Int. J. Dev. Biol. 45: 457-467 (2001)

A history of mammalian embryological research

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Forerunners

The birth of modern reproductive and developmental biology took place in the 17th century. Up to then prevailed the theory of "seeds", belonging to the pluralistic current of the Pythagorean school led by Anaxagoras of Clazomenae and Empedocles of Acragas (5th century B.C.). Applied to human reproduction, pluralism means that a foetus results from the mixing of two parental seeds. According to Hippocrates (~460-370 B.C.), these seeds flow from all parts of the body and each contains both the masculine and the feminine principle. For Aristotle (384-322 B.C.), only the male's seed contributes to forming the foetus and the female's only role in procreation is to contribute menstrual blood. These two rather contradictory notions are present already in the Manava-Dharma-Sastra (sacred law-code of the Hindus), written in the 13th or 14th century B.C. (Rostand, 1950). Galen (~130–201 A.D.), seen as the spiritual heir of Hippocrates, asserts that the seeds of both man and woman contribute to procreation, but that each contains only one principle. Galen also benefited from the knowledge produced by the prestigious school of medicine of Alexandria. He was notably inspired by the work of Herophilus (~340–300 B.C.), a true anatomist from whom he took the notion that women also have testes. He boldly went a step further, asserting that woman's genitalia are identical to man's, but turned inward. Andreas Vesalius (1514–1564), father of modern anatomy, still echoed this interpretation, drawing a parallel between the female's «uterine tubes» and the male's «semen-conveying ducts» (*ductus deferentes*) (Herrlinger and Feiner, 1964). The latter were described correctly in 1561 by Gabrielis Fallopius (1523-1562), student of Vesalius, but their true function was not understood until a century later, thanks to the outstanding work of Reinier De Graaf (1641-1673). The name of this Dutch scientist remains associated with the follicles, which he described remarkably but which he viewed as eggs (De Graaf, 1672). Many authors see De Graaf as the founder of modern reproductive biology (Setchell, 1974). This is due essentially to his use of convergent scientific methods: meticulous dissections, clinical observations and critical analysis of the available literature (Ankum *et al.,* 1996). De Graaf is the one who discovered the source of the eggs which, according to William Harvey (1651), engender all animals, both oviparous and viviparous, including humans: the 'testes', which we now call ovaries. He also asserted that the human egg transits through the Fallopian tubes, viewed at the time as « chimneys enabling the smoke to rise from the matrix into the abdominal cavity» (De Graaf, 1672). The Netherlands were also the home of Antoni Van Leeuwenhoek (1632-1723), a draper from Delft, who five years later observed, with a microscope he had designed himself, what he called «animalcules» or «spermatic worms». This discovery, confirmed by others, gave rise to heated debates opposing ovists, who believed in generation from eggs, and animalculists, who saw in these animalcules the germs from which animals and humans arise (Andry, 1700).

Not until the 19th century did scientific knowledge of mammalian reproduction and development make further significant progress. The century of the Enlightenment had witnessed a clash between preformationists favouring the seed theory or the pre-existence of germs in eggs or animalcules, and epigenesists asserting with Kaspar Friedrich Wolff (1733-1794) that the parts of the body do

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0214-6282/2001/$25.00

Printed in Spain
www.ijdb.ehu.es

not exist from the start but form gradually (Wolff, 1766-1767). In 1825, the Swiss physiologist Jean-Louis Prévost and the French chemist Jean-Baptiste Dumas, having repeated the experiments of Lazzaro Spallanzani (1729-1799) on the fertilisation of amphibian eggs (see De Felici and Siracusa, 2000) and interpreted correctly the role of animalcules, demonstrated this role in fertilisation in both dogs and rabbits (Sarton, 1931). They also tended to interpret a small spherical body observed occasionally in canine De Graaf vesicles as being an egg, but it wasn't until two years later that the ovum was truly discovered in the mammalian follicle. Karl Ernst von Baer (1792-1876) gave a precise microscopic description of the ovum, first in the dog and then in other species (von Baer, 1827). In 1834 Adolph Bernhardt, student of Jan E. Purkinje (1787-1869), one of the founding fathers of modern histology, observed in the ovum a «germinal vesicle». The notion that it might be a cell nucleus could then hardly be avoided. Of course these discoveries were closely linked to the emergence of cell theory, in 1828 (Harris, 1988).

Descriptive studies of the early stages of mammalian development

K. von Baer certainly played a determining role in the development of scientific embryology. His meticulous observations, described at length and abundantly illustrated in his famous letter on egg formation in humans and mammals (von Baer, 1827), led him to notice a resemblance between dog and bird embryos during embryogenesis. Not until half a century later, however, were the early phases of egg development described in detail. This was done by the Belgian zoologist Edouard Van Beneden (1845-1910), who made universally famous the discovery of chromatic reduction during gamete production in the nematode worm *Ascaris megalocephala* (Van Beneden, 1883). He devoted the start of his scientific career to the study of fertilisation and the early development of mammalian eggs (Hamoir, G, 1992, 1994). This led him to describe, for the first time and both in the rabbit (Van Beneden, 1875, 1880) and in bats (Van Beneden and Julin, 1880, 1884), the formation of the three basic layers. He thus demonstrated that the cleavage of the zygote lead to formation of a 'blastodermic vesicle'. This was the first description of what we now call a blastocyst. Van Beneden's remarkable drawings of this entity can still be used today to illustrate the last pre implantation stage in mammals (Fig. 1). Yet it must be said, as noted by J. Mulnard in his remarkable historical survey of some basic contributions to experimental mammalian embryology (Mulnard, 1986), that Van Beneden interpreted this vesicle erroneously, his interpretation leading to a major clash with his mentor in Würzburg, the eminent embryologist Kölliker (1817-1905). For Van Beneden, the blastocyst was a triploblastic gastrula, whose primary ectoderm (according to today's nomenclature) was the mesoderm. He thus believed, contrary to Kölliker (1879), that the mesoderm existed before the primitive streak (Van Beneden, 1880). He finally acknowledged his error, however, accepting that the middle layer of the blastodermic vesicle is not the mesoderm, but that the whole layer serves to form the ectoderm of the bilaminar stage (Van Beneden and Julin, 1884). This episode may seem purely anecdotal for

Fig. 2. Albert Brachet (1869-1930). *Courtesy of Lise Brachet.*

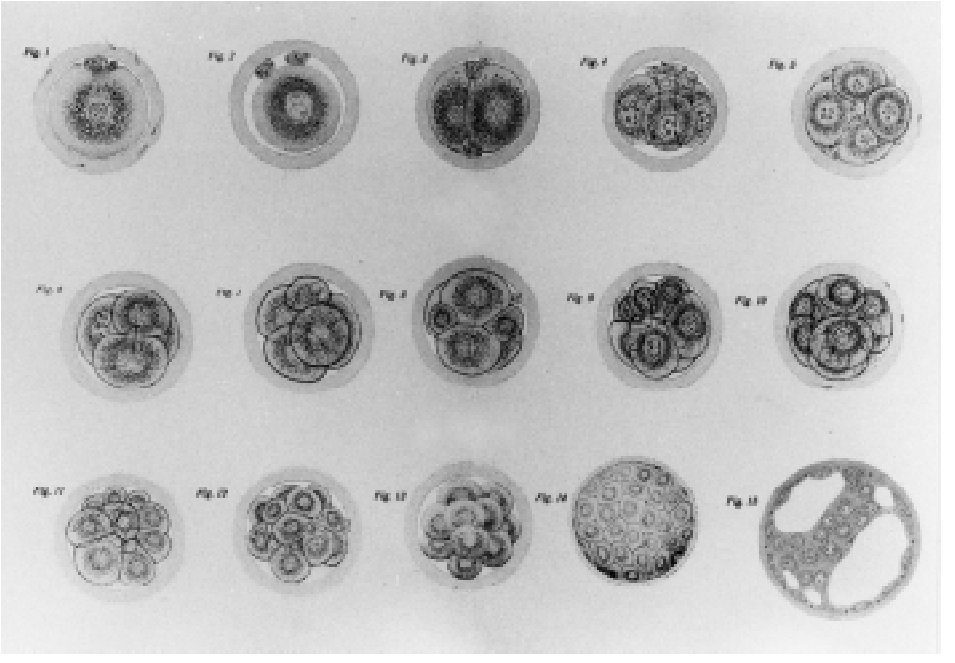

Fig. 1. Plate showing the successive preimplantation stages of the egg in two bat species, *Vesperugo dasycnema* *(Fig. 1)* **and *Myotis sp.*** *(Fig. 2-15)***.** *The eggs were observed in March and April from 1876 (Fig. 1) through 1888 (Figs. 12-15). Reproduced from E. Van Beneden (1911).*

modern embryologists, but it shows how hard it is to establish with certainty a cell lineage, not to mention developmental causalities, on the basis of a morphological description only, however detailed and accurate it may be. We must remember that the end of the nineteenth century saw the emergence of a «new science» called *developmental mechanics* (*Entwicklungsmechanik*) by Wilhelm Roux (1850-1924). Its aim was to use the laws of chemistry and physics to explain developmental events, an aim attainable only by experimentation. W. Roux is therefore generally considered the founder of experimental embryology (Sander, 1991, Counce, 1994), a discipline immediately championed by a handful of embryologists such as Hans Driesch (1867-1941), originator of the concept of embryonic regulation (Sander, 1992).

Albert Brachet (1869-1930, Fig. 2), a student of E. Van Beneden and the founder of the famous Brussels school of embryology (Mulnard, 1991), was a staunch partisan of the «new science». He called it "embryologie causale" (causal embryology) and regretted that the mammalian egg had so far eluded direct experimentation ("L'œuf de mammifère s'est complètement dérobé jusqu'ici à l'expérimentation directe": Brachet, 1912). When he wrote these words, phylogenetic embryology inspired by Darwin's «law of embryonic resemblance» and Haeckel's «biogenetic law» (Churchill, 1991) was still making new adherents. The multiplication of descriptions of early developmental stages in mammals other than the rabbit and bat, such as the mole, sheep, pig, goat, hedgehog, tarsier and later several others (reviewed in Pincus, 1936; Rossant and Papaioannou, 1977; Mulnard, 1986) came mainly within the scope of this comparative or phylogenetic embryology. As noted by J. Mulnard, this trend was to continue until 1940. Mulnard thus attributes the late emergence of the experimental embryology of mammals to a lack of interest coupled with some very real technical difficulties (Mulnard, 1986).

Fig. 3. Professor Robert G. Edwards receiving the title of doctor *honoris causa* from the Rector of the Université de Mons-Hainaut *(march 1994).*

Experimental manipulation of mammalian eggs

The first egg to be manipulated with success was again a rabbit fertilised egg. It was transferred from its biological mother, an Angora rabbit, to a foster mother of a Belgian line. Walter Heape presented this extraordinary experiment in 1890 before the Royal Society of London and published an account of it a year later (Heape, 1891). The method was then systematically applied to other species. By 1956, when Whitten succeeded in developing 8-cell mouse embryos to the blastocyst stage *in vitro* in a defined culture medium, intraspecific transfer had already been performed on the rabbit, goat, rat, mouse, cow, and pig (Hammer, 1998). Later it was applied to species such as the hamster, ferret, mink, horse, baboon, cat, dog, and water buffalo (Kraemer, 1983). Several attempts to transfer embryos between closely related species were also successful: between *Bos gaurus* and *B. indicus* (cattle), between *Bos gaurus* and *B. taurus* (cattle), between *Ovis musimon* and *O. aries* (sheep), between Equus *asinus* (donkey) and *E. cabalus* (horse) (Kraemer, 1983), between *Equus prezwalskii* (Prezwalski's horse) and *E. cabalus* (horse), between *Equus burchelli* (Grant's zebra) and *E. cabalus* (horse) (Allen and Short, 1997). Although seldom mentioned in reviews on the spectacular advances of mammalian embryology, these results open exciting prospects for people like myself who worry about the disappearance of endangered species. In combination with cloning, this strategy might save the big panda, the cheetah, the Bongo antilope, the ocelot, the bucardo…(Lanza *et al.,* 2000). It is permitted to dream!

The year 1956 thus witnessed a historical step in the «domestication» of the mammalian egg, an achievement making it possible, as A. Brachet had dreamed (Brachet, 1912), to experiment on embryos of our own zoological group. Albert Brachet had in fact been a pioneer in this area - he was the first to keep a rabbit blastocyst alive and developing for 48 hours outside the mother's body, in blood plasma (Brachet, 1912, 1913). Similar studies on the rabbit, particularly amenable to manipulation, did not multiply until the late 1920's. The main instigators were W.H. Lewis and G. Pincus. At that time the basic culture medium was blood plasma or serum. The greatest success in terms of the duration of *in vitro* development was accomplished by Lewis and Gregory (1929), who were able to film the development of rabbit eggs under the microscope on glass slides from the initial cleavage stages to the blastocyst stage. Lewis and Hartman (1933) observed the development of the macaque egg from the 2-cell to the 8-cell stage, whereas rat, mouse, and guinea pig eggs refused to develop beyond one or two cleavages (Lewis and Wright, 1935; Pincus, 1936). Using the method of Lewis and Gregory and other experimental devices (hanging drop, Carrel flask, watch-glass in a moist chamber…), Pincus obtained results similar to Lewis's with the rabbit egg (Pincus 1930, 1936; Pincus and Enzmann, 1934). He then investigated the metabolic requirements of this egg (Pincus and Enzmann, 1936; Pincus and Werthessen, 1938), a first step towards developing chemically defined and thus perfectly controllable media.

It was unimaginable to develop valid experimental strategies for studying mammalian eggs without a serum- and plasma-free me-

Fig. 4. Professor Albert Dalcq photographed with two of his students: Professors Jean Brachet (left) and Jean-Jules Pasteels (right). *Utrecht, 1947.*

dium, the composition of these complex fluids being unknown and highly variable. By taking into account the metabolic requirements of mouse eggs, Whitten himself (1957) and Brinster (1963) improved culture conditions to the extent that culturing from the 2-cell stage onward became relatively easy and efficient and perfectly reproducible. Readers interested in this exciting episode of the history of mammalian embryology and in later progress in embryo culture are referred to the reviews by R.E. Hammer, J. Arechaga and R. Brinster, and J.D. Biggers in the 1998 special issue of The International Journal of Developmental Biology, devoted to stem cells and transgenesis. This major but long-awaited breakthrough was to lead in the 1960's, i.e. 70 years after the advent of Wilhelm Roux's «new science» developmental mechanics, to mammalian embryology becoming at last experimental. It was also to make possible another great achievement: the birth, in 1978, of a baby conceived by *in vitro* fertilisation, the first of many to be born above all of the love of their parents, but also thanks to the competence and perseverance of two outstanding scientists, biologist R.G. Edwards (Fig. 3) and gynaecologist P. Steptoe (Edwards and Brody, 1995).

First steps in experimental mammalian embryology: predetermination or regulation?

In addition to the development of controlled culture techniques for pre implantation-stage mouse embryos, other contributions were fundamental. Anne McLaren, developing optimal conditions, succeeded in bringing to birth mice which had been cultivated *in vitro* as early embryos and transferred to the uterus of a foster mother (McLaren and Biggers, 1958). In 1959 for the first time, a living mammal (a rabbit) was obtained by *in vitro* fertilisation followed by intrauterine transfer of the cleaving embryo (Chang, 1959).

With these achievements, it was possible at last to check whether a mammalian egg behaved like that of an ascidian or sea urchin, that is whether it was a 'mosaic egg' or a 'regulative egg' as defined at the end of the XIXth century by Laurent Chabry (1887) and Hans Driesch (1891) respectively. A. Dalcq (Fig. 4), successor of A. Brachet, was an authority on experimental embryology just after the Second World War (Alexandre, 2000). He was notably the inventor, with his collaborator J.-J. Pasteels (Figs. 4 and 5), of the morphogenetic potential theory and 'field-gradient-threshold' concept, according to which morphogenetic substances are distributed in gradients in embryos, so that each developmental fate is determined by a typical concentration threshold (Dalcq and Pasteels, 1937; Dalcq, 1938). In the 1950's, essentially on the basis of cytochemical data, Dalcq elaborated his segregation theory to explain the early differentiation of the trophectoderm and inner cell mass (ICM). He postulated the existence, in the zygote, of a dorso-ventral gradient of morphogenetic substances, principally RNA, with gradual segregation of the «dorsal» material into precursors of ICM cells and of the «ventral» material into the cells destined to become the trophectoderm. This segregation implied early growth of presumptive trophectodermal cells around the presumptive inner cell mass (epiboly) (Dalcq, 1957; Mulnard, 1960). Dalcq's interpretation did not hold up to the experimental evidence. As early as 1942, Nicholas and Hall had shown that rat blastomeres isolated at the 2-cell stage could occasionally become complete embryos, and that conversely, two eggs stuck together formed a chimaera. These results obviously suggested that mammalian eggs belong to the regulative type. They were confirmed and further explained thanks to culturing in controlled medium and embryo transfer to the genital tract of a foster mother. A. Tarkowski (Fig. 6) showed that it was possible to obtain viable young after destruction of one of the first two blastomeres by pricking (Tarkowski, 1959a,b). Yet these results did not truly contradict Dalcq's hypothesis: the first segmentation plane having a random orientation with respect to the gradient or «dorso- ventral» axis, the regulatory power of each blastomere might depend on the presence of a morphogenetic substance. This was expressed very clearly by F. Seidel, who had obtained in the rabbit results similar to those of Tarkowski (Seidel, 1960), and by J. Mulnard (Fig. 5), student of A. Dalcq (Mulnard, 1960). It was thus imaginable that some blastomeres might be exclusively «dorsal» and others exclusively «ventral», the former evolving into pure ICM and the latter into a trophoblastic vesicle devoid of ICM. This was J. Mulnard's reasoning in the early 1960's, and his intention was clearly to demonstrate the pertinence of his mentor's theory (Mulnard, 1960). Yet his delicate experiments involving separation and homologous or heterologous reassociation

Fig. 5. Professors J.-J. Pasteels (left) and Jacques Mulnard (right) *photographed in 1970 in their laboratory in the Faculty of Medicine of the Université libre de Bruxelles.*

Fig. 6. Dr Chris Graham (left) and Professor Andrzej Tarkowski (right) *in the Laboratory of Embryology of the Department of Zoology in Oxford (summer of 1975).*

of blastomeres led him to invalidate decisively the theory that the first two distinct cell populations are predetermined in the zygote in the form of morphogenetic territories (Mulnard, 1966). Dalcq accepted this with philosophy, simply telling his successor that science works that way. What a lesson in intelligence, humility and respect, given by a person who would doubtless be interested in recent papers dealing precisely with specification of embryonic axes in mouse development. The fact that embryonic-abembryonic (Em-Ab) and bilateral axes of the blastocyst which anticipate embryo polarity (Weber *et al.,* 1999) are independent of organisation of the egg, is indeed now questioned mainly because the bilateral axis evidently corresponds to both the animal-vegetal (A-V) axis of the zygote (Gardner, 1997) and the antero-posterior axis of the foetus (Gardner, 1998, Weber *et al.,* 1999, Ciemerych *et al.,* 2000). Using different non-invasive marking techniques, Piotrowska and Zernicka-Goetz (2001) on the one hand, and Gardner (2001) on the other, have very recently demonstrated that both the Em-Ab axis and the plane of bilateral symmetry of the blastocyst are orthogonal to the plane of first cleavage. In addition, Piotrowska and Zernicka-Goetz (2001) have shown that the plane of first cleavage is predicted by the sperm entry position and that the cell inheriting this sperm entry position tends to cleave ahead of its sister and might therefore be incorporated preferentially into the ICM according to previous findings (Graham and Deussen, 1978). The two axes of the blastocyst (A-V and Em-Ab) thus become specified in the single-cell but, in sharp contrast to the theory of Dalcq, these axes are initially not fixed. Embryos are therefore said to be equipped with "regulative flexibility" (Piotrowska and Zernicka-Goetz, 2001).

The study of the potency of cleavage-stage blastomeres led Tarkowski and Wroblewska (1967) to propose a positional theory for the early differentiation of the blastocyst. The theory suggests that the fate of a blastomere is imposed by its position in the morula, the outer cells becoming the trophectoderm and the inner ones becoming pluripotent ICM. This remarkable contribution, known as the inside-outside or epigenetic hypothesis, was the starting point of an important and very fertile line of research, reviewed in many papers (see for instance Gardner and Rossant, 1976; Pedersen, 1986; Pedersen, 1988; Gardner, 1989). Ingenious experiments in which blastomeres were spatially rearranged provided final proof of the totipotent character of blastomeres up to the time they occupy an internal or an external position (Hillman *et al.,* 1972; Kelly, 1977). They further proved that blastomeres are not irreversibly committed until the morula stage (Ziomek *et al.,* 1882). In blastocyst reconstitution experiments, furthermore, R. Gardner (Fig. 7) demonstrated the determined state of the ICM and trophectoderm (Gardner, 1970).

Seeking to better understand how the two types of cells acquire positional information, Martin Johnson and some young coworkers who were to develop their own school of thought brilliantly demonstrated the role of cell polarisation, which occurs in the mouse embryo at the 8-cell stage when the blastomeres stick together (Johnson and Maro, 1986). This phenomenon, known as compaction, had been described with care by J. Mulnard thanks to high-quality filming (Mulnard, 1965: Fig. 8). Mulnard was also the first to call attention to the appearance at the 8-cell stage of membrane polarity in the distribution of alkaline phosphatase and to the probable role of membrane polarity in the early determination of trophectoderm and ICM (Mulnard, 1955, 1974; Mulnard and Huyghens, 1978). Yet M. Johnson and coworkers are undeniably the ones who, by combining blastomere dissociation and reassociation techniques with immunocytochemical detection of membrane and cytoplasmic constituents, contrib-

Fig. 7. Dr Richard Gardner *sailing near Oxford (July 1975).*

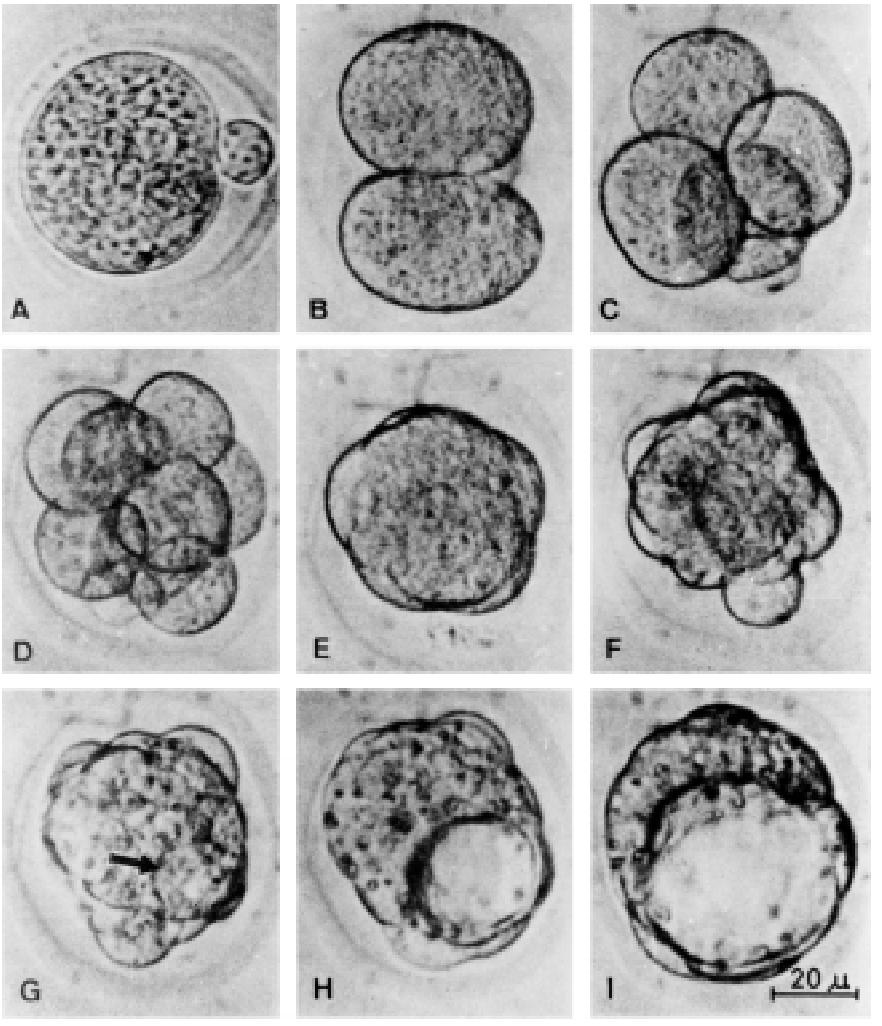

Fig. 8. Successive preimplantation stages in the mouse. *Images B through I are from an accelerated cinemicrographic film. Reproduced from Mulnard, 1967.*

uted most to understanding polarisation and its morphogenetic role in blastocyst differentiation. The theory of polarisation gave substance to the inside-outside hypothesis. More importantly perhaps, it also gave rise to a new and particularly fertile discipline, the cell biology of preimplantation development, the results of which apply to many problems not exclusively related to the reproductive biology of mammals (Johnson and Maro, 1986; Fleming and Johnson, 1988).

The sixties saw experimental mammalian embryology develop into a major discipline in the field of developmental biology. Contributions in this area stimulated research that gave rise to widely publicised findings. Transgenesis and cloning would not have been possible without the delicate micromanipulation techniques developed by a few talented scientists aiming only to acquire more basic knowledge on the reproduction of mammals, so long ignored by scientists. It is only fair to pay homage to pioneers A. Tarkowski and R. Gardner, who were the first to obtain mouse chimaeras, the former by morula aggregation (Tarkowski, 1959; 1998) and the latter by injecting ICM cells into blastocysts (Gardner, 1968). Production of allophenic mice (Fig. 9) by the first technique was rapidly exploited to elucidate the clonal basis of cell diversification during development (Mintz, 1971). Furthermore, mouse chimaeras were an invaluable tool for solving diverse problems in genetics, immunology and other areas (Mintz, 1974; McLaren, 1976; Tarkowski, 1998). The second approach was used for precise determination of early cell lineages in the mouse (Fig. 10) (Gardner, 1975). It is fair also to mention the brilliant work of Kirstie A. Lawson who, using clonal analysis, obtained a fate map of the mouse embryo epiblast. Her cell lineage analysis of the post-implantation embryo has been clearly described in a personal reminiscence recently published in the *Int. J. Dev. Biol* (Lawson, 1999). She notably clearly established the position of the precursors of primordial germ cells (PGCs) at the onset of gastrulation and estimated the size of the associated founding population (Lawson and Hage, 1994), a particular interest of Anne McLaren, whose outstanding contribution to this field (see for instance McLaren 1984, 1988, 1991, 1992, 1995, 1998, 2000) and to most other aspects of mammalian reproduction and development are soundly highlighted in other introductory papers as well as in reviews and research articles in this present issue.

The advent of mammalian molecular embryology

For the technical reasons already mentioned, experimental embryology was not applied to mammals until 80 years after its advent. Meanwhile, the discipline had become chemical (Needham, 1931; Brachet, 1944), then molecular (Brachet, 1960, 1974). Quite naturally, therefore, the remarkable work described in the previous sections was paralleled by research into the synthesis of macromolecules during the first stages of development. A comprehensive description of this research is beyond the scope of the present historical introduction, but its importance is highlighted by the multitude of reviews already devoted to the subject by 1975 (Biggers and Stern, 1973; Epstein, 1975; Graham, 1973; Manes, 1975). The strategies imagined in the early sixties to study DNA, RNA, and proteins and their respective roles in early embryonic development were applicable to mammals as well as invertebrates and lower vertebrates. These methods were essentially autoradiographic (Mintz, 1962, 1964; Hillman and Tasca, 1969), pharmacological (inhibitors of biosynthesis: Monesi *et al.,* 1970), and biochemical (Woodland and Graham, 1969; Pikó, 1970). This work was to provide most of our knowledge on general trends in gene expression during the first stages of development, paving the way for current research on the molecular aspects of events such as the maternal-to-zygotic transition of the 1-cell stage (Schultz *et al.,* 1999), X-chromosome inactivation (Goto and Monk, 1998), and genetic imprinting, research which now exploits the methods of genetic engineering.

Genetic imprinting is a concept that should be familiar to every student. It owes its existence to the experimental and molecular embryology of the mouse. Continuing a line of research that began at the beginning of the 20th century, investigators subjected mamma-

Fig. 9. Adult mouse chimaera obtained by aggregation of two 8-cell embryos and born in Brussels on March 1985.

lian eggs *in vitro* to diverse physical treatments (handling, pricking, heat shock) and chemical treatments (ethanol, hyaluronidase, ionophores) in order to induce their activation (Graham, 1974; Tarkowski, 1975, Kaufman, 1978). The hope was to promote artificial parthenogenetic development like that of the echinoderms and amphibians (Loeb, 1913; Delage and Goldsmith, 1913; Bataillon, 1929). These attempts are described and analysed in several excellent reviews (Graham, 1974; Tarkowski, 1975, Kaufman, 1978), but all of them failed. In their efforts to explain the apparent impossibility of producing a parthenogenetic mammal, McGrath and Solter on the one hand (McGrath and Solter, 1984) and Barton, Surani and Norris on the other (Barton *et al.,* 1984), reached the conclusion that the maternal and paternal genetic complements are qualitatively complementary. In some very elegant pronucleus transplantation experiments, these investigators showed that both gynogenetic and androgenetic embryos are as incapable as parthenogenotes of developing to term. Not only was the interpretation proposed by these authors verified, but it also opened a new and particularly fertile field of investigation in modern genetics: the study of the molecular mechanisms underlying complementary imprinting of the gamete genomes by the two sexes and its erasure post-fertilisation (see Arney *et al.,* this issue). Genetic imprinting now has a place in all developmental biology and genetics textbooks.

A non-egg model for studying the developmental biology of mammals

Obviously embryos are the best material for studying embryo development, but the scarcity and requirements of mammalian eggs can be daunting. Never discouraged, biologists have developed models to avoid such difficulties. This explains why specialists of mammalian development were so interested in a very rare tumour, teratoma, and its malignant variant teratocarcinoma (Pierce's nomenclature - 1967). The first to describe this model in the mouse were Stevens and Little (1954). Their focus was a testicular teratoma found to be abnormally frequent in mouse line 129. It contains many tissues corresponding with different stages of differentiation. All of its cells, whatever their type, derive from a population of pluripotent stem cells called embryonal carcinoma (EC) cells, themselves issued from germinal cells. The resemblance of EC cells to pluripotent embryo cells, as regards both their morphology and their developmental potency, is what made them a favoured model and substitute for embryos. The model was given a determining impetus by Barry Pierce (Pierce, 1967; Arechaga, 1993), to whom the International Journal of Developmental Biology paid a justified homage by publishing in his honour a special issue on Developmental Aspects of neoplasia (Vol. 37, No. 1, 1993).

It became the sole subject of a 743-page book containing the talks presented at a meeting held at Cold Spring Harbor Laboratory in September 1982 (Teratocarcinoma Stems Cells. Cold Spring Harbor Conferences on Cell Proliferation, edited by L.M Silver, G.R. Martin and S. Strickland). This was the second symposium devoted to these cells, the first having been organised in 1975 by Michael Sherman and Davor Solter, at the Roche Institute in Nutley. Leroy Stevens, who had discovered and characterised the providential tumour 28 years earlier, was surrounded by some 150 scientists, including geneticists, molecular biologists, virologists, immunologists, cytologists, oncologists, and more in addition to the main pioneers of the experimental embryology of mammals. All of them were fascinated by the immense potential of «the model» (see for instance Jacob, 1978, 1983). Meanwhile, the model had become emancipated from its initial context. L. Stevens had demonstrated the existence of ovarian teratomas resulting from the activation and parthenogenetic development of oocytes in line LT/Sv, which became as famous as line 129 (Stevens and Varnum, 1974). More importantly, several methods for producing teratomas experimentally had emerged. For this, credit is again due to L. Stevens, who was the first to obtain experimental teratomas by transplanting foetal genital ridges, tubal mouse eggs, or post-implantation mouse embryos into testes of isogeneic adult recipients (Stevens 1964, 1968, 1970).

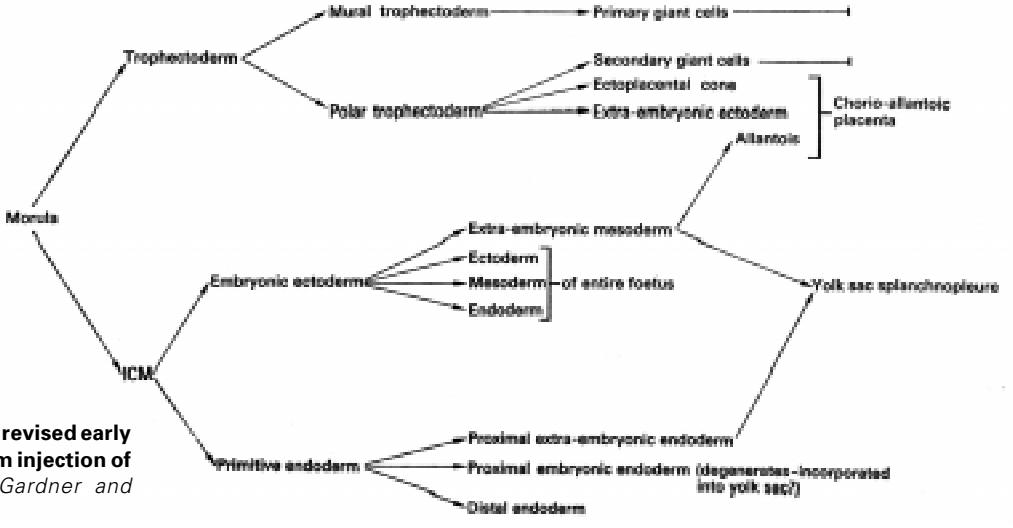

Fig. 10. Diagrammatic representation of revised early cell lineages in the mouse, resulting from injection of embryonic cells into blastocysts *(Gardner and Papaioannou, 1975).*

The famous Zagreb School of mammalian embryology, founded by Nikola Skreb (Svajger, 1991) and mentioned at length in the special issue that the International Journal of Developmental Biology devoted in 1991 to Developmental Biology in Yugoslavia (Vol. 35, No. 3), was also closely associated with the emergence of experimental teratomas. N. Skreb's first contact with mammalian embryology took place in the early 1950's. A visit to A. Dalcq's laboratory in Brussels aroused his interest in the morphogenetic gradient hypothesis. He began to work on it upon returning to Zagreb, but failed to confirm the existence of such gradients in bat eggs and became one of Dalcq's first contradictors on the subject (Skreb, 1953; 1957). He then focused on gastrulation in the rat, showing that this crucial developmental stage is very sensitive to X-rays (Skreb and Bulic, 1962). Ten years later this work attracted my attention when, drawn into the radiobiology current which was then generously funded by Euratom, I did some work on the effects of X-rays on the preimplantation stages of the mouse (Alexandre, 1974). The Zagreb school became known mainly for its work on the developmental potentialities of the three germ layers (definitive ectoderm, mesoderm and endoderm) isolated from rodent embryonic shields and transferred to various extrauterine sites (Skreb *et al.,* 1976, Skreb *et al.,* 1991; Levak-Svajger *et al.,* 1991). These experiments were preceded by transplantation of whole egg cylinders into the anterior eye chamber or beneath the kidney capsule, where they developed into teratocarcinomas (Damjanov *et al.,* 1971; Skreb *et al.,* 1971). These were studied in detail by Damjanov and Solter (1974).

In the wake of this remarkable work, there have been a number of reports describing the establishment of EC cell lines (Martin and Evans, 1974). A complete list compiled in 1983 by Gail Martin included nearly a hundred cell lines and sublines issued from mouse teratocarcinomas (Martin, 1983). Although these lines remained malignant, some were nullipotential and others retained the power to differentiate into a many cell types. This was shown both *in vitro* and *in vivo* (following injection into syngeneic adult mice: Damjanov and Solter, 1974; Jacob, 1978). Under these conditions, however, differentiation followed no set order. It was therefore tempting to study the behaviour of these cells in association with normal embryonic cells. Applying the blastocyst injection technique developed by R. Gardner (1968), R. Brinster obtained the first mouse chimaera from EC cells (Brinster, 1974). The teratocarcinoma cells had thus been «normalised». These experiments were continued by K. Illmensee in B. Mintz's laboratory in Philadelphia and by V. Papaioannou and M. McBurney in R. Gardner's lab in Oxford, where I spent the summer of 1975. I admired this little group of enthusiastic researchers to which Janet Rossant also belonged. So patiently, their hands busy with the two micromanipulators and their eyes glued to the microscope, they delicately introduced primitive ectoderm cells, primitive endoderm cells, or teratocarcinoma cells into an impressive number of blastocysts, then transferred the blastocysts into pseudopregnant females. Then they waited - and the wait seemed always too long - for confirmation of chimaerism. Despite remarkable successes yielding papers that are now historic (Mintz and Illmensee, 1975; Papaioannou *et al.,* 1975), they soon had to accept that most established teratocarcinoma cell lines were aneuploid and had lost their pluripotential character (Kahan and Ephrussi, 1970; Evans, 1972). Furthermore, it proved impossible to transmit a mutation to the germ line of any of the rare chimaeras obtained (Papaioannou, 1979). This precluded using this strategy as a means of producing genetically transformed mouse lines. Yet the road had been opened: the developed methods would later be applied to pluripotential cell lines called embryonic stem (ES) cells, derived directly from blastocyst ICM. This was achieved independently by M.J. Evans and M.H. Kaufman (1981) on the one hand, G. Martin (1981) on the other. It coincided with the first steps in transgenesis by injection of DNA into the zygote (Gordon *et al.,* 1980; Costantini and Lacy, 1981; Palmiter *et al.,* 1982; see also Papaioannou, 1998). In 1986, ES cells were shown to allow the derivation of transgenic strains with pre-determined genetic changes (Robertson *et al.,* 1986). A few months later, site-directed mutagenesis of ES cells by homologous recombination (Doetschman *et al.,* 1987; Thomas and Capecchi, 1987) was to usher in the era of knock-out mice. Then came cloning by nuclear transfer into oocytes (Wilmut *et al.,* 1997). These spectacular advances opened the prospect of exciting applications in basic and medical research and biotechnology.

Such is the legacy of those few pioneers of the experimental embryology of mammals who, in the late fifties, were striving to make the wish expressed by A. Brachet in 1912 come true at last. Anne McLaren was among them. Her major contributions to the vast field of reproductive and developmental biology fully justify the homage paid to her in this special issue of IJDB. I join in this homage with respect and admiration.

Summary

Although Reinier DE GRAAF (1641-1673) can be considered the founder of modern reproductive biology, scientific knowledge of mammalian development did not progress significantly until the XIXth century. Determining contributions to this progress were the discovery of the ovum by Karl von BAER (1792-1876), his meticulous observations of the stages of embryogenesis, and, half a century later, the remarkable descriptions made by Edouard VAN BENEDEN (1845-1910) of egg development in rabbits and bats.

Yet mammalian embryology remained a purely descriptive discipline until the second half of the XXth century, when a handful of exceptional scientists (notably including John D. BIGGERS, Ralph BRINSTER, Anne McLAREN, and W. WHITTEN) managed to obtain reproducibly the development of mouse eggs in a chemically defined medium and to transfer the eggs to the uterine horns of pseudopregnant females. Around the same time (1959), M.C. CHANG was the first to obtain a mammal (a rabbit) by *in vitro* fertilisation, thus opening the way to assisted procreation. This was achieved in our species in 1978, by Robert EDWARDS and Patrick STEPTOE. With these feats, mammalian embryology could at last become causal, as A. BRACHET already in 1912 had hoped it would. New concepts soon emerged from the delicate manipulations performed on mouse eggs by scientists such as A. TARKOWSKI, B. MINTZ, J. MULNARD, and R. GARDNER, concepts such as the oustside-inside hypothesis proposed to explain the determination of the ICM and trophectoderm or the clonal theory of cell determination during development. These new ideas were soon to become the focus of intense study. Other investigators, interested in the synthesis and roles of macromolecules, contributed in the late 1960's most of our knowledge on global trends in gene expression during the first stages of development. As for the many unfruitful attempts to obtain artificial parthenogenetic development in mice, these would lead to the discovery of parental genetic imprinting.

In the 1950's, Leroy STEVENS and Barry PIERCE made famous a very rare tumour, the teratocarcinoma. This tumour soon became a model for studying mammalian development, adopted by an increasing number of research groups. It became the source of a first generation of pluripotent cells culturable *in vitro*: embryonal carcinoma (EC) cells. In the 1980's came the next generation: embryonic stem (ES) cells derived from the ICM of blastocysts, whose advent coincided with that of the first transgenic mice. Then came the era of knockout mice and cloning.

Scientists now envisage with enthusiasm applications that were unimaginable just a few years ago. Such is the legacy of those few pioneers of the experimental embryology of mammals who, in the late fifties, were striving to make the wish expressed by A. Brachet in 1912 come true at last.

Acknowledgement

I wish to express my profound gratitude to Professor Jacques MULNARD, who guided my first steps in the marvelous adventure of mouse embryology, who later accepted me as a coworker, and who today honours me with his friendship. I also thank him for his help in writing this historical account. I thank Mr. D. Franckx (IBMM, Université libre de Bruxelles) for his help in preparing the illustrations.

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