Distribution of proliferating cells and *vasa*-positive cells in the embryo of *Macrostomum lignano* (Rhabditophora, Platyhelminthes)

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ABSTRACT. The neoblast stem cell system of flatworms is considered to be unique within the animal kingdom. How this stem cell system arises during embryonic development is intriguing. Therefore we performed bromodeoxyuridine labelling on late stage embryos of *Macrostomum lignano* to assess when the pattern of proliferating cells within the embryo is comparable to that of hatchlings. This pattern can be found in late embryonic stages (stage 8). We also used the freeze cracking method to perform *macvasa* embryonic labelling. *Macvasa* is a somatic and germ line stem cell marker. We showed macvasa protein distribution during the whole embryonic development. In the *macvasa*-positive blastomeres the protein is localized around the nucleus in the putative chromatoid bodies. However, at a specific embryonic stage, it is also ubiquitously present in the cytoplasm of some blastomeres. We compare our data with what is known from Schmidtea polychroa of the expression of the *vasa*-like gene *SpolvlgA* and the protein distribution of the chromatoid body component *Spoltud*-1. The embryonic origin of the somatic stem cell system and the germ line is discussed.

KEY WORDS: Macrostomorpha, embryonic development, neoblasts, markers, germ line.

INTRODUCTION

The neoblast stem cell system of flatworms is considered to be unique within the animal kingdom because it is responsible for the formation of somatic cells as well as cells of the germ line. In all other animals, such totipotency can only be found in the very early embryo. The question thus rises whether neoblasts are embryonic totipotent stem cells that persist in adulthood.

In their study of the embryonic development of *Macrostomum lignano* Ladurner et al., 2005, Morris et al. (2004) were unable to elucidate the intriguing issue of the origin of this unique stem cell system. It could be that it descends from neoblast-like cells found early in embryogenesis (Le Moigne, 1963) or, alternatively, that it arises at a later stage of development or is renewed from differentiated cells (Peter et al., 2004). The distribution of neoblasts is also unclear. Are they located randomly within the embryo or are there specific clusters associated with specific organ primordia?

A substantial number of studies have previously suggested that platyhelminths do not seem to have a separate embryonic germ line, yet it is formed epigenetically during post-embryonic development (references in Zayas et al., 2005). However, Pfister et al. (2008) recently suggested an embryonic segregation of the germ line in *M. lignano*, based on their observation of a cluster of germ line-specific *macvasa*-positive cells (termed gonad anlage) in freshly-hatched worms. To answer the question when exactly the segregation happens, more embryonic data are needed.

To resolve all these issues we decided to perform a study to clarify the embryonic nature and origin of neoblasts. We used the freeze cracking method (Willems et al., 2009) to perform *macvasa* embryonic labelling. *Vasa* is highly conserved in all animals (Sato et al., 2006; Wang et al., 2007, see references in Pfister et al., 2008) and seems to be a specific germ cell marker. It is responsible for establishing and maintaining the dichotomy of germ line and soma in animal development (references in Rebscher et al., 2007). Therefore, this marker can be used to elucidate the evolution of germ cell specification (Rebscher et al., 2007).

In *M. lignano*, *vasa* is distributed in male and female gonads and in somatic stem cells in juvenile and adult worms, suggesting that *vasa* also plays a role in neoblast maintenance and differentiation (Pfister et al., 2008). In a broad variety of species, *vasa* has been shown to be present in a perinuclear germ line-specific organelle called nuage (Ikenishi, 1998; Shibata et al., 1999; Knaut et al., 2000; Carre et al., 2002; Findley et al., 2003; Bilinski et al., 2004; Parvinen, 2005; Johnstone et al., 2005) — an evolutionarily conserved structure of unknown function. In flatworms a chromatoid body – a structure similar to nuage – has been found in stem cells (Morita et al., 1969; Coward, 1974; Hori, 1982). Chromatoid bodies disappear during neoblast differentiation but remain in the germ line (Coward, 1974; Hori, 1982; Sato et al., 2001, 2006; Pfister et al., 2008). Recently, Solana & Romero (2009) identified *SpolvlgA*, a *Schmidtea polychroa* homolog of the DDX3/PL10 DEAD-box RNA helicase *DjvlgA* from the planarian species *Dugesia japonica* (Shibata et al., 1999).

SpolvlgA mRNA expression was observed: 1) in blastomeres and embryonic cells in early developmental stages; 2) in embryonic cells during stage 5 of planarian development highlighting massive embryonic cell differentiation and 3) in proliferating and differentiating cells during late developmental stages (Solana & Romero, 2009). Moreover, these authors observed a change in localization of this *vasa*-like gene expression during embryonic development, from perinuclear to cytoplasmic.

In this contribution we show, for the first time, true *vasa* protein distribution in the embryo of *M. lignano* (*macvasa*). We also performed bromodeoxyuridine (BrdU) labelling experiments on embryos during late development to study the distribution of proliferating cells.

MATERIALS AND METHODS

Cultures

Cultures of *M. lignano* were reared in Petri dishes following the protocol of Rieger et al. (1988) and fed with the diatom *Nitzschia curvilineata*. They were maintained in a temperature-controlled chamber at 20°C, 60% humidity, and a photoperiod of 13 h light and 11 h dark.

Staging system

Embryonic stages are named according to the staging system of Morris et al. (2004). The total developmental time (± 120 h at 20 °C) was subdivided in intervals of 15h. Eight stages were assigned to each interval. Stage 3 is characterized by the expansion and diversification of the embryonic primordium. Anteriorly and laterally, cells of smaller size form the primordium of the body wall and nervous system (somatic primordium); large, yolk-rich cells in the centre represent the primordium of the gut (Morris et al., 2004).

BrdU labelling

BrdU is incorporated into the S-phase of the cell cycle. Living embryos at 80% and 95% of their total development time were therefore freed from their egg-shell with electrolytically-sharpened tungsten needles allowing the BrdU to penetrate the embryo during the short (30 min) pulse period. BrdU concentration (5 mM) and protocol were as described by Ladurner et al. (2000).

Vasa labelling

Eggs of all stages were collected and washed (3x) in phosphate-buffered saline (PBS). The egg shell of embryos was permeabilized using the freeze cracking procedure (Willems et al., 2009). Primary antibody and secondary antibody concentrations were 1/200 and 1/150, respectively.

RESULTS AND DISCUSSION

Proliferating cells

Embryos at 80% of their developmental time (stage 7 according to Morris et al., 2004), were the earliest stage at which proliferating cells were found in a specific pattern consisting of two lateral bands (Fig. 1). The number of proliferating cells in embryos at this stage was 57.4 (SD 6.50, n=5). This is in accordance with the neoblast distribution in the juveniles and adults (Rieger et al., 1994 for *Macrostomum hystricinum marinum* ; Ladurner et al., 2000; Bode et al. 2006; Egger et al., 2006 for *M. lignano*) and coincides with the position of the main lateral nerve cords. Remarkably, contrasting with the distribution of proliferating cells in a one-day-old hatchling, we also found BrdU-labelled cells anterior to the eyes. In most embryos, proliferating cells were also found perpendicularly to the longitudinal lateral bands, at the level of the post-pharyngeal commissure. This further corroborates the hypothesis that the nervous system might exert a guiding function on proliferating cells via cell-cell connections or via neurosecretion, as proposed by Baguñà et al. (1989) and Bode et al. (2006), even in the embryo. In embryos at 90% of their total developmental time (stage 8; close to hatching), proliferating cells were found in the same pattern as in one-day-old hatchlings with the exception of a few cells occurring perpendicularly to the lateral bands, at the level of the post-pharyngeal commissure (Fig. 1B). In conclusion, the juvenile pattern of BrdU cells, as observed in hatchlings, can already be found in embryos at 80% of their total developmental time. This suggests that already by this stage, only neoblasts are able to proliferate.

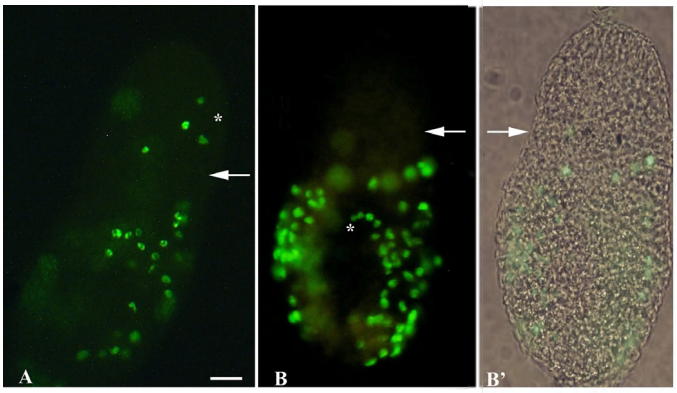

Fig. 1. – Bromodeoxyuridine (BrdU) labelling of stage 7 and stage 8 embryos (80% and 90% of total developmental time). Anterior is to the top. Arrow indicates eye level. Proliferating cells are in green. (A). Stage 7 embryo. Asterisk indicates 3 proliferating cells in front of the eye level. The low number of proliferating cells is due to the organism lying on its side and the epifluorescence image only showing proliferating cells present on the right side. (B). Stage 8 embryo. Asterisk indicates proliferating cells at the position of the post-pharyngeal commissure. (B') Light microscopical image of the embryo shown in (B). Scale bar for all pictures: 20 µm

Macvasa cells

Macvasa labelling shows different patterns in successive embryonic stages. In the ripe oocyte, stained in adults, the *macvasa* protein is only located in the perinuclear chromatoid bodies (Pfister et al., 2008). The same pattern can be found in a restricted number of cells in stage 1 and 2 embryos (Fig. 2A'-B'). At this stage *macvasa* is located in perinuclear granules in most but not all blastomeres. These granules are probably chromatoid bodies as this

localization is extremely similar to subcellular localization of the *Spoltud*-1 protein, a chromatoid body component in *Schmidtea polychroa* (Solana et al., 2009).

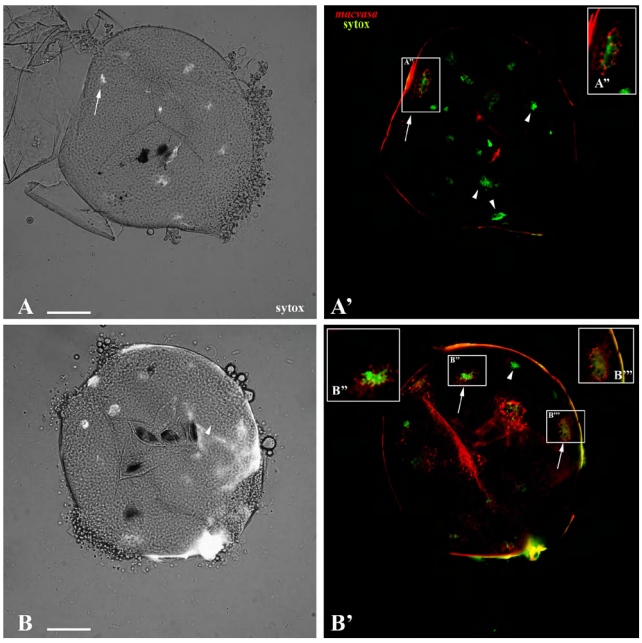

Fig. 2. – *Macvasa* staining of *Macrostomum lignano* stage 1 and 2 embryo (red). Embryos were counterstained with sytox to label nuclei (green). (A) and (B) represents the light microscopical image of the embryo. Sytox is pseudo-coloured white. (A') and (B') show the *macvasa*/sytox labelling as a single confocal stack (one plane) corresponding to the respective embryos depicted in (A) and (B). Insets (A'-A") and (B"-B"') depict magnification of *macvasa*/*sytox* positive cells. Arrows point to *macvasa*-positive cells; arrowheads, point to cells that were only labelled with sytox. Note that the *macvasa* protein is perinuclearly expressed in the chromatoid bodies.

In stage 3 embryos, *macvasa* staining is restricted to cells located in an area in the anterior part of the developing embryo (Fig. 3A). The fate of all other cells has probably already been determined. In some of the *macvasa*-positive cells only chromatoid bodies are stained, but in the majority of these cells the entire cytoplasm is stained and single chromatoid bodies cannot be discerned (inset of Fig 3A'-A"). These *macvasa*-positive cells are the cell pool that will form the neoblast system and the gonads. This immediately poses the intriguing question: why, in stage 3 embryos, do the majority of *macvasa*-positive cells have the complete cytoplasm stained instead of only the chromatoid bodies? Solana & Romero (2009) found a similar cytoplasmic distribution for the gene *SpolvlgA* during stage 5 embryos of *Schmidtea polychroa*. Two possible reasons can be conjectured: 1) *vasa* is generally seen as essential for germ line development but it could also have a broader function in stem cells. The *vasa*-positive cells in the early embryo would then represent embryonic stem cells. *Vasa* up-regulation would function as a restrictor, preventing these cells from differentiating during early embryonic development, when most other tissues and organs start to differentiate. In this way, a population of embryonic stem cells is set aside. These cells would later in development give rise to a separate germ line (see further). 2) *macvasa* could have a totally different function during early development. A similar situation is observed with *nanos* expression (a gene similar to *vasa*) in the acoel *Isodiametra pulchra*. *Nanos* is a germ-line marker but also a dorsal determinant during early embryogenesis (De Mulder, pers. comm.).

After stage 3, the *macvasa* protein could only be found in the perinuclear chromatoid bodies (Fig. 3B). From stages 6-8 we observed a separate cluster of *macvasa*-positive cells that showed strong staining intensity in comparison to the other cells (Fig. C-D). Probably, these cell clusters correspond to the gonad anlage found by Pfister et al. (2008) in one-hour hatchlings. Here up-regulation of *vasa* during stages

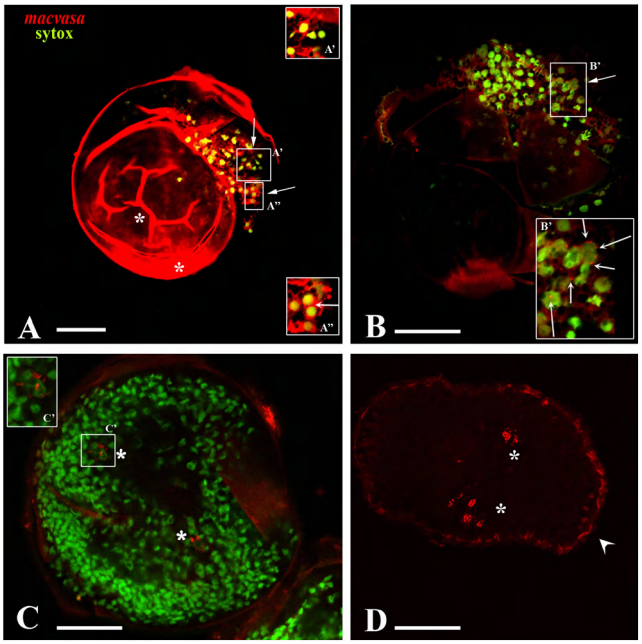

Fig. 3. – *Macvasa* staining of *Macrostomum lignano* stage 3 (A), stage 5 (B), stage 6 (C) and stage 8 (D) embryos (red). Embryos were counterstained with sytox to label nuclei (green). (A) represents a confocal image (one plane) of a stage 3 embryo. Anterior is to the top. Insets (A'-A") are magnifications of labelled cells. Arrows indicate the *macvasa*-positive cells where the *macvasa* protein is ubiquitously localised in the cytoplasm. The strong background of the eggshell should not be taken into account because the secondary antibody tends to stick to the outside of the eggshell. (B) confocal image of a stage 5 embryo (one plane). The inset (B') shows a magnified detail of the *macvasa* positive cells (arrow). Note the localization of *macvasa* in the perinuclear granules or chromatoid bodies (B', arrows). (C) Stage 6 embryo. *Macvasa*-positive cells are, for the first time, located bilaterally in the embryo. A limited number of cells, both on the left and right side of the embryo, show strong *macvasa*-positive signal (asterisks). Note that if we would over saturate the image, other *macvasa*-positive cells would become apparent (not shown). (D). Stage 8 embryo (only *macvasa*). The embryo has already a worm like shape (posterior indicated by arrowhead). Only a cluster of cells shows strong *macvasa* signal in chromatoid bodies (asterisks). The fact that no somatic *macvasa*-positive neoblasts are visible can be attributed to the weaker signal of these cells in comparison to the cluster of cells corresponding to the putative germ line precursors. This low signal may not have been significantly detected by our protocol, although we used the same primary and secondary antibody concentrations as in Pfister et al., 2008). Scale bar: 20 µm, except D: 50 µm

6-8 could represent a restriction mechanism to prevent the precursors of the germ line from differentiating into somatic tissues (Fig. 3 C-D). Possibly, the cell cluster corresponds to a separated germ line during late embryogenesis. These results suggest that by stages 6- 8 of embryogenesis, the specification of the germ line has already taken place and primordial germ cells are in their normal position, where the mature gonad will develop. Recent data on *Smednos* in *Schmidtea meditteranea* stage 8 embryos (Handberg-Thorsager & Salo, 2007) indicate a similar mechanism. Our results of *macvasa* expression during *Macrostomum* embryogenesis provide new evidence that strengthens the hypothesis of Pfister et al. (2008), who stated that the germ line in *Macrostomum* is segregated embryonically.

One should be very cautious when comparing gene expression data with protein localization. Yet, the changes in subcellular localization of *macvasa* bear some similarities with the expression pattern of the *SpolvlgA* gene during the embryonic development of *Schmidtea polychroa* and with the localization of the *Spoltud-1* protein (Solana & Romero, 2009; Solana et al., 2009). Distribution of the *macvasa* protein changes during early development from being in chromatoid bodies to a cytoplasmic distribution pattern, coinciding with waves of cellular differentiation later on, and finally again to a location in chromatoid bodies. However, there are also some notable differences: 1) distribution in the early embryo was restricted to some but not all blastomeres; 2) a specific distribution pattern of the *macvasa*-positive cells was observed only in the anterior of the embryo at stage 3 of development, and finally 3) a stronger label was observed in two clusters of cells in comparison to the remaining embryonic neoblasts, after stage 6. These clusters could be the precursor of the germ line.

In conclusion we identified, for the first time, the distribution of a true *vasa* protein in blastomeres of a flatworm embryo. Unfortunately, *macvasa* labels both germ line and neoblast, which makes it very difficult to discern both cell lines. In the future this type of study awaits a true neoblast or germ-line marker before any final conclusions on the germ line specification mechanism in *M. lignano* can be drawn.

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