Université de Liège

Long term culture and characterization of chicken primordial germ cells.

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Introduction:

As precursors of germ cells, avian primordial germ cells (PGCs) are foreseen as promising tools to develop avian transgenesis and preservation of genetic resources of endangered species. PGCs can be isolated from blood during their circulating phase, expanded in vitro and genetically manipulated while maintaining their germ cells properties and can colonize the genital ridges of recipient embryos. We developed original culture methods that allow long term propagation of undifferentiated PGCs and their efficient cryopreservation.

Material and methods:

PGCs culture and cryopreservation:

Blood samples were collected from embryos originating from three commercial layer breeds (White Leghorn, Isa Brown and Dekalb White) and two Belgian endangered breeds (Herve and Ardennes breeds). Samples were pooled and cultivated on cell culture inserts in selective medium in the presence of mitotically inactivated Buffalo Rat Liver cells.

PGCs were cryopreserved using either slow freezing (KnockOut-DMEM containing 50% FBS and 5% DMSO) or a newly developed vitrification method. Vitrification was performed as follow: cells were successively submitted to three vitrification solutions (proprietary formulation – Dr F. Ectors), gradually increasing the cryoprotectants concentration. Cells were resuspended in the third solution (vitrificant solution *sensu stricto*), loaded in a straw and rapidly immersed in liquid nitrogen.

PGCs characterization:

PGCs were labelled for the ES cells marker, SSEA-1, whose expression was evaluated by flow cytometry. Expression of germ line markers (CVH, CDH, DAZL), pluripotency markers (PouV, Sox2, Nanog), telomerase and CXCR4 receptor was evaluated by RT-PCR.
The proportion of female cells in stabilized cell lines was estimated by means of a quantitative PCR method, based on the amplification of a chromosome W specific sequence.
Migratory ability of PGCs was assessed by injecting cell-track labelled cells in recipient embryos.

Results:

Upon primary culture of pooled blood samples, PGCs emerge in 3 to 4 weeks. Undifferentiated cells display a round morphology and grow as unattached single cells or small clusters (Fig. 1a). Adherent cells are systematically eliminated. Overall, 35% of pooled samples give rise to cells lines.
Cells lines expressed SSEA-1 marker (Fig, 1b). Flow cytometry analysis showed expression rates varying from 90 to 99% (Fig. 2).
Results of RT-PCR are shown in Fig. 3. As expected, PGCs expressed all the tested markers.

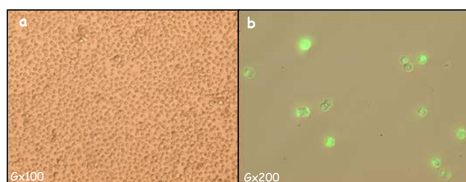

Fig. 1: Chicken primordial germ cells cultivated on a PET cell culture insert (a) (line p 12/2011 – day 151). Chicken primordial germ cells labelled for SSEA-1 expression (b) (line 05/03 – day 245). Positive cells appear in green.

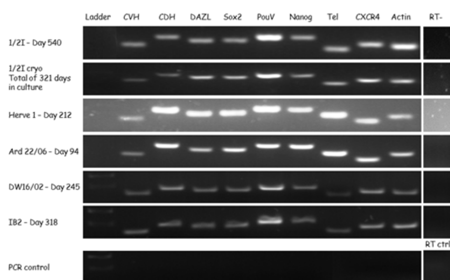

Fig. 3: Expression profile, tested by RT-PCR, of some representative PGCs lines from different breeds: White Leghorn, before and after slow freezing cryopreservation (1/2I), Herve and Ardennes breeds (Herve 1 and Ard 22/06), Dekalb White (DW16/02) and Isa Brown (IB2).

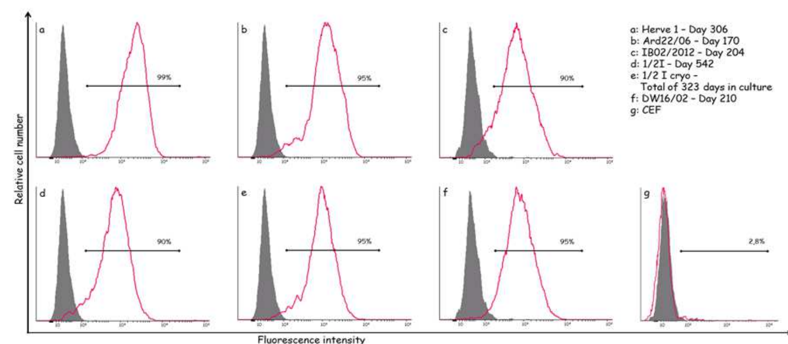

Fig. 2: Analysis of SSEA-1 expression of some representative PGCs lines by flow cytometry. PGCs are originating from different breeds: Herve and Ardennes breeds (Herve 1 and Ard22/06), Isa Brown (IB02/2012), White Leghorn before and after slow freezing cryopreservation (1/2I) and Dekalb White (DW16/02). Chicken embryonic fibroblasts are used as negative control cell line. Negative control (secondary antibody alone) appears in grey, labelled cells in rose.

Sex determination experiments on all our White Leghorn PGCs lines showed a systematic drift towards the male sex, while they were initially isolated from pooled blood samples with statistically equivalent numbers of male and female embryos. Twelve stabilized PGCs lines, originating from 64 pooled embryos (35 females and 29 males), were tested at day 245. Female DNA could not be detected in any of them. A small proportion of female cells was detected in line 05/03 tested at day 75, but the percentage was under 0,01%. Female DNA became undetectable at day 103. The same phenomenon was observed in Isa Brown tested lines.

Analysis of injected embryos, 48 to 72 hours post-injection showed evidences of PGCs migration in genital ridges (Fig. 4).

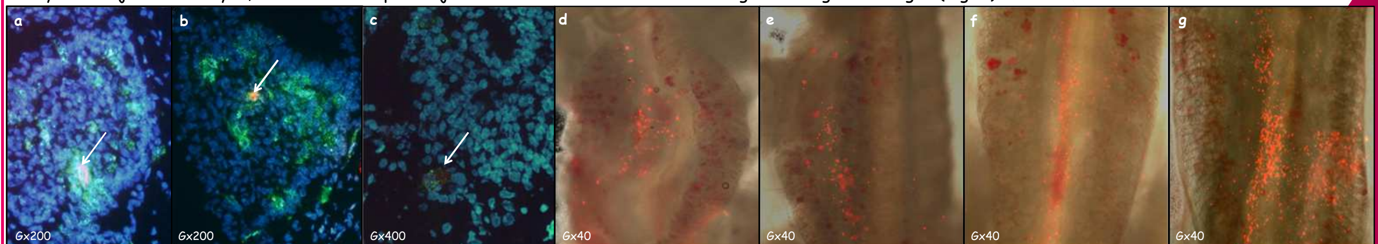

Fig. 4: Migratory ability of PGCs.
a-b-c: Cryosections were performed on injected embryos 72 h post-injection. Cell-track labelled cells appear in red, endogen and injected PGCs appear in green after anti-SSEA-1 immunostaining. Injected cells are observable into the genital ridges (a and b: line 1/2I – day 353; c: line 1/2I after slow freezing cryopreservation – total of 442 days in culture).
d to g: Observation of embryos 48 to 72 h post-injection of cell-track labelled cells (red). Injected cells are observed in the genital ridges region (d: line Herve 1 – day 408; e: line 05/03 – day 98; f and g: line 05/03 – day 98, after slow freezing and vitrification, respectively).

Conclusion:

In conclusion, we provide here an original method allowing to efficiently promote PGCs expansion in an extended period of time. Long-term cultured PGCs still expressed all tested specific markers and showed high ability to colonize embryonic gonads even after one cryopreservation step. In addition, we demonstrated that all our stabilized cell lines present a male phenotype, confirming published observations (van de Lavoir *et al.*, Nature, 2006).