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 chicken embryos originating from three commercial layer breeds (White Leghorn, Isa Brown and Dekalb White) and two Belgian endangered breeds (Herve and Ardennes breeds) and from turkey embryos. 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.

PGCs transfection:
PGCs were transfected using lipofection (Lipofectamine® LTX or Fugene ®). The circular pCAGGSeGFPNeo plasmid was used for transient transfection and was linearized for stable transfection.

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). Doubling time is generally around 2 days. Adherent cells are systematically eliminated. Overall, 35% of pooled samples give rise to cells lines.

Cells lines express SSEA-1 marker (Fig. 1b). Flow cytometry analysis on chicken PGCs 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.

Turkey PGCs were recently isolated, are morphologically similar to chicken PGCs and express the SSEA-1 marker (Fig. 1, c and d).

Sex determination experiments on all our stabilized 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. Female DNA was still detectable after 2 month of culture, but the proportion was under 0,01% in all cells lines submitted to a kinetic study. It became undetectable after 3 month of culture. The same phenomenon was observed in PGCs from other breeds.

A stable GFP-expressing cell line was isolated after transfection with linear DNA and neomycin selection (Fig 4).

Analysis of injected embryos, 48 to 72 hours post-injection, showed evidences of PGCs migration in genital ridges (Fig. 5).

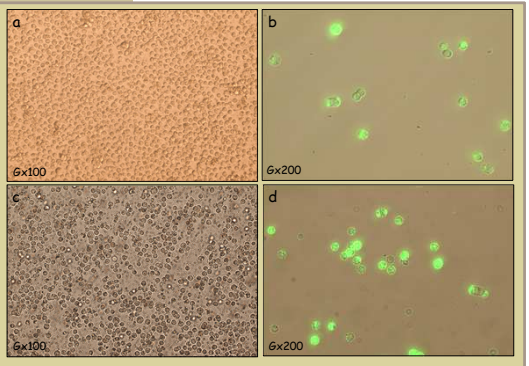

Fig. 1: Chicken (a: line p 12/2011 – day 151) and turkey (c: day 60) primordial germ cells cultivated on a PET cell culture insert.
Chicken (b: line 05/03 – day 245) and turkey (d: day 60) primordial germ cells labelled for SSEA-1 expression. Positive cells appear in green.

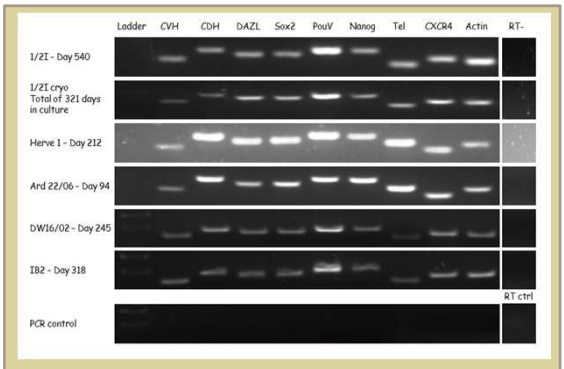

Fig. 3: Expression profile, tested by RT-PCR, of some representative chicken PGCs lines from different breeds: White Leghorn, before and after slow freezing cryopreservation (1/2I), Herve and Ardennes breeds (Herve 1 and Ard 22/06), Dekalb White (DW16/02) and Isa Brown (IB2).

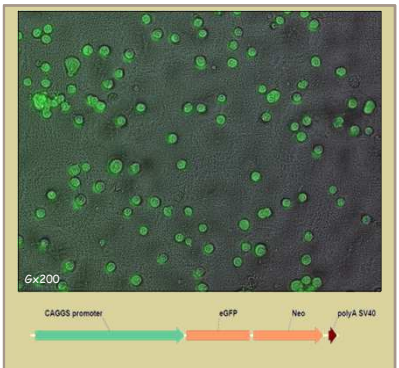

Fig. 4: Chicken PGCs line stably transfected with the CAGGSeGFPNeo construct and expressing GFP (line Ard11/10/12-F02-09).

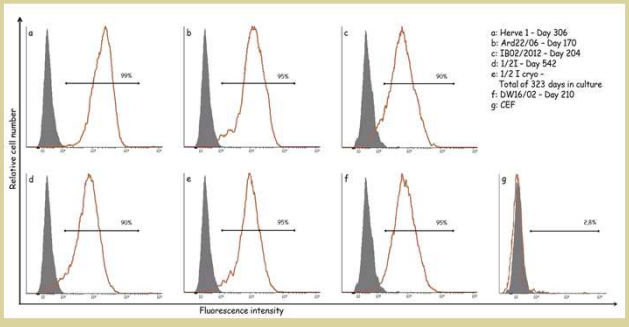

Fig. 2: Analysis of SSEA-1 expression of some representative chicken PGCs lines by flow cytometry. PGCs are originating from different breeds: Herve and Ardennes breeds (Herve 1 and Ard22/06), Isa Brown (IB02/2012), White Leghorn before and after slow freezing cryopreservation (1/2I) and Dekalb White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

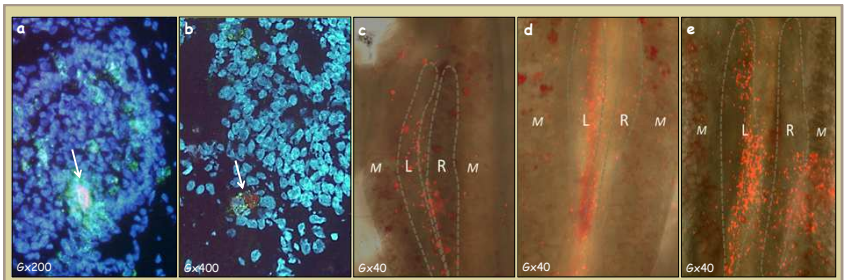

Fig. 5: Migratory ability of long-term cultured chicken PGCs.

a-b: 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: line 1/2I – day 353; b: line 1/2I after slow freezing cryopreservation – total of 442 days in culture).

c to e: Observation of embryos 48 to 72 h post-injection of cell-track labelled cells (red). Injected cells are observed in the genital ridges region (M: mesonephros; L and R: left and right genital ridges) (c: line 05/03 – day 98; d and e: 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).
Our culture method seems easily replicable to other avian species, as we demonstrated isolation and expansion of turkey PGCs. Further characterization of this cell line is in process.