic (as might be expected at a time of the sweeping overall changes in methylation – Fig. 4A) and that regulation relies on the probability of methylation of a number of sites, with some sites being more important than others (Goto and Monk, 1998).

Following the closure of the Mammalian Development Unit at the time of Anne's retirement from the MRC, I continued my research on the molecular mechanisms of imprinting at the Institute of Child Health. Here, the work was extended to show (by transfection and transgenesis studies, band shift assays and Western blots) that the differentially methylated region in the *Xist* promoter identified by Maurizio was essential for transcription, that a sequence-specific DNA-binding protein bound to the differentially methylated motif only when it was methylated at, at least, one of its three CpG sites (Huntriss *et al.,* 1997), and that differential methylation of the *Xist* promoter in reporter transgenes in sperm is correlated with expression of the sperm-transmitted transgene in preimplantation embryo progeny (Goto *et al.,* 1997). Our current research on imprinting at the Institute of Child Health is now mainly in the context of human development, and on the expression, and mono-allelic expression, of imprinted genes associated with genetic diseases such as Prader Willi, Angelman's and Beckwith Wiedemann, syndromes, and childhood tumours (Huntriss *et al.,* 1998; Salpekar *et al.,* 2000).

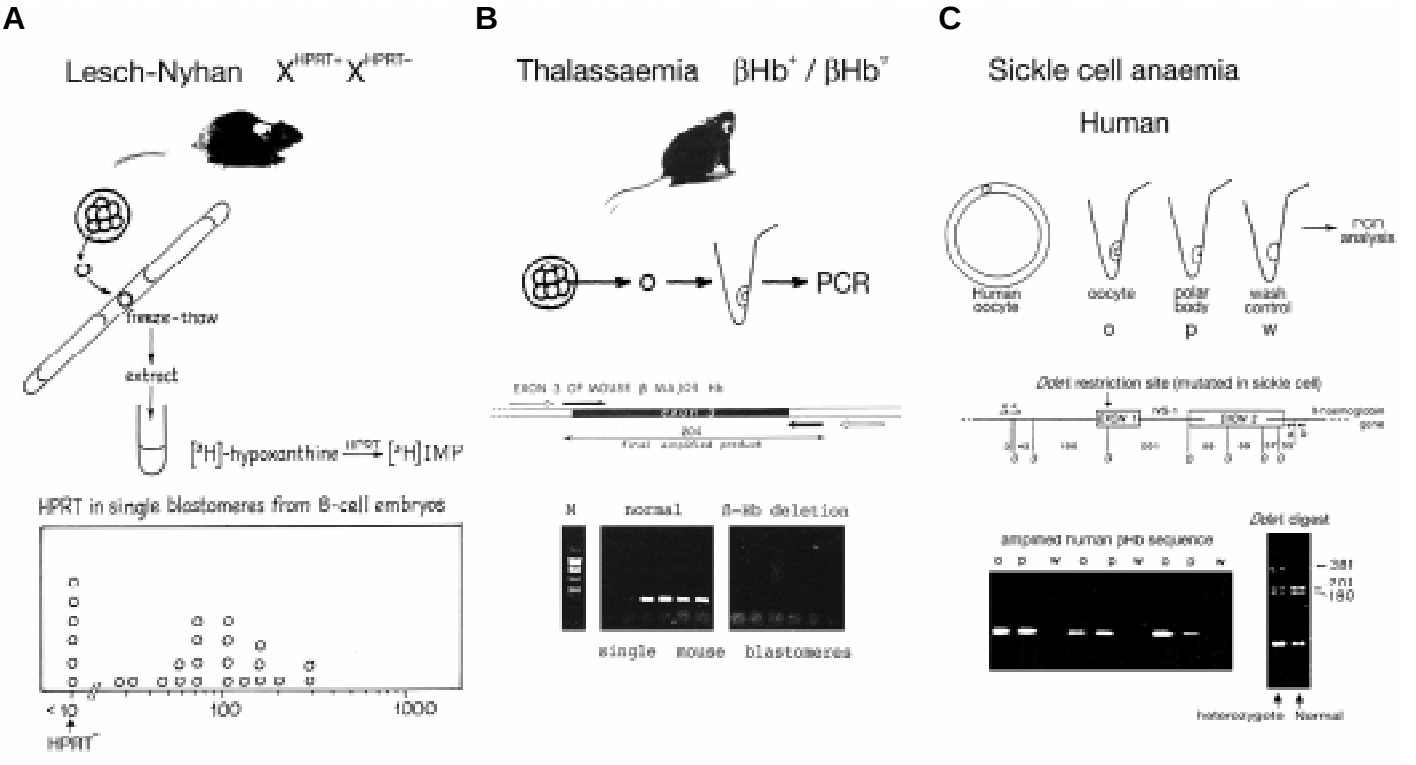


Fig. 5. Pioneering the procedures for preimplantation diagnosis of genetic disease. (A) *Diagnosis of HPRT-deficient male mouse embryos from an heterozygous mother by assay of HPRT enzyme activity in single blastomeres from 8-cell embryos.* **(B)** *Diagnosis of mutant thalassaemia mouse embryos by nested PCR detection of the presence or absence of a deletion in the haemoglobin gene DNA in single blastomeres of mouse 8-cell embryos.* **(C)** *Diagnosis of the Sickle cell mutation site in single polar bodies of human oocytes by nested PCR. (The Sickle cell mutation alters the DdeI restriction pattern digest of the PCR product).*

A new view of mammalian development

The results on X-inactivation and reactivation in the female germ line, the postimplantation origin of the primordial germ cells from around 50 cells, and the erasure of methylation modification of the DNA in preimplantation development and into the germ line, have led to a new view of mammalian development. The ground state of development is not represented by the gametes but by the primordial germ cell. The profound undermethylation we observed in the primordial germ cells at the time of onset of meiosis (in the female embryo), or mitotic arrest (in the male embryo), and X-reactivation in female germ cells at this time, are compatible with this hypothesis. Also in keeping with these findings is the derivation of the immortal, totipotent EG (Matsui *et al.,* 1992) and ES cell lines from the primordial germ cells and their precursor lineage, the ICM. Given that primordial germ cells represent the ground state of development, it might be considered appropriate to draw our Y axis of development at the PGC and not at fertilisation (Fig. 4B). This has important influences on our thinking about development in all sorts of ways.

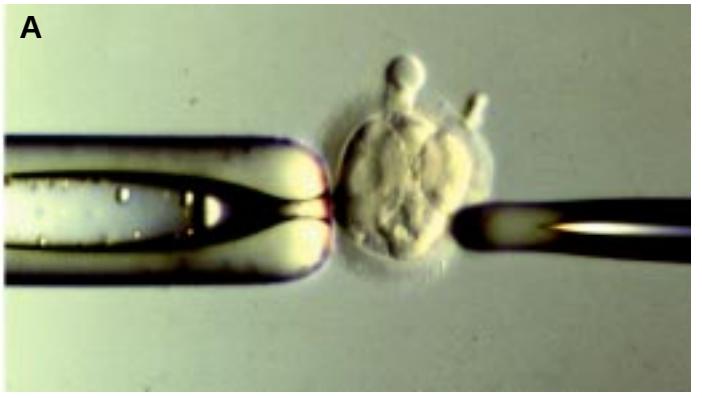
Of man

Preimplantation diagnosis

One of the most significant contributions of my work in Anne's Unit beginning in the 70's was the development of procedures for preimplantation diagnosis of genetic disease (Monk *et al.,* 1987; Monk *et al.,* 1988; Benson and Monk, 1988; Holding and Monk, 1989; Monk and Holding, 1990; Monk, 1990c and d; Monk, 1991).

During the time I was in Anne's laboratory exciting developments were taking place in human embryology. The critical event was the work of Steptoe and Edwards on *in vitro* fertilisation (IVF) and the first test-tube baby born in 1978. However, the idea of molecular analysis of human embryos was not uppermost in my mind until I attended a meeting in 1985 at the Ciba Foundation where people were discussing the possibility of diagnosing genetic disease in a human preimplantation embryo. This would be a significant breakthrough for couples at high risk of having a child with a severe genetic disease. Such a couple could start a pregnancy knowing that the embryo was free from the disease-related gene and that they would not have to undergo a later abortion of an affected fetus. One idea would be to biopsy some cells from an embryo, check them for the presence or absence of the disease-related gene, then replace only the unaffected embryo in the uterus to start the pregnancy. It was generally agreed at that meeting that the biopsied cell(s) would have to be cultured *in vitro* to obtain sufficient cells for diagnosis as there were no single cell techniques available at that time. The revolutionary PCR procedure had just been developed but at that time even PCR did not work at the level of sensitivity required.

At this meeting, I offered the information that my X-linked enzyme assay procedures were single cell sensitive and that I was diagnosing the sex of mouse preimplantation embryos routinely (the evidence that two X chromosomes were active in female preimplantation mouse embryos was based on the finding that my assay distinguished two classes of embryos with respect to HPRT activity; the



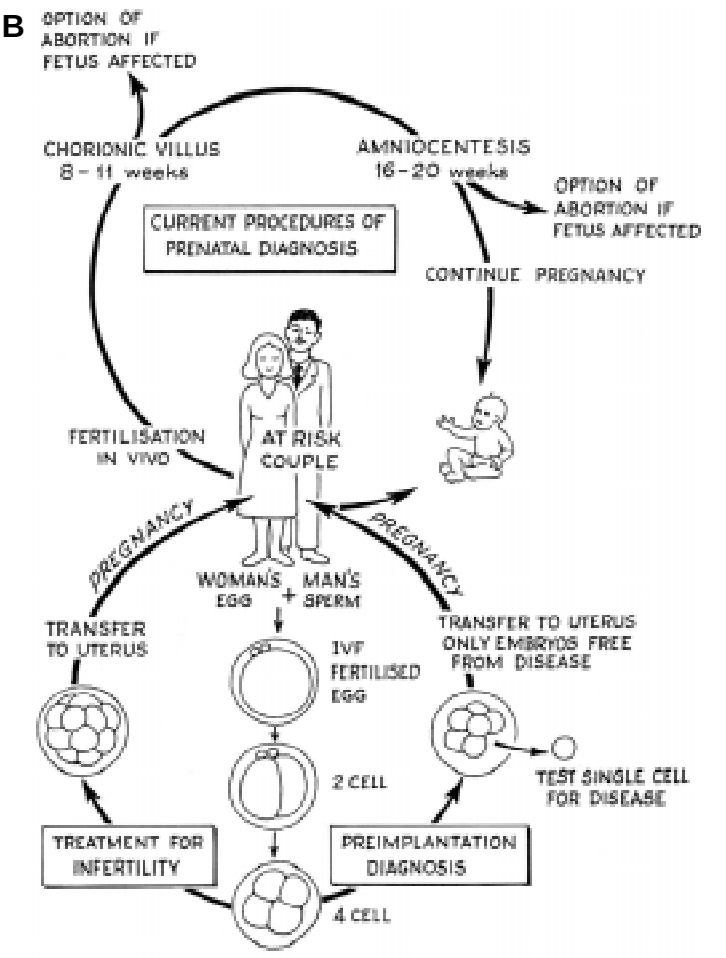


Fig. 6. Clinical preimplantation diagnosis of genetic disease. (A) *Biopsy of a blastomere form a human 8-cell embryo. A single blastomere is gently pushed out through a hole made in the zona pellucida (picture courtesy of Dr. Tetsuya Goto).* **(B)** *Scheme of IVF treatment of infertility and preimplantation diagnosis.*

females had double the HPRT/APRT ratio compared to the males)(Monk and Harper, 1978). Moreover, my assay would tell me whether the embryo had a normal or mutant HPRT gene and, in the human, HPRT deficiency causes Lesch-Nyhan syndrome. So the procedures for preimplantation diagnosis of this deficiency were already in existence in my lab in Anne's Unit. However, this information was not taken on board at that meeting probably due to the fact that my work was seen as firmly ensconced in the academic X-inactivation arena. I decided it was necessary to re-frame my work in the language of preimplantation diagnosis to show that this approach to prevention of inherited genetic disease was feasible.

The experiments required biopsy of the 8-cell mouse embryos, assay of HPRT in the single cell and replacement of the operated embryos back into a foster mother to produce healthy live born young. The work-load was quite demanding so I asked Anne if she would like to join me in these experiments. However, at that time, Anne herself belonged to the camp that claimed that single cell preimplantation diagnosis was not possible (she was also very busy running the unit and doing her own experiments). Thus competitively activated and undaunted, I then asked Paul Burgoyne in the Unit to help me in this endeavor but Paul's interests and time were already firmly placed in his exciting experiments on the influence of sex chromosomes on early growth and development. So it was that I next asked Alan Handyside, then at the Hammersmith Hospital, if he would do the biopsies since his previous work in Cambridge had been based on biopsy of cells from preimplantation embryos. Alan turned out to be a reliable and efficient collaborator, and he readily came to Anne's Unit on the appropriate days to help me with the biopsies and transfers. The excitement of this work was that its timing coincided - perhaps not accidentally - with the years of the debate leading up to the passage of the Human Fertilisation and Embryology Bill through Parliament in 1990. It was also exciting because of the amount of interest it generated and in the life lessons about the differences between relating to scientific colleagues in academia and interested parties in the "real world" of clinical research heralding a new medical breakthrough.

The first tests were done on one of the first, if not the first, genetically engineered mouse models - the "Lesch-Nyhan" mouse. This mouse was created by Martin Hooper and co-workers (Hooper *et al.,* 1981) from an embryonic stem (ES) cell line with a mutation of its X-linked HPRT gene. Male chimeras were produced with sperm derived from the mutated ES cells from the culture dish. Daughters of these males are heterozygous for the HPRT mutation. We were able to use offspring from this heterozygous female mouse to complete the picture and show that we could diagnose the mutant embryos (half the males) carrying the mutation by assay for HPRT in single cells taken from 8-cell embryos (Fig. 5A) and then produce live born progeny from the embryos diagnosed in this way to prove the accuracy of our procedures. Although the sensitive microassays for HPRT in single embryos and single embryonic cells had been part of my work for over ten years, this was the first complete demonstration that preimplantation diagnosis by biopsy, analysis of a single blastomere and replacement of the operated embryo would work (Monk *et al.,* 1987). Audrey Muggleton-Harris and I quickly followed this report with a demonstration that preimplantation diagnosis could also be done by biopsy and analysis of a few trophectoderm cells extruded through the zona pellucida (Monk et al. 1988) - a technique perfected by Audrey whilst working with David Whittingham (David had moved from Anne's Unit by this time and had set up his own Unit at St. George's Hospital).

Cathy Holding had joined my laboratory in Anne's Unit in the 80's and we set out to create further single cell enzyme assays for common inherited genetic diseases. One of these was adenosine

deaminase (a deficiency in this enzyme is the basis of SCID, severe combined immunodeficiency disease, which had caused the death of a baby in my family in Australia). Again we showed that it was relatively easy to make our methods sensitive to the single cell (Benson and Monk, 1987). However, from the advent of the polymerase chain reaction (PCR) in 1984, Cathy and I (encouraged by Cathy Abbott at the Galton Laboratory) had taken up the relaxing past-time of moving racks of tubes between water baths at different temperatures. We had set out to develop the procedures for single cell PCR amplification (at the same time testing out the new PCR machine which was being developed by Martin Evans and BioCam in Cambridge). We wanted to use PCR to look directly at the actual mutation in the DNA of a single cell.

Again we used a mouse model - the thalassaemia mouse - and developed a modification of the powerful PCR procedure to make it even more sensitive. This modification was the use of nested primers – first amplifying the larger sequence and then, in a new reaction, amplifying an inner sequence with the inner primers. This vastly increased the specificity and sensitivity of the reaction so we were able to analyse single cells and publish the first nested PCR on a single cell detected by a simple agarose gel assay (Fig. 5B; Holding and Monk, 1989) and, at the same time, show that preimplantation diagnosis of thalassaemia is accurate and reliable in this model. Following our success, others were trying single cell PCR and we met with some difficulty with claims from other labs in Chicago and London that single cell PCR was impossible without contamination. In fact, our controls could not have been more rigorous in that we used a control of a drop of medium from the wash drop of every single cell that we analyzed. Subsequently, others became able to do the single cell PCRs without contamination. I suspect it was our care in UV sterilizing the pipettes and my earlier training in sterile technique working with bacteria that made it relatively easy for us to pioneer single cell PCR without contamination.

And finally, in 1989, coinciding with the final stages of the debate on embryo research, we were the first to show that it would be possible to diagnose genetic disease (sickle cell anemia in the first experiments) in the tiny polar body cell of a human unfertilised egg, thus avoiding working on the human embryos themselves (Fig. 5C; Monk and Holding, 1990). The passage of the Bill through Parliament, which was to allow embryo research under license in Britain, was thus greatly influenced by the clear medical significance of preimplantation diagnosis and the proof provided by our genetic experiments that this was possible. These were exciting times, for me and Cathy at the bench, and I suspect for Anne and others who were doing the front line work at Westminster. For all of us it was front page of the newspapers, a seemingly unwarranted accolade for this academic scientist for whom the breakthrough was not in the science (single cell sensitive assays applied to embryos were the basis my research for well over a decade before preimplantation diagnosis) but in its new significance.

Cathy and I and others in my Unit at the Institute of Child Health continued with the development of new single cell procedures for genetic diseases including Lesch-Nyhan, SCID, thalassaemia, Sickle cell, myotonic dystrophy, Fragile X and Kennedy's disease (Daniels *et al.,* 1995: 1996). We also extended our single cell procedures to new and more refined approaches, such as cell recycling (analysis of chromosomes and genes in the same single cell, Thornhill *et al.,* 1994; Thornhill and Monk, 1996) and to the imprinted Prader-Willi, Angelman, Beckwith-Wiedemann and Silver Russell syndromes (Huntriss *et al.,* 1998; Salpekar *et al.,* 2000).

Clinical preimplantation diagnosis is not difficult once the procedures are developed and there is confidence that they can work efficiently and accurately. It requires access to embryos outside the womb (routine in IVF treatment of infertility), efficient biopsy of a single cell of the embryo (Fig. 6A) without compromising further growth and development, a sensitive diagnostic test on the biopsied single cell and replacement of the diagnosed embryos into the uterus to initiate a pregnancy (see Fig. 6B showing the routes for IVF treatment of infertility, preimplantation diagnosis of genetic disease and alternative prenatal diagnoses). Today, the origin of preimplantation diagnosis is said to be the first clinical preimplantation diagnosis in 1990 when six out of seven babies (approx. 25% error rate) were born as females after a repetitive sequence was used for diagnosing sex at the Hammersmith Hospital (Handyside *et al.,* 1990). Nevertheless, our earlier single cell diagnostic procedures in the Mammalian Development Unit combined with the embryological expertise learnt from Anne heralded the real breakthrough in this procedure. However, our interests have always been more with the interesting biological questions rather than the clinical manifestation of our research.

Gene expression and its regulation in human development

Mouse development is an excellent model for the study of human embryology since for the first week of development mouse and human embryos are very similar. So the many years of study in the mouse and my training in Anne's Unit have paved the way for similar studies in the human over the last decade at the Institute of Child Health. Mouse and Man may have the vast majority of their genes in common but we now know that there are many differences in gene expression and its regulation between the mouse and the human, e.g., in the expression and regulation of *Xist* in the mouse and *XIST* in the human (Daniels *et al.,* 1997; Goto and Monk, 1998). It is essential to study the human embryo directly.

Our current academic studies are concerned with elucidating the genetic programme of human development and understanding the regulation of gene expression, gene silencing and the hemizygous expression of imprinting in the human. One of the major limitations to research in the human is the scarcity of human embryos and the associated ethical concerns. Therefore, a major part of our recent research has been the creation of cDNA libraries representing the genes expressed in human oocytes, preimplantation embryos, primordial germ cells and human embryonal carcinoma (EC) cells (and their differentiated derivative cells) (Goto *et al.,* 1999; Holding *et al.,* 2000, Monk *et al.,* 2001; Salpekar *et al.,* 2000, Monk and Salpekar, 2000). These libraries have proved to be an excellent resource for the study of expression of a range of known genes, the identification of new members of known gene families, and the identification of novel human developmental genes not expressed in somatic tissues. The immortality, pluripotency and motility of early embryonic and germ cells suggests that the human developmental genes identified may be candidate genes for a role in tumourigenesis. A panel of cDNAs prepared from cancer cell lines and fresh tumor samples is being screened for expression of these genes with a view to the identification of embryo/cancer genes that could be targeted in cancer therapy and the preparation of DNA vaccines. Our preliminary work has produced exciting results as we have identified several such genes (Monk *et al.,* unpublished).

The clinical significance of research into the molecular basis of human development is many-fold - if we know how the human embryo develops normally we can have a better understanding of

what can go wrong to cause congenital abnormalities and genetic disease. There is the need to improve the efficiency and safety of treatment of infertility, for quality control and the assessment of the safety of new procedures (e.g., a perturbation of imprinting would be associated with disease and tumourigenesis), to develop new methods of contraception, and further developments in the field of preimplantation diagnosis and prevention of genetic disease. There is also the developing field of human ES cell research. By a combination of production of an "embryo" cloned from a patient's somatic cell nucleus, derivation of an ES cell culture from that "embryo" and directed differentiation, there is the exciting possibility of immunologically matched tissue transplantation for the treatment of disease or injury. And finally there is our current interest in the study of the re-appearance of embryonic gene expression in the process of tumourigenesis and the identification of development genes that may be targeted in the treatment of cancer.

Concluding remarks

Looking back over the multifarious research projects during my path through science, it does seem that circumstances directed the changes from microbes to moulds to mice to Man. In another way it also seems that they did not. Big decisions are made of a multitude of little choice steps so that when one finds oneself suddenly in a new place it seems fortuitous because one is not aware of a single decision having been made. If I were to try to define a common influence on these small choices, it would be my interest always in what is just around the corner. I think I might have learnt some of this trait from Anne; it was certainly in me and encouraged by her. Whatever the driving forces, I am always finding myself caught up in the latest and "hottest", and often most ethically controversial, field. Inevitably, as one changes from one field to another, big changes like microbes to moulds, or moulds to mouse, or mouse to Man (and associated changes from DNA replication and repair, to cell signalling, to mammalian embryology, to gene expression, to preimplantation diagnosis, to ES cell research and cancer), there is a tendency to take what one was last doing and try to do it in the new system. This led to some rather daft experiments, like investigating whether amoebae would crawl towards the origin of a primitive streak in a mouse egg cylinder (as a sensitive indicator of a possible cAMP signal), or whether a source of cAMP would induce a second axis in a mouse embryo. (Daft because embryos and amoebae do not like the same temperature, pH, salt concentrations and so on.) But the real gift for the research scientist is the freedom to experiment and to discover something new. I will be forever grateful for the freedom and encouragement which Anne always provided and for her wise and brilliant guidance in all matters, whether it be lessons in letting go ("water under the bridge" she would say), adolescent kids, or acute understanding concerning the significance of one's research findings and the priorities for the way to go on.

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