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Twenty years of research on primordial germ cells

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ABSTRACT Just twenty years ago I was preparing a research project centred on establishing methods for the isolation and culture of mouse primordial germ cells (PGCs). The project had been suggested to me by Anne McLaren and was to be developed at the Medical Research Council (MRC) "Mammalian Development Unit" in London under the direction of Anne herself. At that time I was a young postdoctoral researcher at the Institute of Histology and Embryology of the University of Rome "La Sapienza" and did not imagine that my decision to be involved in this project would signal a profound switch in my scientific life. From then on my research would mostly concentrate on primordial germ cell biology. I feel like saying that the modern history of mammalian primordial germ cells began twenty years ago at the MRC Mammalian Development Unit under Anne McLaren's impulse. It is not surprising that among the most active researchers in the last twenty years in studying mammalian primordial germ cells, three, namely Chris Wylie, Peter Donovan and myself, began their studies under Anne McLaren's guidance. Over the years, Anne's suggestions and encouragement were always precious for my studies and her presence marked my most important findings on PGC biology. She often invited me to present the results obtained in my laboratory to workshops and congresses. In the present article some of these results particularly influenced by Anne's teaching and suggestions will be briefly reviewed.

KEY WORDS: *primordial germ cell, gametogenesis, stem cell factor, leukemia inhibitory factor, meiosis.*

The long saga of purification and culture of mouse primordial germ cells

Primordial germ cells (PGCs) are the founders of the gametes. They arise at the earliest stages of embryonic development and migrate to the gonadal ridges, where they differentiate into oogonia/oocytes in the ovary and prospermatogonia in the testis. When I first became interested in mammalian PGCs, mainly morphological studies describing the main stages of PGC development had been performed. Thanks to their high alkaline phosphatase activity, PGCs had been identified in an extraembryonic region near the yolk sac, early in embryogenesis, and traced in their migratory route towards the gonadal ridges (for a review, see Buher, 1997). In the mouse, PGCs first appear in the early 7-day embryo as a cluster of 50-100 alkaline phosphatase positive cells at the base of the allantois (Ginsburg *et al.,* 1990). From this region, PGCs translocate into the endoderm of the yolk sac and hind gut. At 10 days *post coitum*, they begin to move by active migration up the dorsal mesentery and into the gonadal ridges where, around 12.5 days *post coitum*, they differentiate into oogonia/oocytes and prospermatogonia in the ovary and testis, respectively (for a review, see De Felici, 2000).

The main goals of my early experiments carried out in Anne McLaren's laboratory at the MRC Mammalian Development Unit was to develop methods for the isolation, purification and culture of mouse PGCs at different developmental stages. We anticipated that the possibility of studying PGC behaviour *in vitro* would allow uncovering some of the mechanisms underlying their development. Using a relatively simple method based on EDTA and mechanical disaggregation of gonadal tissues, we obtained an enriched PGC population from post-migratory stages (12.5-13.5 days *post coitum* embryos). Moreover, the use of a discontinuous Percoll gradient allowed us to increase the purity of such PGC populations up to 80-90% (De Felici and McLaren, 1982). Their very low numbers, their migration throughout different tissues and the lack of specific antibodies, rendered difficult the task of isolating PGCs during their migratory stages. Nearly thirteen years later in my laboratory in Rome, the availability of monoclonal antibodies able to bind to PGC surface molecules (SSEA-1, Fox *et al.,* 1981; EMA-1, Hahnel and Eddy, 1986; TG-1, Donovan *et al.,* 1986) and the introduction of magnetic cell sorting techniques, allowed us to develop efficient methods for purifying PGCs from both migratory (10.5-11.5 days *post coitum*) and post-migratory (12.5-13.5 days *post coitum*) embryonic stages (De Felici and Pesce, 1995; Pesce and De Felici, 1995) (Table 1). Afterwards

Abbreviations used in this paper: EG cells, embryonic germ cells; ES cells, embryonic stem cells; LIF, leukemia inhibitory factor; PGCs, primordial germ cells; SCF, stem cell factor.

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Massimo De Felici with Anne McLaren at the CIBA Foundation Symposium on Germline Development held in 1993, London.

efficient purification of early mouse PGCs was also obtained using transgenic mice expressing *lacZ* under the control of the *Akp2*^{tm1Sor} mutant allele of the tissue nonspecific alkaline phosphatase (TNAP) and fluorescence activated cell sorter (FACS). It was curious that Anne visited my laboratory in Rome the same year we published our paper on the magnetic cell sorter purification of mouse PGCs. She was happy about our results and eager to know and see all the procedures of our new method. That completed one of the purposes of our original research project on PGCs. However, the other one, the *in vitro* culture of isolated PGCs, remained unfulfilled. Our first attempts at the Mammalian Developmental Unit had little success. In fact, we observed that germ cells isolated from embryonic gonads of different ages have different abilities to survive *in vitro* and that 11.5-12.5 days *post coitum* PGCs did not survive at 37°C in any of the several culture conditions employed (De Felici and McLaren, 1983). I remember the frustration in seeing PGCs invariably undergo rapid degeneration whatever culture condition, media and substrates we used. However, when I reported my failure to Anne on her return from her frequent participation in meetings and congresses, she had always words of encouragement and was able to see a positive side to such negative results. The main conclusions of these early studies are well summarised in the sentences we wrote in publishing our first paper on PGC culture (De Felici and McLaren, 1983):

> *" In their normal environment, PGCs do not at any time exist as an independent tissue, but are always closely associated with other cells from which they may derive nutrients as well as developmental signals. Such factors may prove critical in controlling the survival of germ cells in vitro and perhaps also their proliferation and development in vivo."*

Such prediction turned out to be true and some of these factors have now been identified. Although conditions for culturing isolated PGCs have not been achieved yet, the use of feeder layer culture for PGCs, has led to the establishment of *in vitro* culture conditions that allow their survival and proliferation, and the identification of survival and/or proliferation factors needed for PGC development (Table 1). The leukemia inhibitory factor (LIF) was the first growth factor discovered in my laboratory in Rome as able to significantly increase PGC survival *in vitro* (De Felici and Dolci, 1991). A second growth factor found to exert a very important action on PGCs was the stem cell factor (SCF). In 1990, SCF was identified as a novel growth factor ligand for the tyrosine kinase receptor c-kit encoded by the *White* (*W*) locus in hematopoietic cell lines. SCF was found to be encoded by the *Steel (Sl)* locus and produced from two alternatively spliced mRNAs as either transmembrane or soluble forms (for a review, see Besmer, 1991). Immediately afterwards, Matsui et al. (1990) demonstrated that SCF is produced by the somatic cells surrounding migratory and post-migratory PGCs, and that the c-kit receptor is expressed on the surface of PGCs. In 1991 Susanna Dolci, at that time a Ph.D. student in my laboratory, working with Peter Donovan's group at Frederick, showed, simultaneously with other groups, that SCF is essential for PGC growth *in vitro*, and that the PGC life-supporting activity of certain feeder cells is partly due to the production of this growth factor, in particular of the SCF membrane-bound form (Dolci *et al.,* 1991; Matsui *et al.,* 1991; Godin *et al.,* 1991). Experiments carried out in my laboratory in Rome confirmed and extended these results. In particular, we showed that recombinant mouse SCF added to PGCs growing on their own gonadal somatic cells, significantly increased the number of PGCs without directly stimulating their proliferation (Dolci *et al.,* 1993). Moreover, antibodies against c-kit receptor inhibited PGC survival on STO cell feeder layers (De

TABLE 1

MAJOR EVENTS IN THE DEVELOPING OF METHODS FOR PURIFICATION AND CULTURE OF MOUSE PRIMORDIAL GERM CELLS
(for references see text).

1982-1983	De Felici and McLaren: culture of PGCs purified by EDTA/Percoll gradient
1986	De Felici *et al.*: culture of PGCs on Sertoli and follicle cell feeder layers
	Donovan *et al.*: culture of PGCs on STO cell feeder layers
1987	McCarrey *et al.*: purification of PGCs by FACS
1991	De Felici and Dolci: LIF sustains the survival of PGCs in culture
1991	Dolci et al.; Godin *et al.*; Matsui et al.: SCF is required for PGC survival in culture
1992	Matsui et al.; Resnick *et al.*: production of embryonic germ cells (EG cells) from PGCs in culture
1993	De Felici *et al.*: stimulation of PGC proliferation in culture by cAMP-dependent pathways
	Pesce *et al.*: SCF and LIF promote PGC survival in culture by suppressing apoptosis
1995	Pesce and De Felici: purification of PGCs by magnetic cell sorting
1996	Capel *et al.*: establishment of putative immortalized PGC cell lines
1996	Abe *et al.*: purification of PGCs from TNAPβ^{-geo} mouse embryos using FACS-gal

Felici, unpublished observations). Most importantly, we found that the addition of SCF to the culture medium markedly reduced the number of apoptotic PGCs during the first hours of culture (Pesce *et al.,* 1993). More recently, we have shown that the effect of SCF on PGC apoptosis is, at least in part, due to a reduction of the expression of the pro-apoptotic gene *bax* (De Felici *et al.,* 1999). The finding that PGCs begin to undergo apoptosis a few hours after isolation from the gonadal ridges, gave finally an explanation for their rapid degeneration in culture. Clearly, PGCs must be prevented in the embryo from undergoing apoptosis by soluble factors and/or direct contact with the surrounding somatic cells. Interestingly, we have recently reported that the membrane form of SCF also promotes PGC adhesion to somatic cells (Pesce *et al.,* 1997). Most of these results on PGC apoptosis were the object of my presentation at the CIBA Foundation Symposium on Germline Development held in 1993 in London and chaired by Anne McLaren. Some years earlier Anne had invited me to the ESHRE Congress in Paris to present the results about the important role that cAMP plays in stimulating PGC proliferation *in vitro* (De Felici *et al.,* 1993).

Thus, two aspects of PGC development, their strict dependence on growth factors for survival and a cAMP-dependent pathway for proliferation, were eventually discovered thanks to *in vitro* culture studies. Both findings were to have important implications for explaining the germ cell deficiency characteristic of *Sl* and *W* mutations in the mouse (Russel, 1977) and for production of pluripotent ES-like cell lines (EG cells) from mouse and human PGCs *in vitro* (Matsui *et al.,* 1992; Resnick *et al.,* 1992; Koshimizu *et al.,* 1997; Shamblott *et al.,* 1998).

An important aspect of PGC *in vitro* culture that remains to be investigated is the possibility to obtain immortilized PGC lines. Although we failed to obtained immortalized PGCs from transgenic SV40 large T-antigen (De Felici and Rassoulzadegan, unpublished observation), others reported long term survival of putative mouse PGCs from transgenic mice carrying a temperature sensitive SV40large T-antigen (Capel *et al.,* 1996). However, only preliminary characterization of the derived clonal lines was performed and no further use of such cell lines were reported.

Regulation of germ cell meiosis and differentiation of germ cells in the foetal gonads

What determines the phenotypic sex of primordial germ cells, i.e., whether they undergo oogenesis or spermatogenesis? Until the time when primordial germ cells enter the gonadal ridges their appearance and behaviour seem identical regardless of whether they are in a female or a male embryo. However, around 13.5 days *post coitum* in the female, primordial germ cells enter the prophase of meiosis; in the male they undergo mitotic arrest and differentiate as prospermatogonia in Go stage of the cell cycle.

The mechanisms of primordial germ cell sex differentiation and entering into meiosis has long interested Anne McLaren who has made important contributions to understanding the problem (McLaren, 1984; McLaren and Buher, 1990, McLaren and Southee, 1997). In 1983, when I left her laboratory one of the things she suggested to me was to devise an *in vitro* system for studying the influence of the tissue environment on the beginning of meiosis in primordial germ cells. Do the female gonadal somatic cells induce prenatal entry into meiosis, or does the male ridge inhibit it, or both?

Several years later in my laboratory in Rome we developed a method to reaggregate *in vitro* PGCs and gonadal somatic cells of the opposite sex to make chimeric gonads and to culture them *in vitro* in order to study the effect of the somatic environment on this process. The results obtained showed that 12.5 days *post coitum* PGCs isolated from female gonads enter and progress through meiotic prophase in chimeric gonads made with somatic cells from ovaries but not those made from testis. On the other hand, male PGCs of the same age were unable to enter into meiosis in either type of reaggregates (Dolci and De Felici, 1990). These results strongly support the existence of a meiosis-preventing substance in the fetal testis, but not of a meiosis inducing substance in the fetal ovary. Using an approach similar to ours, McLaren and Southee (1997) reached similar conclusions. Very recently, we have made a further contribution to this problem. We found that both female and male primordial germ cells express the meiotic specific synaptonemal complex SCP3 protein and that in the male such expression is rapidly down regulated as PGCs undergo Go arrest (between 13 and 14 days *post coitum*, Di Carlo *et al.,* 2000). This strongly supports the hypothesis that PGCs are programmed to enter meiosis irrespective of their sex and that foetal testis produces a factor that inhibits this programme. Not surprisingly Anne has been the first to send me her congratulations on this paper.

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