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Short Report

Primordial germ cell characterization by immunohistochemistry of *vasa*-homologue protein in preimplantational rabbit embryos

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ABSTRACT Primordial germ cells (PGC) present alkaline phosphatase (AP) activity that make easy their localization in different species. Nevertheless in rabbit, like in mouse, AP shows positive reaction in different tissues. New markers allow a more specific localization of PGCs. In this work, we present the identification of PGCs in the development of pre-implantation rabbit embryos using mouse-*vasa*-homologue protein (MH-*vasa*) in young rabbit embryos (6, 7 and 7.5 dpc.) and their analyses in culture for 48 hours of 7.5 dpc. embryos. Colocalization of label with AP and TEC-1 was made. The results indicate that MH-*vasa* is more specific for the identification of PGC in young rabbit embryos. MH-*vasa* label a high number of cells in 6 dpc. rabbit embryos, probably not all of them PGC but in 7.5 dpc embryos after 48 h in culture, only a group of cells in the trophoectoderm close to the caudal pole of the embryo is labeled. This localization may correspond to the specific position of PGCs. MH-*vasa* is described as a specific marker of RNA helicase that maintains the undifferentiated state of PGC during development and that is present in pre-implantation rabbit embryos. Only PGCs maintain the MH-*vasa* label in 7.5 dpc. embryos in culture for 48 h in specific cells located outside the embryonic disc, facing its caudal aspect (Project ECOS-SUD/CONICYT C97B03).

Introduction Primordial germ cells (PGCs) are founders cells of the gametes and, as such, they carry forward the phenotype of each individual, including all its defects, into future generations. A molecular description of germ cell differentiation will lead to a better understanding of fertility, childhood diseases, germ-cell tumors and methods of contraception. It is amazing that no germ cells exist in the gonads of vertebrates at the beginning of gonadal development. Their ancestors, primordial germ cells (PGCs) are originated just before and during early gastrulation, around the proximal part of the adjacent epiblast, in an extra-gonadal and extra-embryonic region, near the yolk sac site in the base of the allantois. Later they migrate to the gonads to form the germ line in the ovaries or testicle. Many approaches have been used to investigate the origin of germlines; however, because of the lack of reliable molecular markers, it still remains unclear how primordial germ cells (PGCs) originate during early embryogenesis. Alkaline phosphatase (AP) is described as a classical marker for PGC but in mouse show positive reaction in different tissues of embryos (MacGregor, 1995). The development of new markers should make possible the identification of the beginning of differentiation of PGC in young embryos and their possible isolation for clonage and "in vitro" study. The *vasa* gene is one of the genes responsible for maternal-effect mutations that cause a deficiency in the formation of germline precursor cells, pole cells in *Drosophila* (Hay *et al.,* 1988). The *Vasa* protein is a member of an ATP-dependent RNA helicase of the DEAD-box family protein. Structural conservation allows us to isolate the conserved sequences of *vasa* homolog genes using a PCR cloning technique. Many vertebrate *vasa* homolog genes have been reported (Fujiwara *et al.,* 1994; Komiya and Tanigawa, 1995). Immunohistochemical analyses demonstrated that *Vasa* homologue protein was exclusively expressed in PGCs just after their colonization of embryonic gonads and in germ cells undergoing gametogenic processes until the post-meiotic stage both in mouse males and females (Toyooka, *et al.,* 2000). The aim of this study is the primordial germ cell characterization during the early embryogenesis by immunohistochemistry of *Vasa* homologue protein in preimplantational rabbit embryos.

Material and Methods Embryos of rabbits were obtained of the INRA farm (Jouy-en-Josas, Paris, France) under techniques of artificial insemination of New Zealand white rabbit females. Embryos of 6, 7 and 7.5 dpc were removed by flushing pressure of 40 mL of PBS to each one of uterine corn. Older embryos were obtained by dissection of each uterine horn. The embryos were processed following the protocols for immunohistocemistry (IHC) in whole embryos, fixed 2 hours in PAF 4% and processed for Confocal and Fluorescent Microscopy. Monoclonal antibodies, Tec-1 marker of two different cells surface embryoglycans (Dowsing *et al.,* 1998) and the polyclonal MH-*vasa* protein marker for maternal gene (Toyooka, 2000) was obtained from the above referred. 7.5 dpc whole rabbit embryos were incubated for 48 h in DMEM with 10% of FBS, streptomycin and penicillin 1%, non-essential amino-acids (L-alanine 8.9 μg/ml, L-asparagine 13.2 μg/ml, L-aspartic acid 13.3 μg/ml, L-glutamic acid 14.7 μg/ml, glycine 7.5 μg/ml, L-proline 11.5 μg/ml, L-serine 10.5 μg/ml.) (GIBCO BRL), insulin 0.1 μg/ml, transferrin 0.06 μg/ml, selenium 0.05 μg/ml (ITS SIGMA). The embryos fixed in PAF were treated with Triton 0.5% in PBS-BSA 0.2%, rinsed and incubated with goat serum 10% supplemented with pure goat anti-rabbit IgG for blocking non-specific binding of immunoglobulins. Mouse anti Tec-01 was diluted 1/100 and rabbit anti MH-*vasa* 1/1000 in PBS-BSA 2%-Saponine 0.05% and incubated all night at 4°C. Tec-1 further was incubated with 1/200 goat anti-mouse IgM-FITC or RT and MH-*vasa* with goat anti-rabbit IgG-FITC (Dowsing *et al.,* 1998; Toyooka, 2000). Staining for Alkaline Phosphatase (AP) was done with NBT/BCIP following their standard protocols (Roche Germany).

Results MH-*vasa* labels a high number of embryonic epiblastic cells in 6 dpc rabbit embryos and present a granular label in the

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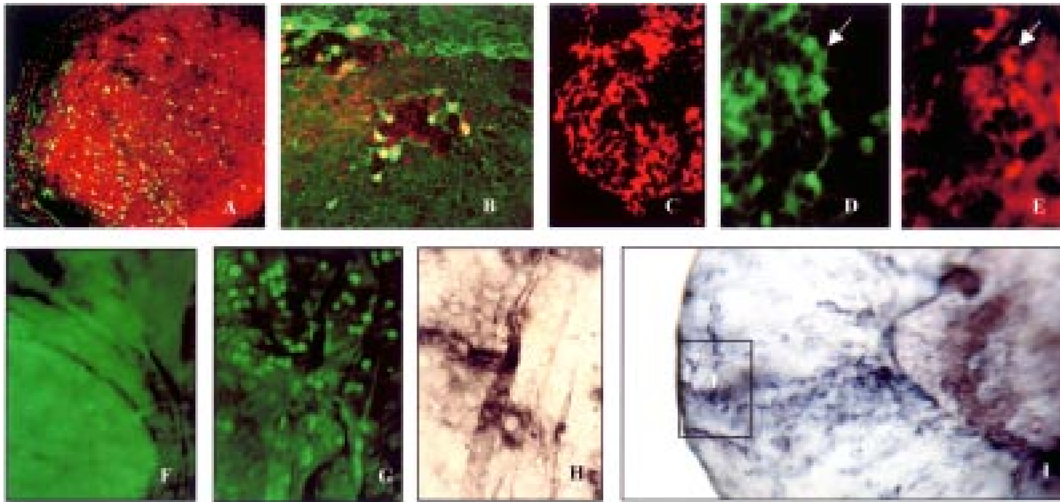

Fig. 1. Labeling of PGCs in different stages of preimplantational rabbit embryos. *Immunohistochemistry of MH-vasa* **(A, B, D, F, G)**, *TEC-1* **(C,E)** *and PA cells* **(H,I)**. *D and E are the same cellular population, only some cells reveal double labeling with MH-vasa and TEC-1 (arrows). MH-vasa is absent in the posterior pole of 7.5 dpc embryo with 2 days in culture (F) and we found a specific cellular group that reveals MH-vasa label in the extraembryonic region (G), not all these cells have a dark PA reaction (H). I, Microphotograph that shows the whole embryo, the inner box (J) shows the position of specific cellular group MH-vasa positive cells, seen in G.*

trophoectoderm limit cells (Fig. 1A). The embryonic epiblastic cells with vasa label are big cells that may be migratory cells that differentiate in specialized tissue and lost the vasa expression, seen in 7 dpc embryos in culture. Only a group of cells confined to the border of the posterior pole in the embryonic disc, with the expression of vasa in the specific mark of PGC are seen (Fig. 1B). In embryos of 7.5 dpc Tec-1 mark the embryo epiblast cell layer and big cells in extra-embryonic region adjacent to the posterior pole, they correspond to PGC that change the position to an extra-embryonic region (Fig. 1C). Co-localization vasa and Tec-1 is only evident in some cells (Fig. 1 D,E). Whole embryo with 9.5 days, 48 hrs. in culture show alkaline phosphatase mark in a high number of cells in the trophoectoderm near the posterior pole (Fig. 1I). Of these cells, a specific group of MH-vasa positive cells was observed at a considerable distance of the posterior pole (Fig. 1 G,J). This location may correspond to the yolk sac and allantoid fold position in implanted embryos. This specific mark of undifferentiated cells group is indicative of PGC location and not all of these have AP activity (Fig. 1H). Posterior pole do not present Vasa positive cells in whole embryo with 9.5 days, 48 hrs. in culture (Fig. 1F). This result indicates that only a few number of cells maintain a non-differentiated state important for preserving the toti-potentiality of PGC.

Discussion and Conclusions

Alkaline phosphatase is a classical marker for PGC but in rabbit it shows mark in different tissues, mesonephros, neural tube and secretor cells like in mouse embryos and is a controversial marker for the PGCs (MacGregor, 1995). Transplantation experiments showed that before and during early gastrulation, these cells are not yet committed to the germ-line, and when grafted into distal positions they can develop into somatic tissues. Consistent with the notion of germ-cell induction through cell-cell interactions, distal cells that would develop as somatic cells can develop into germ cells when grafted into the region where germ cells normally form. Indeed, formation of the founding population of PGCs in the mouse was shown to depend on the function of at least one extracellular factor-BMP4 development (Lawson, *et al.,* 1999). SSEA-1 anti-body for the same protein that TEC-1 and EMA-1 has been described as a marker of undifferentiated PGCs in young embryos of Pig (Takagi, 1997). Vasa homologue protein of *Drosophila* is observed in mouse germinal cells in young embryos after the colonization of the genital ridges (Tooyoka *et al.,* 2000). PG-2 (Schafer-Haas and Viebahn. 2000) present peri-nuclear mark in PGC, however young pre-implantational embryos treated with PG-2 in this work show positive label only 18 dpc gonads (data not shown). Vasa, an evolutionary conserved protein is important for the maintenance of toti-potentiality of PGC and clearly shows the differentiation of PGC (Raz, 2000). The expression of vasa and the more specific mark for identification of PGC in the development of pre-implantational rabbit embryos with mouse-homologue of *vasa* protein marker is demonstrated.

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