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FROM THE GERMINAL CELLS TO THE NEWBORN ANIMAL: THE TRANSMISSION OF GENES AND LIFE THROUGH THE GENERATIONS*

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The technology of reproduction progressed considerably during the last decade, leading to a certain availability of *in vitro* methods for fertilisation, oocyte maturation and embryo culture. The most spectacular manipulations are cloning and transgenesis. This review focuses on the early appearance of germinal cell precursors and the long-standing fate of gametes in mammals. The evident complexity and long-term programming of events in gametes and early embryos explain part of the difficulties encountered during the development of *in vitro* and *in vivo* methods such as multiple ovulation and embryo transfer (MOET), oestrus synchronisation, ovulation induction, superovulation, *in vitro* maturation and fertilisation, cryopreservation, transgenesis, nuclear transfer and cloning) and the occurrence of unexpected alterations of development, e.g. embryonic or fetal mortality, large-weight newborn syndrome and other dysregulations in imprinting or DNA transmission.

Key words: Cattle, germinal cells, follicular growth, *in vitro* methods

Germinal cell precursors (PGC) clearly appear early after the fertilisation of the oocyte. In mice, around Day 7 after conception, a few (probably four to eight) large cells with a cytoplasm rich in ribonucleic acid (RNA) and positive for alkaline phosphatase reaction can be identified in the nascent extra-embryonic mesoderm. The surgical removal of this area leads to an embryo lacking the germinal cells (GC), whereas the graft of the removed cells produces large numbers of germinal cells (Ginsburg et al., 1990).

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So, originating in the extra-embryonic mesoderm, the precursors of germinal cells enter the embryo: first they invade the mesoderm, near the primitive line and then, after regrouping in the allantois around Day 7.5, they enter the endoderm to migrate in the yolk sac (Mintz, 1975). The cells divide into two populations and each of them migrates in the genital area of the embryo (left and right).

Constitution of the germinal cell (GC) reserve

Multiplication of the 4 to 8 primordial germinal cells

Most of the precursors of germinal cells reach the area of the future gonad as early as Day 11 after fertilisation. During the migration process, these cells divide actively: starting from 10 to 100 cells, they rapidly reach 2500 to 5000 cells (in the future gonad around Day 12). The determinism of germinal cells multiplication is still incompletely understood. However, the collaboration between somatic cells and growth factors including *Leukaemia Inhibitory Factor* (LIF) and *basic Fibroblast Growth Factor* (bFGF) seems to be essential for their survival and proliferation: a first gene (called W-gene) encodes a tyrosine kinase receptor which ligand is represented by *the mast cell growth factor* (Dolci et al., 1991) encoded by another gene (the *Sl*-gene; Zsebo et al., 1990). Gene–receptor interaction induces the production of growth factors by somatic cells, essential for GC multiplication.

How the migrating PGC reach their definitive localisation site

The interactions involved in the migration process include rearrangements of tissues (mechanical factor) as well as fibronectin (Ffrench-Constant et al., 1991), which role in PGC migration has recently been studied: a local and developmentally regulated decrease in adhesion of PGC to fibronectin in the hindgut is correlated with the initiation of their migration and permits the cells to leave this area by active migration. These changes in cell-cell adhesion could also play a role in the initiation of the PGC migration by an active down-regulation of cell-cell adhesion molecules. Alkaline phosphatase has also been implied in PGC migration in salamander, suggesting its possible role in mammals (Zackson and Steinberg, 1988). Growth factors (Ffrench et al., 1991; Dolci et al., 1991) and possible chemotactic substances originating from the gonads are also described in the active PGC migration (Godin et al., 1990; Godin and Wylie, 1991).

Some genes are implied in the control of PGC multiplication

Several genes with influence on the setting-up of germinal cell population have been identified. For example, dysfunction of the tyrosine kinase receptor 'kit/Mgf' leads to the death of the cells before they reach the primitive gonads

(Besmer et al., 1993). Other experimental inactivated genes coding for proteins that bind to ribonucleic acid lead to absence of germinal cells, gonadal agenesis and sterility in both sexes (Ruggiu et al., 1997; Beck et al., 1998). Abnormal meiosis was also observed in females with inactivated serine-threonine-kinase-*mos* gene implied in cellular cycles. In these cases, no spontaneous arrest of meiosis appears after ovulation, resulting in spontaneous (parthenogenetic) activation of oocytes and sterility of the females (Colledge et al., 1994).

Constitution of the 'birth primordial follicle pool'

In mammals, birth coincides with or just follows the achievement of the mitosis multiplication stage of the oogonia (Baker, 1963). In cow, this mitotic multiplication occurs from Day 45 till Day 150 of intrauterine life, with ovaries of the young fetal female reaching a maximum of 2×10^6 oogonia (Erickson, 1966*a, b*). The meiotic process begins as soon as Day 80 of fetal development in the cow (Erickson, 1966*a*; Mauleon et al., 1967; Jost, 1972), with a blockade in the prophase of meiosis I (oocyte I) even before complete achievement of mitotic stage. Only the oocytes that will be surrounded by some follicular cells (3–4) and a basal membrane will survive to constitute primordial follicles. This process explains the lower number of oocytes remaining at the birth: 235,000 in cow (Erickson, 1966*a, b*), 160,000 in ewe (Driancourt et al., 1985; Driancourt et al., 1991), 1,000,000 in humans (Gougeon, 1984) and 20,000 in rat (Hirshfield and Midgley, 1978).

Prophase of the meiosis I

Oogonia enter in meiosis earlier than birth. The Meiosis Inducing Substance (MIS) originating from the internal side of the ovary positively influences the process (Byskov, 1979; Westergaard et al., 1985) while oocyte meiosis inhibitors (OMIs) including cAMP, unisolated peptide, hypoxanthine and adenosine are responsible for the meiotic arrest (prophase I) (Eppig and Down, 1988). OMIs are produced by granulosa cells (Sirard et al., 1989) and transmitted to the oocyte by the numerous gap junctions existing between these cells (Szőllősi, 1978). The result is an increase in intra-oocyte cAMP concentrations, responsible for the arrest of cellular cycle (Schultz, 1987).

Endocrine and paracrine factors in the ovary: influence on follicular growth (for a review see Drion et al., 2000; Hanzen et al., 2000)

We can still remember the sentence of Greenwald (1972): 'one of the most intriguing mysteries in the ovarian physiology is what factor determines whether one follicle remains quiescent, another begins to develop but later become atretic, while still a third matures and ovulates'.

In fact, two main phases have been described for follicular growth in mammals. The first one, the 'basal' growth concerns follicles, from the primordial stage till the preantral stage (Hage et al., 1978; Driancourt, 1991; Driancourt et al., 1991). This phase is mainly under paracrine control of growth factors (GF) (Peters et al., 1973). The second phase is described as dependent on gonadotropins and concerns preantral follicles till ovulation (Dufour et al., 1979; Moser et al., 1989). It is also well known that mature Graafian follicles are capable of concentrating steroids and gonadotropins in the follicular fluid. Certain molecules present in the serum are excluded from follicular fluid, leading to the conclusion that the follicular fluid is a semi-protected environment (Aggarwal et al., 1999).

Growth factors are implied in follicular growth (Bendell and Dorrington, 1990; Evain-Brion, 1991; Lobb and Dorrington, 1992; Meidan et al., 1992)

The respective effects of EGF (Epidermal GF), IGF (Insulin-like GF), TGFβ (Transforming GFβ), TGFα (Transforming GFα), FGF (Fibroblast GF), inhibin, activin and follistatin on granulosa cells of follicles have been described for various species *in vitro*. However, due to the ubiquity of these factors, it still remains unclear whether these results can be applied to *in vivo* follicular growth. Only few mutations concerning growth factors encoding genes are compatible with life. This explains the relatively poor information obtained from *in vivo* experiments aimed at modifying gene expression and comparing the observed effects with physiological situations. These experiments could only be applied to some encoding genes (GDF9 – Growth Differentiation Factor 9, MGF – mast/stem cell growth factor, Kit – transmembrane tyrosine kinase receptor gene, and IGF-1). For example, inactivation of IGF-1 gene expression leads to blockade of follicular growth in preantral stage even unblocked by a gonadotropic stimulation (Baker et al., 1996).

Gonadotropins as modulators of folliculogenesis

Numerous studies have permitted to establish/confirm the primordial role played by gonadotropins in terminal follicular growth.

Mice with experimentally deleted distal part of the encoding gene for GnRH (inactivated α- or β-subunit of gonadotropins) present arrest in hypophyseal gonadotropin production and blockade in folliculogenesis (Mason et al., 1986; Kendall et al., 1995; Kumar et al., 1997). If spontaneous abnormalities of encoding gene for gonadotropins lead to a relatively high frequency of infertility in human or murine females, it is rather surprising to note that in the male, perturbations in the levels or action of FSH slightly modify fertility levels, even with affected spermatogenesis.

In the other way, elevations of plasmatic gonadotropin levels dependent on inactivated inhibin α-subunit encoding gene or overexpression of LH lead to

early gonadal tumours (granulosa or theca), lower fertility, ovulation troubles and prolonged luteal phases (Matzuk et al., 1992; Risma et al., 1995; Kumar et al., 1996). These furiously evoke the symptomatology of cystic ovaries as well as a higher frequency of ovarian cancer in menopausal woman presenting a physiological increase in plasmatic pituitary gonadotropins, allowing urinary purification of human menopausal gonadotropins (hMG).

Preovulatory gonadotropin surge

Resumption of meiosis is dependent on follicular oestrogens. Their production by the preovulatory follicle induces positive retroactive control on the hypothalamo-hypophyseal-gonadal axis (Callesen et al., 1986). Most of the gap junctions existing between granulosa cells, between granulosa and cumulus cells and even between cumulus cells disappear after the gonadotropin surge, resulting in the isolation of the oocyte from inhibitor factors (Szőllősi, 1978). The first meiotic division is completed when first polar globule ejection occurs with consecutive blockade of the oocyte in metaphase of second meiotic division (Metaphase II), waiting for a hypothetical fecundation.

Steroid and peptide hormones: endocrine dysregulation leads to abnormal sexuality

The implication of steroid and peptide hormones in the maintenance of morphological and functional characteristics of the genital tract as well as in sexual behaviour of the males and females is well known. Inactivation of oestrogen receptors is associated with uterine hypoplasia, high frequency of follicular atresia as well as cystic and haemorrhagic follicles, depleted in granulosa cells (Lubahn et al., 1993). At the opposite, inactivation of the aromatase-encoding gene leads to unovulatory follicles (Fisher et al., 1998). Inactivated progesterone receptors prevent ovulation even after gonadotropic stimulation (Lydon et al., 1995).

Hyperprolactinaemia (hypophyseal adenoma or treatment-induced; Vitse, 1984) or hypo- or aprolactinaemia (inactivated encoding gene or inactivated receptors) respectively provoke amenorrhoea (negative hypophyseal retroaction) and irregular oestrous cycles, reduced ovulation rates and failures in implantation and growth of mammary gland (Ormandy et al., 1997; Horseman et al., 1997).

In the same way, euthyroid status of the female is necessary to ensure ‘normal’ gonadal function. Primary hyper- or hypothyroidism (Berga et al., 1989) is frequently associated with dysfunctions of the hypothalamo-hypophyseal axis, while secondary hypothyroidism – hypothalamic amenorrhoea in mental anorexia (Schwartz, 1997; Schwartz and Selley, 1997) – associated with reduced T3 circulating levels leads to variations in neurohormonal hypothalamic secretions.

The surprising length of complete follicular growth partially explains the elevated loss rate of ovarian follicles

Numerous questions still remain concerning the growing speed of small follicle in the majority of mammals and in cows. It was, however, established that approximately 100 to 150 days are necessary for a primordial follicle to reach the preantral stage and again 40 to 60 days to develop into a preovulatory one (Hirshfield and Midgley, 1978; Cahill and Mauleon, 1980; Gougeon, 1982; Hirshfield, 1991).

Only few follicles (0.005%) reach the preovulatory stage and most of them undergo atresia, i.e. an atretic follicle can be identified by picnosis and/or apoptosis of granulosa cells and/or degenerative oocyte alterations (Hirshfield, 1989). The loss of expression or activity of P-450 aromatase in granulosa cells and of sensitivity to gonadotropins is frequently associated to atresia of terminal growth follicles, leading to a decrease in oestrogen/androgen levels in the follicular fluid. In the same way, marked elevations in expressions of IGF-binding proteins (types 2, 4, 5) encoding genes, as well as diminution of their proteolysis have been described in atretic follicles (Cataldo and Giudice, 1992; Monget et al., 1993).

Apoptosis or programmed cell death (Willie, 1980; Schwartzman and Cidlowski, 1993) has been demonstrated in follicles and has been proposed as being one of the elements of follicular atresia (Hughes and Gorospe, 1991).

Due to the experimental difficulty to investigate the first period of follicular growth in physiological conditions, the first attempts to control oestrus and ovulation were focused on the latest period of folliculogenesis: oestrus synchronisation or induction, and induction of superovulation were the first successful manipulations of folliculogenesis. More recently, *in vitro* methods have been developed in most countries to better exploit the reproductive capacities of the females. Culture methods have also been developed for oocyte maturation and *in vitro* growth of follicles.

Physiological status of the female: influence on sexual function

Puberty, follicular maturation processes, luteal phases and other reproductive functions are related to the energetic status of the organism. The body weight, body condition score, qualitative composition of the body, distribution of adipose tissue and food intake all influence sexual maturation and function (Bringer et al., 1997).

Every quantitative or qualitative variation in food intake can modulate the hypothalamo-hypophyseal-gonadal activity (Frisch, 1987; Sanborn et al., 1987). These variations are mediated by secondary modifications (Bringer et al., 1999) in metabolism (glucose, free fatty acids, ketone bodies), hormonal signals (leptin,

insulin, IGF-1&2, IGF-binding proteins) and neuropeptides (cortico-releasing hormone – CRH, Y-neuropeptide – YNP, opioids, catecholamines and serotonin).

So, due to the extended period for primordial follicles to reach terminal development and ovulation, it appears to be obvious that the consequences of long-term malnutrition, stress, inadequate management conditions, or breeding can influence numerous following cycles (Butler and Smith, 1989; Lucy et al., 1991).

In cows, suckling negatively affects the recovery of follicular growth. Suckling influences secretion of cortisol by the adrenal gland, inhibiting the hypothalamo-hypophyseal axis and disrupting ovulation of antral follicles *via* inhibition of LH release (Derivaux et al., 1984). Moreover, numerous studies have demonstrated the influence of postpartum negative energy balance on the resumption of physiologic ovarian cycles in high-producing dairy cows (Butler, 1998). In these cows, the lactation peak frequently precedes recovery of the maximal ingestion capacity. These females go through a period of negative energy balance, positively correlated with the milk production level. Insulin, hypothalamic opioids and ketosis negatively interfere with the gonadotropic activity of the hypothalamus and directly affect ovarian follicles (MacMillan et al., 1996). It has been proposed to supplement the feed with (poly-) unsaturated fatty acids that could increase the plasma levels of GH, insulin, IGF-1 and lipoproteins, all responsible for the stimulation of ovarian metabolism (Williams, 1996).

From maternal transcripts to embryonic transcripts: fecundation just precedes a long-term period of subtle gene regulation

The newly ovulated egg contains maternal messenger ribonucleic acid capable of serving as a template for protein synthesis (Braude et al., 1979). Changes in protein synthesis following fertilisation are related to both post-translational modifications (Van Blerkom, 1981; Van Blerkom and Runner, 1984) and utilisation of the maternal stored mRNA (Howlett and Bolton, 1985). It appears that the rate of protein synthesis does not increase within 24 hours after fertilisation (Brinster et al., 1976) which could be explained by a relative deficiency in oocyte ribosomes (Bachvarova and De Leon, 1977).

During the early cleavage mammalian embryos begin to rely on products of the zygotic genome. Protein synthesis before the 2-cell stage of mouse embryo is transcription independent (Braude et al., 1979; Petzoldt and Hoppe, 1980). On the other hand, development beyond the 2-cell stage requires protein synthesis (Bensaude et al., 1983). A progressive increase in mRNA synthesis is then observed, with the total amount of mRNA accumulated at the 8-cell stage approaching that present in the ovulated egg and the amount of histone still being only one-fourth that of the egg (Giebelhaus et al., 1985).

The transition from 'maternal transcripts' to embryonic transcripts (zygotic genome activation) occurs at the 2-cell stage in hamster embryo, between 2-cell and 16-cell stages in rabbit embryo, 4- to 8-cell stage in human embryo, 8- to 10-cell stage in pig embryo and 8- to 16-cell stage in sheep and cow embryo (Braude et al., 1988; Telford et al., 1990; Seshagiri et al., 1990; Jarrell et al., 1991). It seems that e.g. in the mouse some early transcripts resulting from transcription of paternal pronucleus occur between fertilisation and 2-cell stage embryo (Ram and Schultz, 1993).

In vitro manipulations: possible consequences on gene expression and embryo formation

Multiple ovulations (MO) and embryo transfer (ET), oestrus synchronisation, induction of ovulation, superovulation, *in vitro* maturation and fertilisation, and cryopreservation constitute expanding techniques in the management of reproduction. They also allow the realisation of procedures like transgenesis, nuclear transfer and cloning (Ectors et al., 1975).

As in mice (Eppig, 1976), rats (Daniel et al., 1989), pigs (Hirao et al., 1992) and cats (Jewgenow and Pitra, 1993) the isolated preantral follicles had been cultured and growth of the follicles was observed, such cultures were also attempted in the bovine species (Nuttinck et al., 1993; Figueiredo et al., 1994; Hulshof et al., 1994; Hulshof et al., 1995).

All these investigations describe techniques usable for isolating large numbers of preantral follicles, media for *in vitro* culture of morphologically unaltered follicles and media suitable for increasing follicle growth *in vitro*.

However, some mysteries still remain: it has been established (Fair et al., 1995) that bovine oocytes are not fully grown when the follicle reaches the antral stage. This suggests questions about the ability (obtained *in vivo*!) of oocytes punctured from preantral follicles to acquire complete competence without spontaneous resumption of meiosis while cultured, to be fertilised and develop into a normal embryo.

The last unsolved limiting factor in MOET is the individual variability affecting the number of fertilised ova and the quality of the recovered embryos. It remains difficult to classify the possible sources of variation: repeat-breeder cows, previous repeated treatment using various xenogenous gonadotropins such hMG, equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) leading to active immunisation against the exogenous hormones (Drion et al., 1998), nutritional conditions or possible ovulation of incompletely matured oocytes in such cows. If the higher variation in the weight of newborn calves derived from *in vitro* fertilisation (IVF) may be explained by an incomplete maturation of oocytes at the time of fertilisation, it is also possible that MO concerns smaller fol-

licles yielding incompetent or partially competent oocytes. Alternatively, the higher variability in the weight of calves originating from cloning (Wilson et al., 1995) may be due to the *in vitro* exposure of the gametes and (or) the embryos. These questions are worthy of an experimental confirmation in a contemporary comparison of ongoing pregnancies after transfer of embryos produced in normal non-stimulated cycle and after superovulation induction, using of course the same combination of donor, bull and recipient because these parameters influence the endocrinological pattern of pregnancy (Guilbault et al., 1990).

All the considerations found in the literature on the reproductive physiology of mammals sometimes let us imagine that a newborn animal has survived two pregnancies: the first one would be a high-risk pregnancy that represents the oocyte development and survival inside the follicle with a tetraploid genome and the zona pellucida and cumulus cells as placenta-like structures. Around the 'first birth' (ovulation), oocytes (individuals) accept *in vitro* conditions for maturation, fertilisation and culture. Afterwards, a 'second pregnancy' will be necessary in the mother or in the recipient uterus; a low-risk pregnancy depending on the acquisition of oocyte competence and on the conditions or length of exposure *in vitro* (Beckers et al., 1996).

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