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Production and characterisation of cell- and tissue-specific monoclonal antibodies for the flatworm *Macrostomum* sp.

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Abstract Monoclonal antibodies (mABs) against various cell types of the basal free-living flatworm *Macrostomum* sp. were produced by immunising Balb/c mice with cell suspensions of disintegrated animals. We identified 360 positive supernatants with specific staining of various tissues, cell types, patterns or structures. Here we report immunocytochemical characterisation, histological stainings and isotyping of 11 mABs specific for muscle cells (MMu-1, MMu-2, MMu-3, MMu-4), digestive and prostate glands (MDr-1 and MDr-2, MPr-1), epidermal cells (MEp-1), the ventral nerve cord including neuron clusters (MNv-1), gastrodermal cells (MDa-1) and spermatids (MSp-1). Confocal microscopy, histological techniques, electron microscopy and immunoblotting were applied to demonstrate stainings in juveniles, adults, starved or well-fed animals. Considering the current lack of specific markers the obtained mABs will be particularly helpful studying embryonic and postembryonic development, pattern formation, cell differentiation, regeneration and reproductive allocation in *Macrostomum* sp., and possibly other basal flatworms. The small size, ease of culturing, short generation time, transparency and the basal phylogenetic position specify *Macrostomum* sp. as a suitable

model organism for comparative analyses within Platyhelminthes and to *Drosophila* and *C. elegans*.

Keywords Platyhelminthes · Differentiation · Stem cells · Neoblasts · Turbellaria

Introduction

During embryogenesis cells become determined and differentiate to form tissues and organs. Differential gene expression leads to the establishment of a cell type-specific protein composition necessary for morphological, biochemical and functional characteristics of the respective cell type. Besides molecular approaches, monoclonal antibodies (mABs) are successfully employed to identify and analyse morphological, physiological and developmental regulated antigens in a broad variety of species.

Free-living Platyhelminthes are characterised phenotypically by a simple morphology with a characteristic body wall musculature, multiciliated epidermis, a primitive nervous system, an blind-ending gut and a remarkable stem cell system. Within the animal kingdom Platyhelminthes are known to be the only major body plan possessing apparently totipotent stem cells (neoblasts) in adult animals (Baguña et al. 1989; Ladurner et al. 2000).

In *Macrostomum* sp. the lack of specific markers for individual cell types or tissues limited the potential for studies on embryonic development, regeneration and cell differentiation. A recent survey on the embryonic development of *Macrostomum* sp. (Morris et al. 2004) was based on histochemical stainings (fuchsin), semithin serial sections, stainings with a commercially available antibody for tubulin and electron microscopy. The availability of specific mABs for various cell types will provide new means to follow the embryologic origin and differentiation of tissues and organs in *Macrostomum* sp. Particular questions, such as the formation of the embryonic

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muscle pattern and the nervous system, the differentiation of the epidermis and the formation of the gastrodermis, can be studied in detail. Postembryonic differentiation of reproductive organs can now be followed by applying spermatid- and prostate gland-specific antibodies.

With the availability of new cytological and molecular markers there is revived interest in the regeneration of planarians (Sanchez and Newmark 1998; Kato et al. 1999; Sanchez 2000, 2003; Agata 2002; Newmark and Sanchez 2002; Salo and Baguna 2002; Agata et al. 2003). Monoclonal antibodies were produced for different planarian species, such as *Dugesia* (*Girardia*) *tigrina* (Romero et al. 1991; Bueno et al. 1997), *Phagocata vivida* (Shirakawa et al. 1991; Asano et al. 2001) and *Dugesia japonica* (Asano et al. 2001; Orii et al. 2002). Physiological regeneration was recently studied in an acoel species (Gschwentner et al. 2003). Muscle pattern reorganisation after cutting was analysed in *Macrostomum* sp. (Salvenmoser et al. 2001).

In flatworms, cell differentiation can be traced from a pool of pluri- or totipotent stem cells. Neoblasts (Baguña 1981) remain mitotically active throughout adult life and are responsible for physiological cell renewal and the exceptional regenerative abilities of most free-living forms ("Turbellaria") and in parasitic Platyhelminthes (Gustafsson 1976; Gustafsson and Eriksson 1992). Recent studies have demonstrated the differentiation potential of neoblasts in planarian and acoel species (Gschwentner et al. 2001; Newmark and Sanchez 2000). We showed that a comparable stem cell system exists in the primitive rhabditophoran *Macrostomum* sp. (Ladurner et al. 2000; Nimeth et al. 2004). We applied bromodeoxyuridine (BrdU) labelling to show the distribution, migration and differentiation of S-phase neoblasts in this species. The results put forward the stem cell system of Platyhelminthes as an appropriate model system to study basic processes such as cell differentiation, cell migration, pattern formation and regeneration.

Modern data on the differentiation of neoblasts are mostly from higher rhabditophoran groups such as planarian species based on light and electron microscopic investigations. In order to further analyse cell differentiation in *Macrostomum* sp. cell- and tissue-specific markers are necessary to assess the differentiation potential of neoblasts. Compared to planarians *Macrostomum* sp. has several advantages when performing such analyses:

1. Its small size of about 1 mm (only 25,000 cells) enables neoblast differentiation to be followed using BrdU incorporation in whole mount preparations. The unique pattern of S-phase cells in two lateral bands allows the migrating and differentiating BrdU-labelled cells to be followed and quantified in pulse chase experiments.
2. Because of its transparency, differentiating tissues can be surveyed easily.
3. *Macrostomum* sp. displays the same general tissue differentiation level as planarian species but structures and organs are at least two orders of magnitude

smaller, and much more concrete (for example, two testes and two ovaries).

4. The morphology of the muscle and the nervous system are known in great detail. The position of nerve cords and nerve cells as well as the organisation of muscle fibres are constant to a certain extent and can be followed during differentiation and regeneration.
5. The embryonic development of Triclad is highly derived (*Blastomeren Anarchie*), while the phylogenetically basal rhabditophoran platyhelminth, the Macrostromorpha, exhibit spiral cleavage during first division phases.
6. *Macrostomum* sp. has a short generation time of about 3 weeks.

A major drawback for studying cell differentiation in *Macrostomum* sp. was the lack of specific markers to identify individual cell types or tissues. Commercially available antibodies such as anti-serotonin (Ladurner et al. 1997) or anti-FMFR-amide (Ladurner unpublished) were applied to study the morphology of the nervous system or phalloidin to visualise the organisation and regeneration of the muscle system (Reiter et al. 1996; Ladurner and Rieger 2000; Hooge 2001; Salvenmoser et al. 2001; Gschwentner et al. 2003). Very few mABs from planaria that we tested showed positive crossreactivity (Ladurner unpublished observations). Therefore, specific markers for studying cell differentiation in intact and regenerating macrostomid species were available in a very limited number. In order to explore further the differentiation potential of stem cells we have, therefore, produced mABs against cells and tissues of *Macrostomum* sp. by immunisation with cell suspensions of dissociated worms. To investigate the nature of stem cell differentiation and the morphology and organisation of cells, tissues and organs we report here 11 mABs specific for muscles, nerve, gland cells, gut, epidermis and spermatids of *Macrostomum* sp.

Materials and methods

Animal culture

Macrostomum sp. is a new member of the *Macrostomum tuba* species group of the Macrostromorpha-Macrostromida, Platyhelminthes (Ladurner et al. 2000). The description of this species is accepted (Ladurner et al., see note added in proof). Animals were held in petri dishes with nutrient-enriched artificial seawater (Guillard's F2 medium) and fed the diatom *Nitzschia curvilineata*, and they were maintained in a climate chamber at 20°C, with 60% humidity and a 14:10 day-night cycle (Rieger et al. 1988). Animals were starved for 2 days before antigen preparation. A schematic midsagittal section drawing of *Macrostomum* sp. is shown in Fig. 1.

Preparation of antigen and immunisation

For starvation about 2,000 animals were transferred to petri dishes with autoclaved artificial seawater. The medium was changed several times per day to remove debris and digested algae. In order to obtain a cell suspension animals were dissociated by incubation

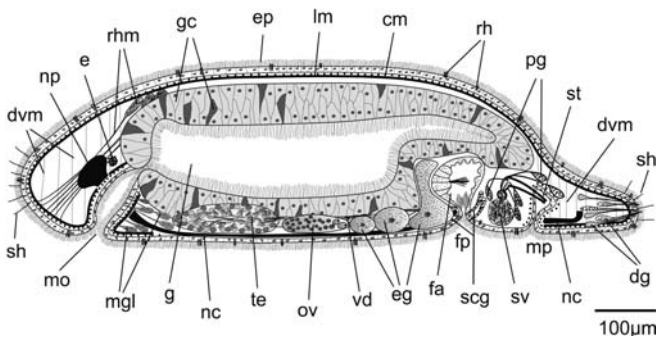


Fig. 1 Schematic drawing of *Macrostomum* sp. midsagittal section. *cm* Circular muscles, *dg* dual gland adhesive system, *dvm* dorso-ventral muscles, *e* eye, *eg* eggs, *ep* epidermis, *fa* female antrum, *fp* female genital pore, *g* gut, *gc* gastrodermal cells, *lm* longitudinal muscles, *mgl* mouth gland cells, *mo* mouth opening, *mp* male genital pore, *nc* nerve cord, *np* neuropile, *ov* ovary, *pg* prostate glands, *rh* rhabdites, *rhm* rhamites, *scg* shell and cement glands, *sh* sensory hairs, *st* stylet, *sv* seminal vesicle, *te* testis, *vd* vas deferens

in calcium magnesium free medium [400 $\mu\text{g/ml}$ $\text{PO}_4\text{H}_2\text{Na}$, 800 $\mu\text{g/ml}$ NaCl , 1,200 $\mu\text{g/ml}$ KCl , 800 $\mu\text{g/ml}$ NaCO_3H , 270 $\mu\text{g/ml}$ glucose, 1% bovine serum albumin (BSA), 3.75 mg/ml HEPES buffer] for 10 min at 37°C and mechanically by pipetting ten times with a 1,000- μl pipette. The suspension was washed by centrifugation at 800 g for 5 min at 4°C and resuspended with 1 ml sterile phosphate-buffered saline (PBS). After the final wash the pellet was resuspended in 300 μl sterile PBS and mixed with 300 μl Freund's complete adjuvant (Stimula). A viscous emulsion was produced using a three-way stopcock (Discifix; Braun) with Luer lock and two 2-ml syringes. Approximately 1×10^7 cells (150 μl emulsion) were injected subcutaneous inguinal into two female Balb/c mice. Incomplete Freund's adjuvant was used for booster immunisations after 4 and 8 weeks.

Generation of *Macrostomum* sp.-specific antibody-secreting hybridoma cell lines

Three days after the final boost the spleen was removed, cut into pieces with scissors and mechanically dissociated into single cells by passing through a 100- μm mesh. Spleen cells were fused with P3-X63-Ag8.653 myeloma cells in the presence of polyethylene glycol. Fused cells were plated out in 24-well tissue culture plates in 1 ml hypoxanthine-aminopterin-thymidine medium (RPMI), 10% FCS, penicillin-streptomycin (Gibco), L-glutamine (Gibco) and hybridoma cloning supplement BM Condimed H1 (Roche). Supernatants from all wells containing visible clones were screened for antibodies using an enzyme immunoassay to determine if mouse IgGs were present in the hybridoma supernatant according to the manufacturer's instructions (Roche Mouse IgG ELISA).

Screening of positive wells and selection of clones for limiting dilution

Ten microlitres of the centre of hybridoma clones of all positive wells were picked and transferred to 12-well or 6-well plates. The supernatant of these wells was used to perform immunocytochemical whole mount (ICC) staining (see below). Three hundred and sixty wells were identified to contain cell- or tissue-specific antibodies. Clones that stained the whole animal were rejected. The staining pattern was described according to the stained structure (for example, muscle, nerve, epidermis, etc.). Because of the high number of obtained ICC-positive wells it was impossible to perform all-encompassing limiting dilutions. Therefore, all cells of 360 positive wells, although likely containing polyclonal lines, were

stored in liquid nitrogen. We have selected 25 clones from these frozen stocks based on our interests in muscle, nerve, epidermis, gut and spermatids. These hybridoma clones were thawed and limiting dilutions were performed. Eleven clones were further characterised and are presented here.

Isotyping of selected hybridoma lines

The isotype of each mAb was determined using a Mouse Immunoglobulin Screening/Isotyping kit (Zymed) according to the manufacturer's instructions except that instead of the initial antigen coating the coating antibody of the Roche Mouse IgG ELISA kit was used to capture immunoglobulins in the supernatant.

Immunocytochemistry

For ICC screening several hundred animals were relaxed in MgCl_2 (7.14%) isotonic with seawater for 20 min and fixed in 4% paraformaldehyde (PFA) for 60 min. They were washed in PBS for 3 \times 5 min, incubated in PBS-T (PBS with 0.1% Triton X-100) for 1 h and transferred into the blocking solution BSA-T (PBS with 0.1% Triton X-100 and 1% BSA) for 30 min. Five to seven animals were divided into each well of 24-well plates and hybridoma supernatant was added to a final dilution of 1:5 and incubated overnight at 4°C. After washing in PBS-T for 3 \times 10 min (washing medium was removed from the wells using a pulled glass pipette attached to a peristaltic pump) specimens were incubated in secondary FITC-conjugated goat anti-mouse antibody (Dako; 1:100 in BSA-T) for 60 min at room temperature and washed in PBS for 3 \times 10 min. After the final wash specimens were covered with 90% glycerol and observed with a Zeiss Axiovert 135.

Standard ICC staining for individual mAbs was performed in embryo dishes. Specimens were mounted on slides using Vectashield (Vector Laboratories) and observed with a Reichert Polyvar epifluorescence microscope or a confocal Zeiss LSM 510 microscope.

Immunoblot assay

Total protein of 10, 20 and 40 *Macrostomum* sp. was extracted in 20 μl Laemmli sample buffer each and loaded onto 15% acrylamide gels (20 μl per lane). The protein was blotted onto polyvinylidene fluoride membranes (Immobilon-P; Millipore) and the membranes were blocked for 2 h with PBS:0.1% Tween 20, containing 5% skimmed milk powder. The blots were incubated overnight at 4°C with the mouse mAbs MMu-1, MMu-2, MDR-1, MPr-1, MEP-1, MNv-1, MDa-1 and MSP-1 diluted 1:250 in dilution buffer (PBS: 0.05% Tween 20:5% skimmed milk powder). After washing the blots for 3 \times 10 min in PBS:0.05% Tween 20, the membranes were incubated with alkaline phosphatase-conjugated anti-mouse immunoglobulin (Sigma), diluted 1:10,000 in dilution buffer, for 1 h at room temperature. The blots were washed and the immunocomplexes were detected using nitro blue tetrazolium:5-bromo-4-chloro-3 indolyl phosphate (LifeTechnology).

Immunohistochemistry

Relaxation and fixation was carried out as described above. After washing in PBS-T for 3 \times 10 min, 3% peroxide was used to block endogenous peroxidase. Non-specific binding sites were blocked with BSA-T for 30 min. Monoclonal antibodies were applied according to the whole mount procedure. After washing in PBS-T, detection of the primary antibody was achieved using a StreptABC detection kit (Dako) followed by visualisation with diaminobenzidine (liquid DAB; Dako). After dehydration with a standard ethanol series specimen were embedded in Spurr's low viscosity resin. Blocks were trimmed and sectioned serially with a Reichert Autocut microtome using Ralph glass knives. Sections were counter-

stained with Richardson dye and mounted in cedar wood oil and examined with a Reichert Polyvar microscope.

Hoechst 33342 staining

Live animals were relaxed with $MgCl_2$ (7.14%) and incubated in 1 $\mu g/ml$ Hoechst 33342 for 10 min. Only epidermal cell nuclei were stained; the dye does not penetrate below the epidermis in live animals.

Electron microscopy and immunogold labelling

For standard electron microscopy specimens were relaxed and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer containing 10% sucrose. After washing in cacodylate buffer animals were postfixated with 2% osmium tetroxide in 0.05 M cacodylate buffer, dehydrated in standard ethanol series and embedded in Spurr's low viscosity resin. Ultrathin sections were double stained with uranyl acetate and lead citrate and examined with a Zeiss TEM 902.

For immunogold staining with the mAB MPr-1, animals were relaxed and fixed with 4% PFA containing 0.5% glutaraldehyde. After excessive washing in buffer, specimens were dehydrated in an ethanol series, embedded in Lowicryl resin and polymerised using UV irradiation for 48 h.

Immunogold staining was carried out using 1% BSA containing 0.1% freshwater fish gelatine to reduce non-specific staining. Primary antibody MPr-1 (dilution 1:40) was incubated for 1 h at room temperature followed by a 1-h incubation of a rabbit anti-mouse 15-nm gold conjugated antibody (dilution 1:40; British Biocell) after washing in 0.1 M PBS. No other antibodies were tested for immunogold staining. Sections were double stained with uranyl acetate and lead citrate and examined with a Zeiss TEM 902 using inelastic imaging for contrast enhancement.

MSp-1 number as a measure of male investment to gametes

The relationship between testis size and the number of MSp-1-positive cells was assessed as follows. Worms were removed from the mass cultures and placed for 1 week in groups of three into wells of 24-well tissue culture plates containing 1 ml of f/2 and an ample supply of fresh diatoms. The testis size of one worm per replicate was then measured morphometrically according to a method described previously (Schärer and Ladurner 2003). Next worms were anaesthetised, fixed, stained and mounted in Vectashield using the described immunocytochemistry procedure (see above).

The number of MSp-1-positive cells in both testes was then determined by two independent observers under epifluorescence illumination on a Reichert Polyvar at 1,000 \times magnification. The two independent measurements allowed to determine the repeatability, i.e. the intraclass correlation coefficient r_1 following Sokal and Rohlf (1995), of the MSp-1-count, which can serve as a measure of the clarity of the signal. Finally, a linear regression was calculated with the sum of the testis area as the predictor variable and the total number of MSp-1-positive cells as the dependent variable.

Nomenclature

Monoclonal antibodies were named using a code of three letters and a number. The first letter indicates the species (M for *Macrostomum* sp.) and the second and third letters represent the tissue or cell type, for example, Mu for muscle, Ep for epidermis, Dr for gland cells (German *Drüsen*). The number indicates the chronological order of identification of a mAB of a certain tissue or cell type.

Results

General results

We report here the immunocytochemical staining pattern by confocal microscopy, in histological sections, immunoblotting, and the isotyping of mABs against muscle (MMu-1, MMu-2, MMu-3, MMu-4), digestive and prostate glands (MDr-1 and MDr-2, MPr-1), epidermal cells (MEp-1), the ventral nerve cord with neuron clusters (MNv-1), phagocytes (MDa-1) and a spermatid-specific mAB (MSp-1).

The classes and subclasses of mouse mABs in culture supernatant were determined using a modified ELISA kit. All mABs described here were found to be members of the IgG₁ subclass.

Western blots were made for the antibodies MMu-1, MMu-2, MDr-1, MPr-1, MEp-1, MNv-1, MDa-1 and MSp-1. MSp-1 recognised a *Macrostomum* sp. protein with the size of 55 kDa (Fig. 9A). No immunocomplexes were formed by the other antibodies and, therefore, no signal was visible in the western blots (data not shown).

Muscle-specific mABs

As known from phalloidin labelling studies (Rieger et al. 1994; Salvenmoser et al. 2001) the muscle system of macrostomid flatworms consists of an outer circular, an inner longitudinal and an intermediate dorsal and ventral diagonal muscle layer. In addition, complex pharyngeal muscles form a part of the feeding system, dorsoventral muscles are necessary for dorsoventral flattening, longitudinal and circular muscles enclose the gut, and specially arranged muscles constitute the male and female reproductive organs. We identified 47 hybridoma clones specific for muscle cells, the majority staining the complete muscle system. In this study we show staining patterns of four mABs, MMu-1, MMu-2, MMu3 and MMu-4. All mABs stained the muscle processes while the cell nucleus was not labelled.

MMu-2, MMu3 and MMu-4 labelled all components of the muscle system including circular, longitudinal and diagonal muscles of the body wall (Fig. 2), dorsoventral muscles, pharyngeal muscles, muscle of the gut, and muscles belonging to the female and male reproductive system. Double labelling with fluorescent conjugated phalloidin revealed that the whole multitude of different muscle cells appeared stained with both phalloidin and each of MMu-2, MMu3 or MMu-4 (Fig. 2C, D). Preliminary experiments demonstrated that all antibodies crossreacted with the muscle system of the closely related species *Macrostomum pusillum* while only MMu-3 crossreacted with other flatworm species including acoels (data not shown).

MMu-1 was highly specific for longitudinal muscles of the body wall musculature (Fig. 2E). Muscle fibres ran parallel from anterior to posterior. An individual longitudinal muscle fibre extended about two-thirds of the

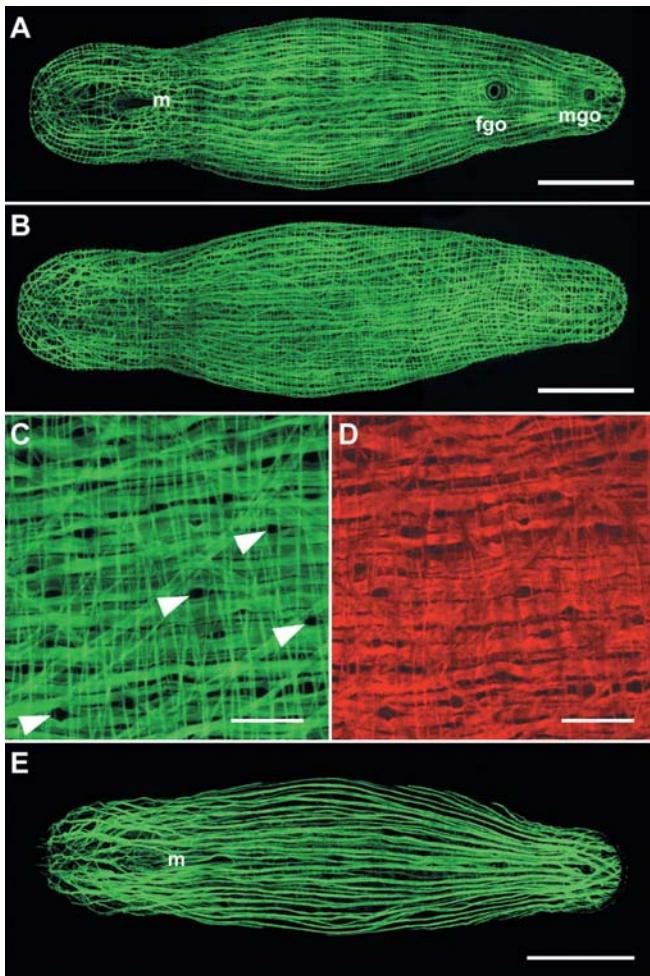


Fig. 2A–E Confocal projections of muscle stainings of *Macrostomum* sp. using monoclonal antibodies (mABs) MMu-1, MMu-2, MMu-3 (A–C, E) and phalloidin (D). Anterior is to the left in all panels. A, B Ventral (A) and dorsal (B) views of body wall musculature labelled with mAB MMu-3. C, D Detail of the body wall musculature double labelled with mAB MMu-2 (C) and phalloidin (D); arrowheads indicate openings of rhabdite glands. Note that all three muscle layers of the body wall, circular, longitudinal and diagonal muscles, are labelled. E Selective staining of longitudinal muscles of the body wall with mAB MMu-1. Outer longitudinal muscles in the posterior and anterior part of the animal were cut through the confocal projection; they actually continue further. m Mouth, fgo female genital opening, mgo male genital opening. Scale bars 100 μ m in A, B, E; 20 μ m in C, D

length of the animal. The ends of the muscle processes frayed and connected to other fibres and the extracellular matrix. In hatchlings, about 15–18 longitudinal muscle fibres were present and at least 50 in adults. Muscles of the pharynx, gut and the female genital opening were not stained. Positive staining was also observed in muscles related to the male reproductive organs and “dorsoventral” muscle fibres in the tail plate.

Gland cell mABs

A large diversity of unicellular gland cell types is present in *Macrostomum* sp. Three gland cell mABs, MDr-1, MDr-2 and MPr-1, are shown here: two gland cell mABs of the gut (MDr-1 and MDr-2) and that of prostate glands (MPr-1) of the male reproductive system. MDr-1 bound specifically to gland cells of the anterior gut, a special subset of the so-called “granular clubs” or “Minotian gland cells”. The cells are embedded within gastrodermal cells of the postpharyngeal region of the gut (Fig. 3A–E). The cells were funnel-shaped with the narrow end directed towards the gut lumen. The cytoplasm contained granules with intense staining of the granule hull (Fig. 3C). In adult animals about 25 glandular cells were present (Fig. 3B) while hatchlings possessed only around 6–7 cells. Well-fed animals had a higher number of gland cells with densely packed granules and intense staining compared to starved ones (Fig. 3B–E).

MDr-2 labelled a similar gland cell type but cells were additionally located in the mid and posterior region of the gut (Fig. 3F).

MPr-1 specifically labelled cytoplasm and secretory granules of prostate gland cells located around the stylet and the vesicula seminalis in the tail plate (Fig. 4). Gland necks were between 50 and 100 μ m long and opened into the prostatic vesicle (vesicula granulorum) that opens into the stylet itself. Up to 35 labelled cells were present in adult animals while no staining was observed in hatchlings and juveniles. In light microscopic sections prostatic glands were seen to fill large areas between the male copulatory apparatus and the epidermis (Fig. 4C).

In TEM preparations these prostatic gland cells consisted of an active nucleus with a prominent nucleolus. In the cytoplasm large areas of rER were dominant with enlarged ER cisternae and granules in various stages of maturation (Fig. 4D). The granules were ellipsoid with an unequal electron density of their internal structure. In immunogold stainings, the signal is increased in the more dense compartments of these granules (Fig. 4E).

Epidermal mAB

The epidermis consists of polygonally arranged multiciliated epidermal cells (8–10 μ m). They are cuboid cells forming a single layer connected by apical zonula adherentes and septate junctions. The epidermal sheath is underlain by an incomplete extracellular matrix network. The nuclei of epidermal cells of *Macrostomum* sp. are highly lobulated and exhibit a complex three-dimensional morphology (Fig. 5C, D). MEP-1 was directed against nuclei and, though much less distinct, against ultrarhabdites in the apical cytoplasm (Fig. 5). Staining of the ultrarhabdites was more frequently observed within epidermal cells around the mouth area. Confocal cross-sections clearly showed that nuclei of the epidermal layer were always stained, while ultrarhabdites that form a

Fig. 3A–F Demonstration of digestive gland cells of the gastrodermis stained with mABs MDr-1 (A–E) and MDr-2 (F). **A** Interference contrast image superimposed with confocal projection of MDr-1 staining of digestive gland cells shown in white. **B** Confocal projection of all labelled gland cells of A. **C** Detail of a single gland cell. Note granules with intense staining of the granule hull. **D, E** Confocal projections of digestive gland cells stained with antibody MDr-1 of 1-week-starved animals. **F** Confocal projection of MDr-2 labelled cells of a 1-week-starved animal. Note the occurrence of labelled gland cells throughout the gastrodermis. *Arrows* indicate level of the eyes. *e* Eyes. *Scale bars* 100 μm in A, F; 50 μm in B, D; 20 μm in C, E

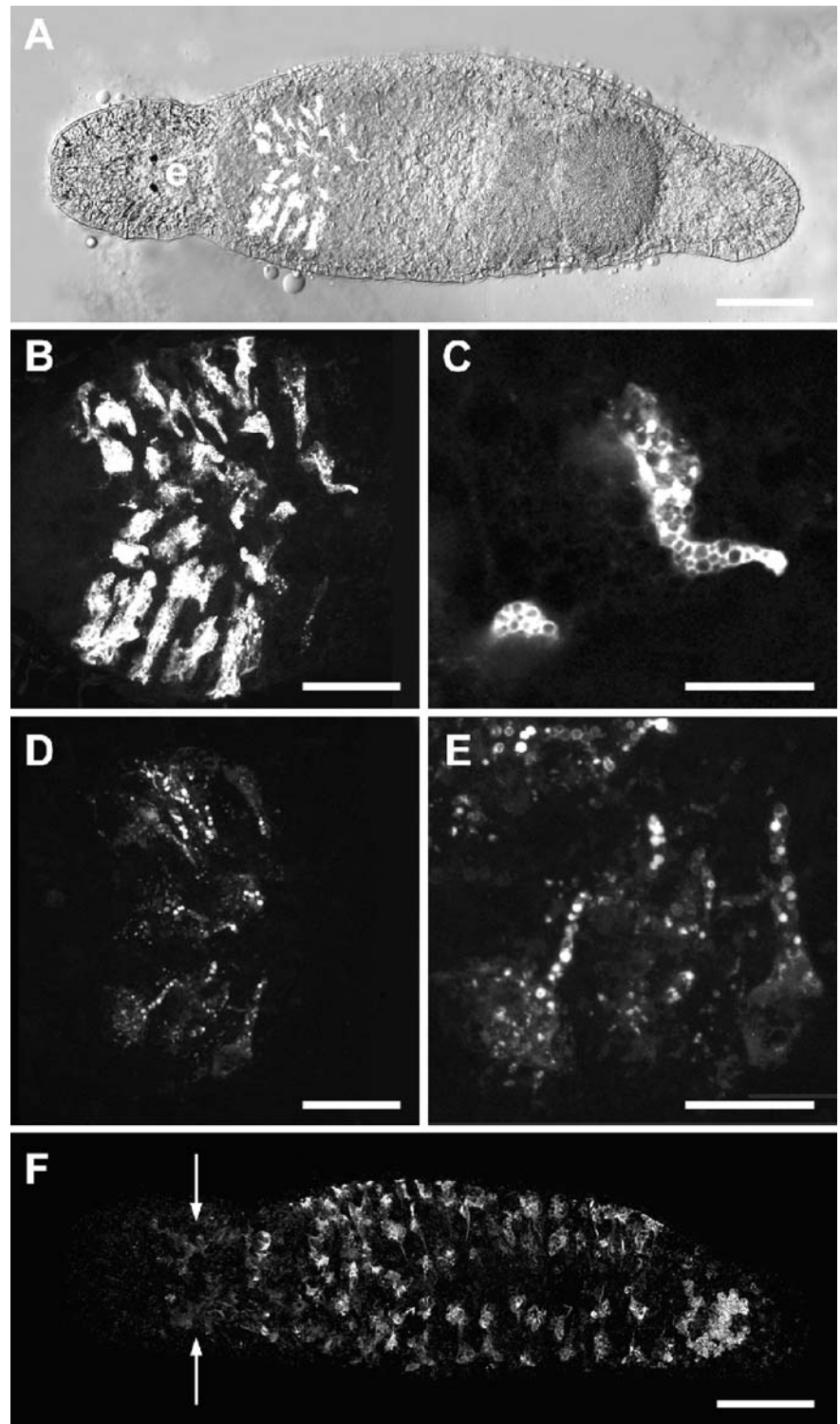


Fig. 4A–E Immunocytochemical demonstration of cytoplasmic content within prostate glands of the tail plate of *Macrostomum* sp. stained with mAB MPr-1 using confocal microscopy (A, B), histological sectioning (C) and electron microscopy (D, E). Anterior is to the left in A and B; C–E are cross-sections through the tail plate. **A** Interference contrast image with confocal projection of MPr-1-stained prostate glands (*prg*, green). **B** Confocal projection demonstrating the location of the vesicula seminalis (*vs*) and the stylet (*st*) surrounded by prostate glands (*prg*). **C** Cross-section of the tail plate. **D** TEM section of prostatic gland cells with nucleus

(*n*), enlarged ER cisternae (*double arrowheads*) and granules of different stages of maturation. A sequential maturation can be seen from early granules with electron-lucent areas surrounded by dense material (*arrowheads*) and mature granules with dense material only (*arrows*). **E** Immunogold staining of prostatic cells demonstrating immunoreactivity in early (*arrowheads*) and mature (*arrows*) granules. Note, electron-lucent areas are not labelled in early granules. *e* Eyes, *ep* epidermis. *Scale bars* 100 μm in A; 10 μm in B, C; 5 μm in D, E

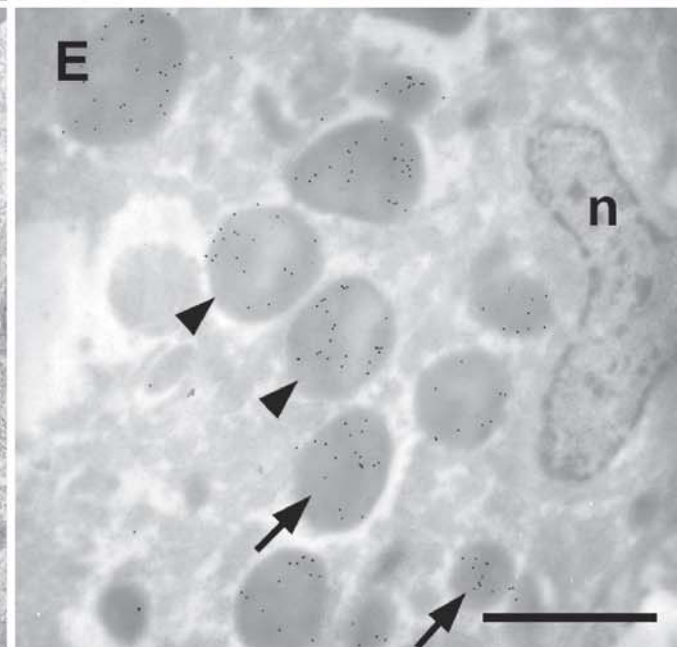
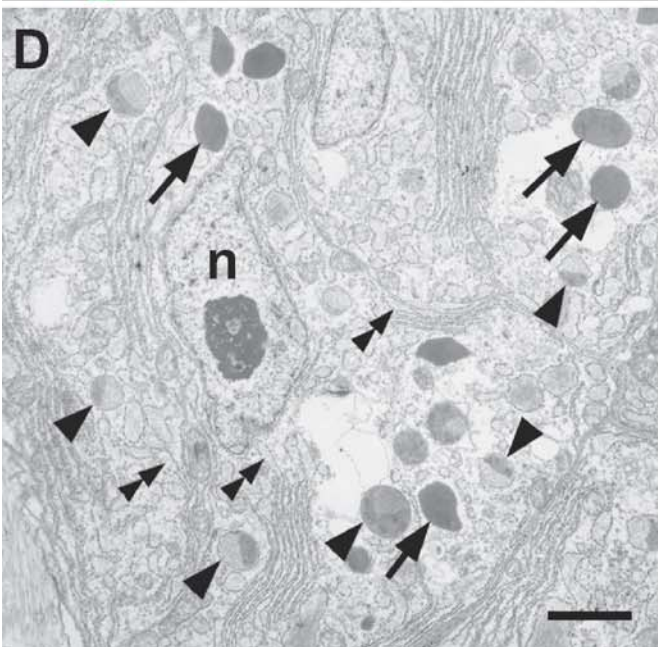
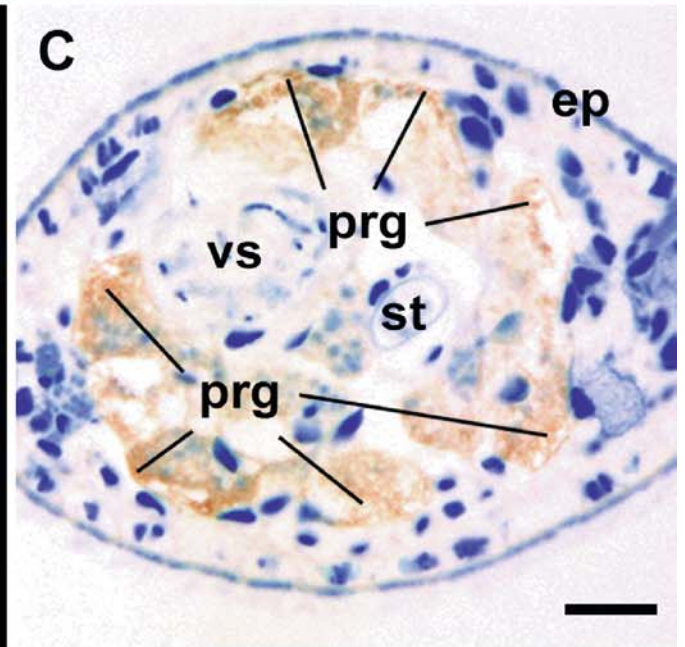
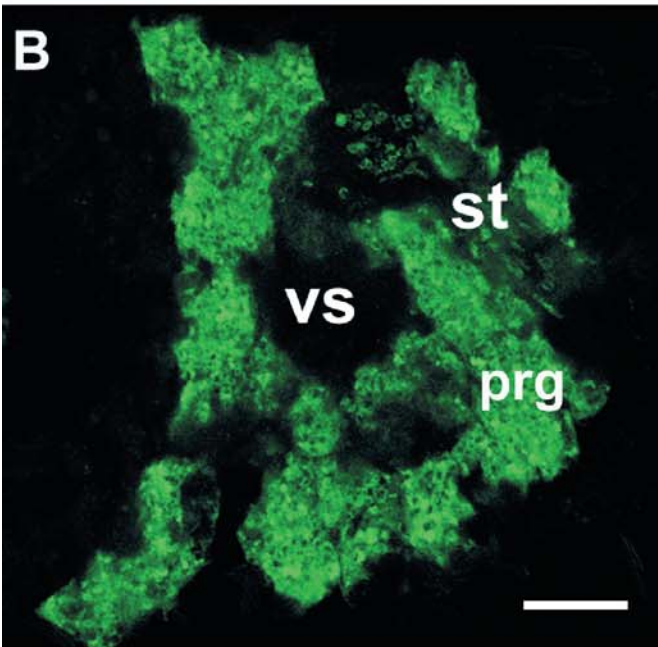
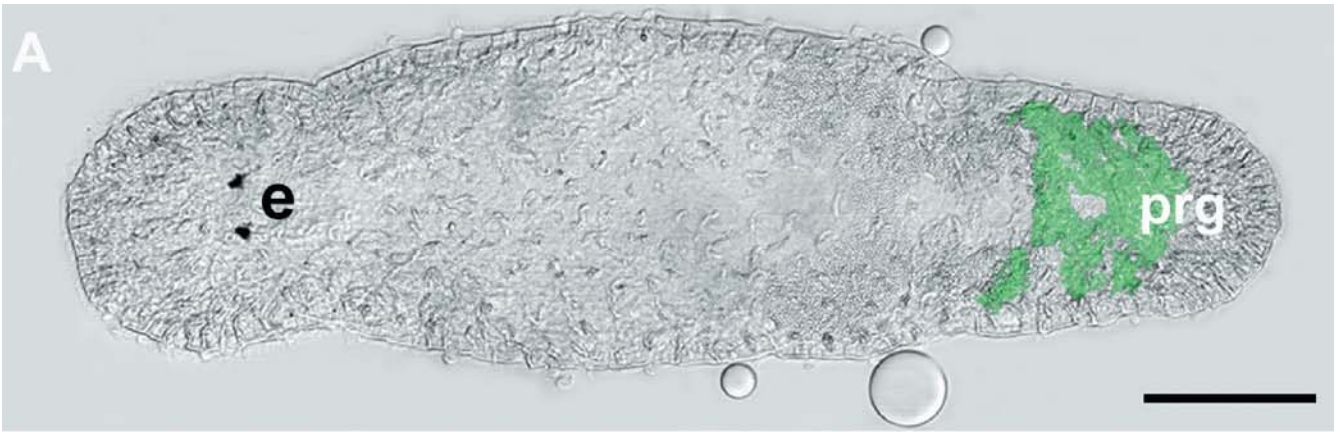
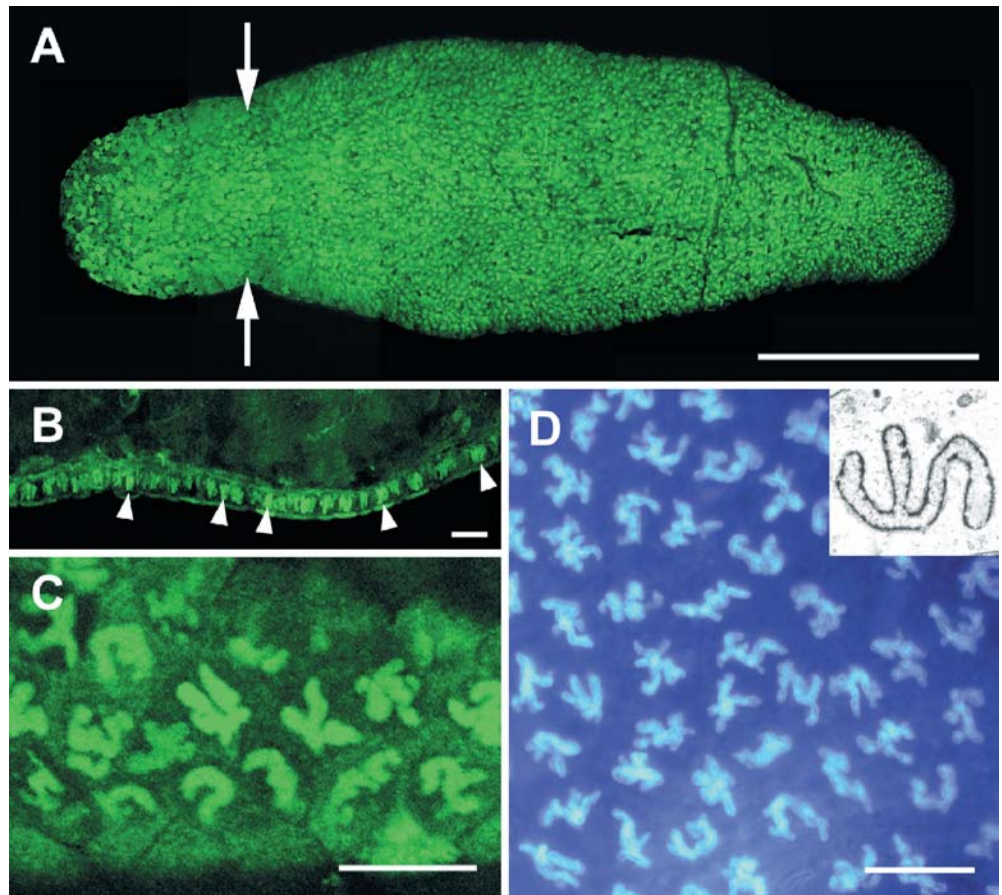


Fig. 5A–D Immunocytochemical staining with mAB MEp-1 (A–C), Hoechst 33342 labelling (D) and ultrastructure of epidermal cell nuclei (*inset* in D) of *Macrostomum* sp. **A** Confocal projection of the dorsal epidermis stained with the mAB MEp-1. Anterior is to the left. Arrows indicate level of the eyes. **B** Optical cross-section of the ventral midbody region with stained nuclei of the epidermal layer (*arrowheads*). **C** Detail (single confocal section) of epidermal cell nuclei at the lateral body margin. Note the lobed morphology of the epidermal cell nuclei. **D** Hoechst 33342 staining of epidermal nuclei of live *Macrostomum* sp. Note the lobed organisation of epidermal cell nuclei. *Inset* shows ultrastructure of lobed epidermal cell nucleus. Scale bars 200 μm in A; 10 μm in B–D



dense layer just below the apical cell membrane were not (Fig. 5B).

Ventral nerve cord mAB

The central nervous system of *Macrostomum* sp. consists of a brain and the main lateral nerve cords connected with a loop in the caudal tail plate. In addition a peripheral nervous system is formed by dorsal and ventral nerve cords interconnected by commissures and some plexus. Subsets of an axon bundle are stained with the commercially available polyclonal antibodies against serotonin or FMRF-amide (Ladurner unpublished observations). MNv-1 specifically labelled nerve fibres and neuronal clusters of the ventral nerve cords (Fig. 6). In adult animals about 6–10 often bilaterally symmetrical stained neuronal clusters were visible along the nerve axons (Fig. 6A, B, D, F). Juveniles and hatchlings possessed only 4–6 stained clusters (Fig. 6D). Some of these clusters near the caudal end of the ventral nerve cord reached a size of approximately 30 μm . In the head region a dorsally located arch (brain loop) was present from which two nerve cords extend towards the ventral side at the level of the postpharyngeal commissure (Fig. 6G, H). From there the axons proceeded within the ventral nerve cord to the tail plate (Fig. 6H). The MNv-1 antibody also showed

weak background labelling of the gut in all stained specimens.

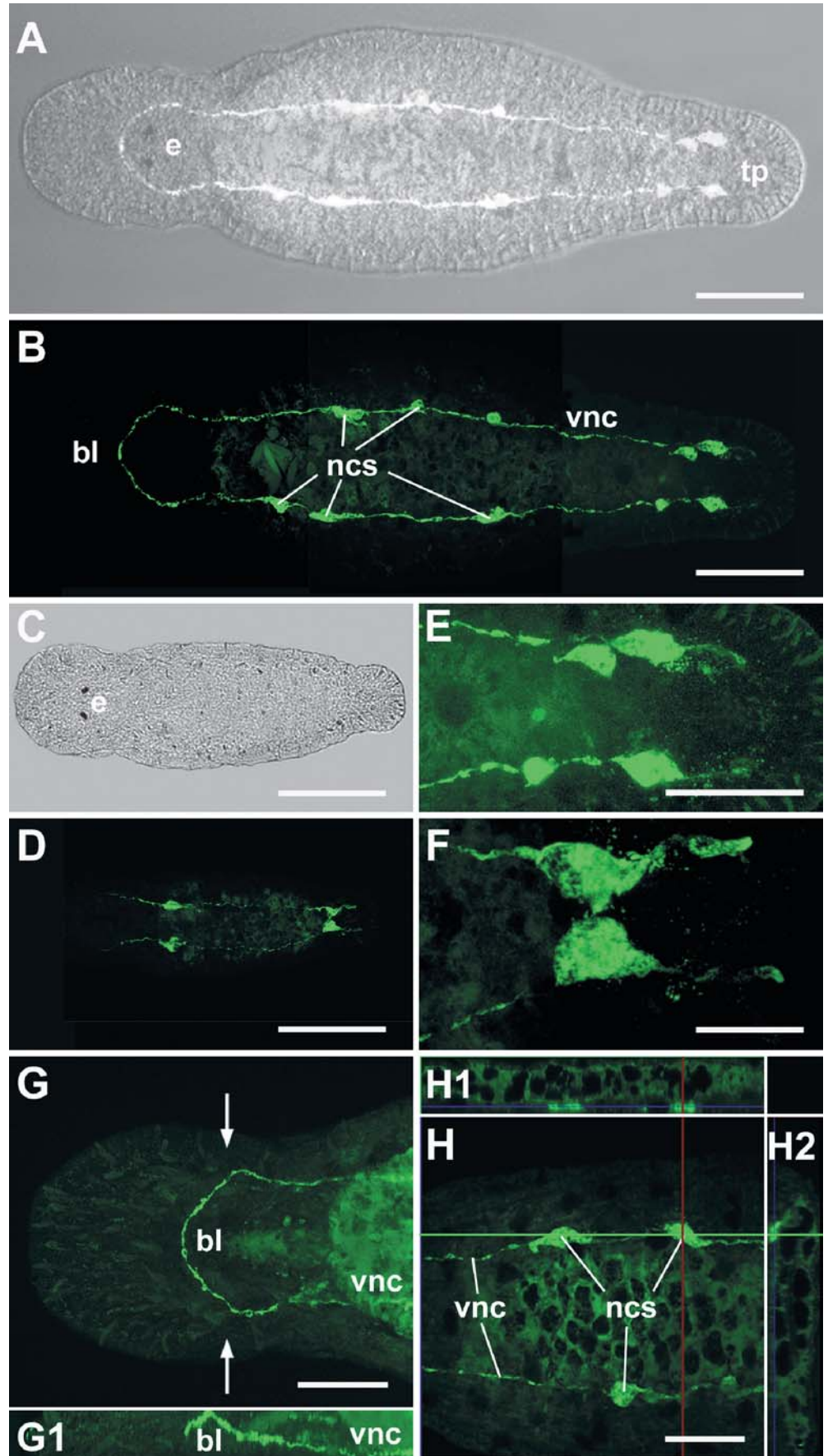
Gut-specific mAB

Macrostomum sp. has a straight, sac-like gut with a single epithelium of relative large gastrodermal cells with long cilia. Two cell types are predominantly present: phagocytes and glandular (granular or Minotian gland) cells. MDA-1 labelled all gastrodermal cells (Fig. 7), the border of gut lumen and pharyngeal glands with different intensities depending on the physiological status of the animals. The cytoplasm of granular gastrodermal cells with inclusions showed specific immunoreactivity (Fig. 7A–E). The apical membranes of phagocytes displayed the strongest labelling (Fig. 7F, G). Phagocytes as a whole showed a stronger immunoreactivity in well fed animals than in starved ones (Fig. 7E). The specific staining resulted in strong immunoreactivity of the gut lumen of well-fed animals while starved animals revealed only faint labelling (compare Fig. 7C–E).

Spermatid-specific mAB

Macrostomum sp. is a simultaneous hermaphrodite with male gonads located anterior to the female gonads. The

Fig. 6A–H Confocal projections of ventral nerve cord and associated neuronal clusters stained with mAB MNv-1 of adult and juvenile *Macrostomum* sp. Anterior is to the left in all panels. **A** Interference contrast image superimposed with confocal projection of MNv-1 staining from **B**. Ventral nerve cord and neuronal clusters are shown in white. **B** Confocal projection of MNv-1 staining of an adult specimen. Note labelling of ventral nerve cord (*vnc*), ten neuronal clusters (*ncs*) and commissural loop in the brain (*bl*). **C** Interference contrast image of a juvenile specimen. **D** Confocal projection of MNv-1 staining of animal shown in **C**. Note that only four neuronal clusters are present in juveniles. **E** Magnification of four neuronal clusters in the tail plate of **B**. **F** Magnification of two neuronal clusters in the tail plate of **D**. **G** Confocal projections of the commissural loop in the brain. Arrows indicate level of the eyes. **G1** Sagittal projection of confocal stack of **G**. Note that the commissural loop arches dorsally over the brain (*bl*) and axons proceeded towards the ventral caudally. Dorsal is top. **H** Projection of midbody region containing ventral nerve cord (*vnc*) and three neuronal clusters (*ncs*). **H1** Virtual sagittal section through two neuronal clusters at the level of the green line in **H**. Note ventral location of clusters. **H2** Virtual cross-section through one neuronal cluster at the level of the red line in **H**. Ventral is left. *e* Eyes, *tp* tail plate. Scale bars 100 μ m in **A–D**; 50 μ m in **E**, **G**, **H**; 20 μ m in **F**



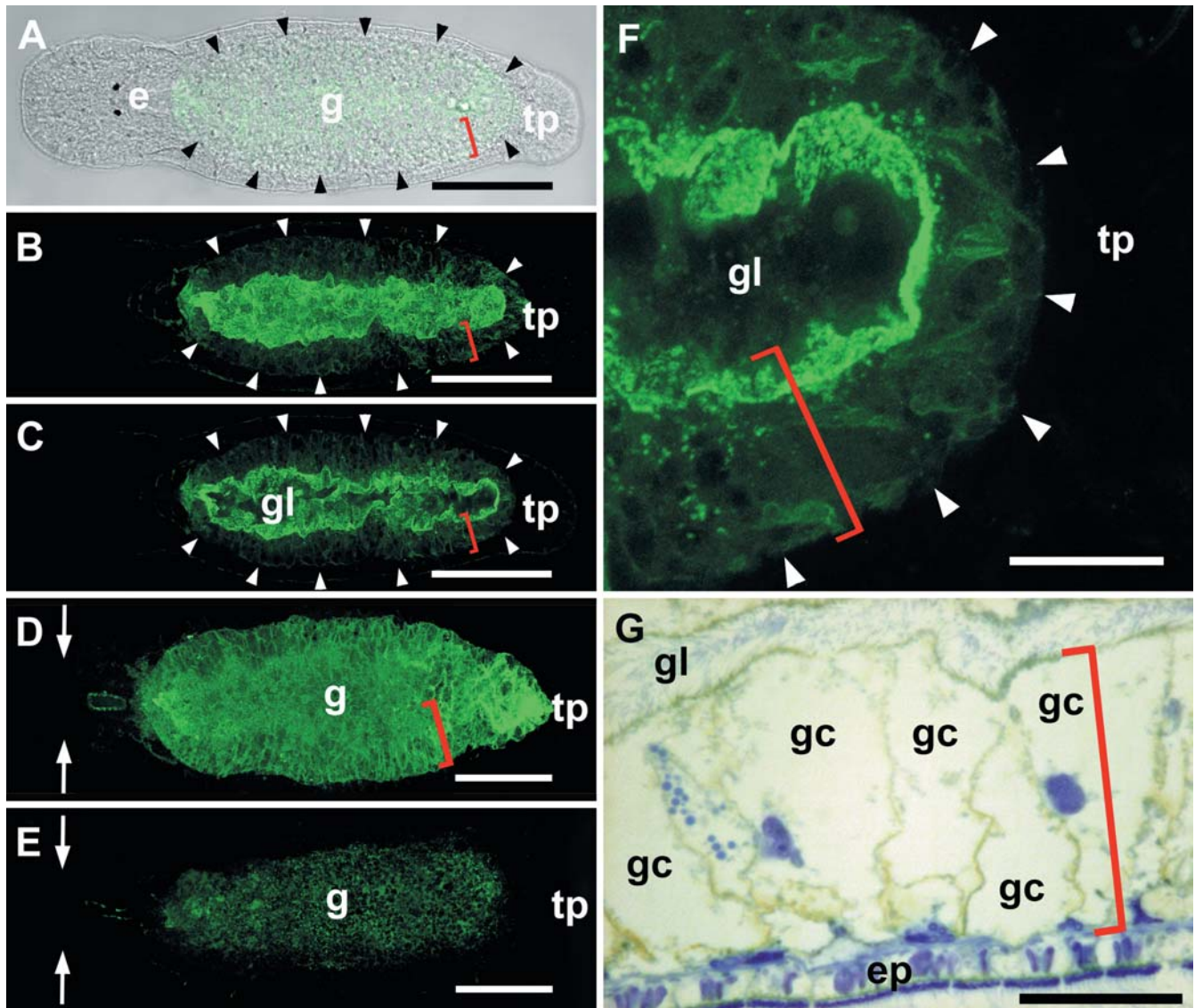
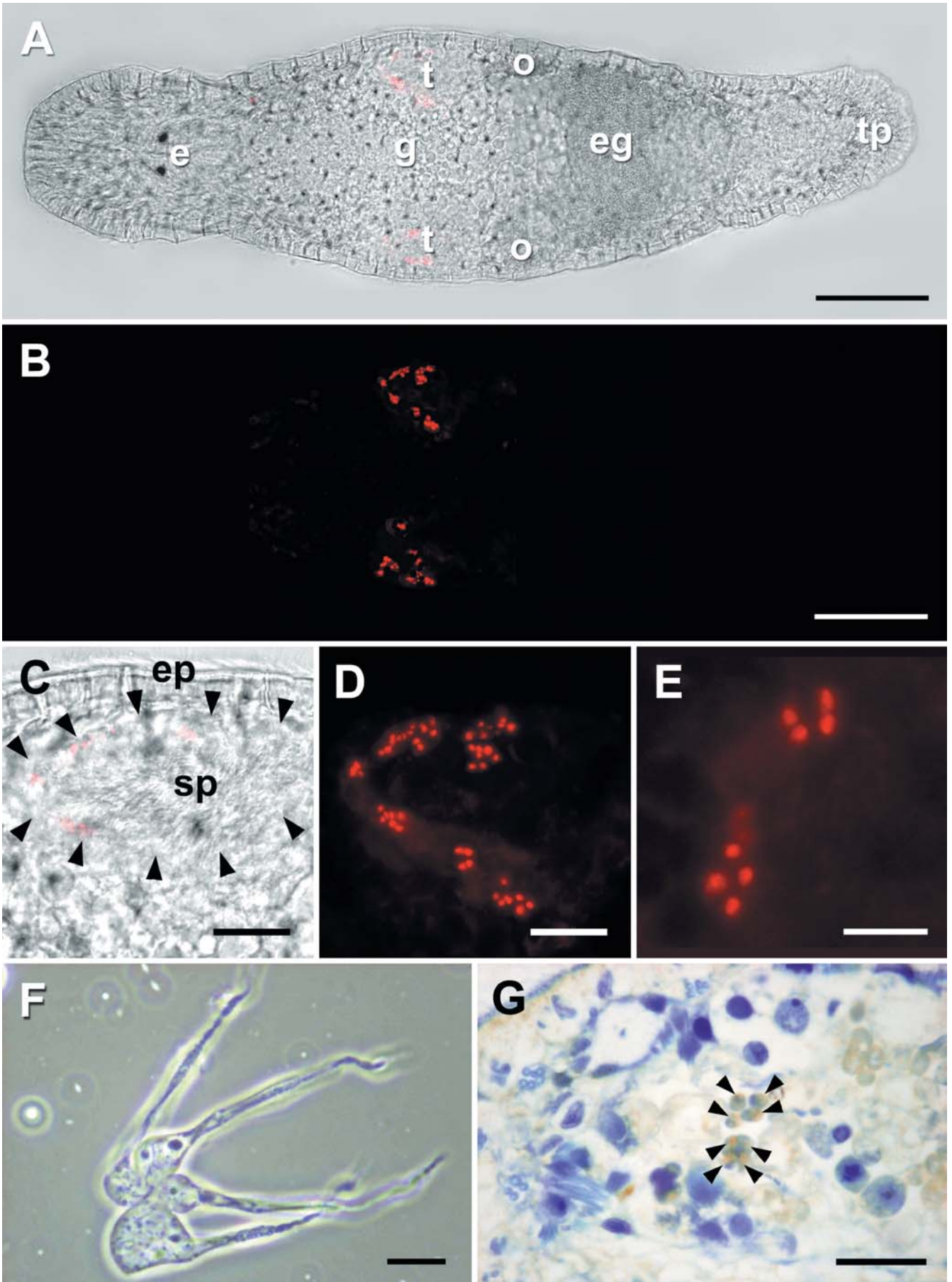


Fig. 7A–G Immunocytochemical staining of the gut with mAb MDa-1. Anterior is to the left in all panels. Red brackets indicate the extension of the gastrodermis. Arrows indicate level of the eyes. **A** Interference contrast with confocal projection of the stained gut. **B** Optical section above the gut lumen of a 2-day-starved animal. The outermost extension of phagocytes is indicated by arrowheads. **C** Optical section through the gut lumen of the same animal as in **B**. Note that only the most apical part (closest to the gut lumen) of phagocytes shows intense staining. **D** Confocal projection of a well-

fed animal. Note that phagocytes show homogeneous staining compared to 2-day-starved animals in **B** or **C**. **E** Confocal projection of a 14-day-starved animal. **F** Optical section of the posterior end of the gut. Only the most apical part of phagocytes shows intense staining. **G** Midsagittal histological section with MDA-1 labelling (brown). Note that the label is concentrated at the apical as well as the basolateral cell membrane of the phagocytes. *e* Eyes, *ep* epidermis, *g* gut, *gl* gut lumen, *tp* tail plate. Scale bars 100 μm **A–E**; 20 μm in **F**, **G**

testis can be divided into distinct functional zones comprising a germinative zone, a growth zone and a differentiation zone. Within the testis of *Macrostomum* sp. all stages of spermatogenesis (spermatogonia, primary and secondary spermatocytes and spermatids) can be identified based on their morphology. MSp-1 stained clusters of spermatids located on the outer margin of the testis (Fig. 8A–D). The cluster included groups of four, eight or twelve labelled spermatids (Fig. 8D, E). In maceration experiments and histological sections differentiating spermatids were often seen as clusters of four (Fig. 8F, G). Based on immunostained sections labelling

Fig. 8A–G Spermatids stained with mAb MSp-1. **A** Interference contrast image with confocal projection of MSp-1-labelled spermatids (red staining) in the testis (*t*). **B** Confocal projection of labelled spermatids of animal of **A**. **C** Detail interference contrast with confocal projection of stained spermatids (red) at the outer margin of the testis. Note differentiated sperm (*sp*) in the centre of the testis. Arrowheads indicate the outline of the testis. **D** Confocal projection of the anterior half of the testis with labelled spermatids (red). **E** Optical confocal section of two four-cell clusters of spermatids. **F** Macerated differentiating spermatids in clusters of four cells. **G** Histological section of the testis stained with MSp-1. Note four-cell clusters of labelled spermatids (arrowheads). *e* Eyes, *eg* egg, *ep* epidermis, *g* gut, *o* ovary, *tp* tail plate. Scale bars 100 μm in **A**, **B**; 20 μm in **C**, **D**; 10 μm in **E–G**



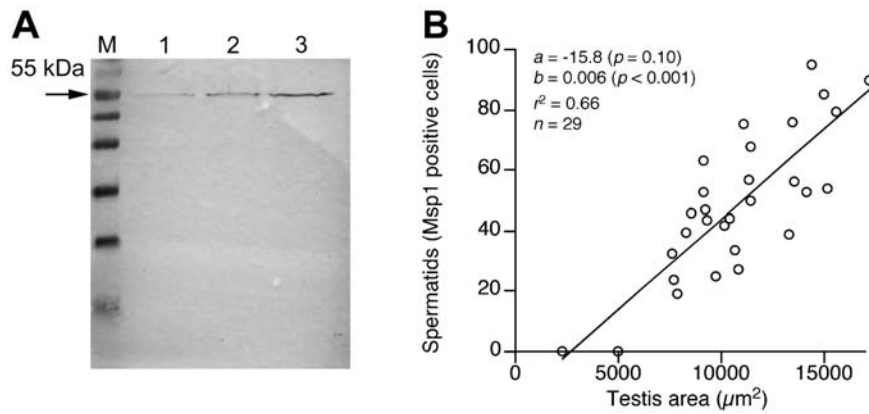


Fig. 9 **A** Detection of *Macrostomum* sp. proteins in a western blot assay with the mAB MSp-1. The bands represent the protein content of 10, 20 or 40 animals in lanes 1, 2 and 3, respectively. The stained protein has a size of 55 kDa. *M* pEqGOLD Protein Marker IV. **B** Relationship between the testis size and the number of

MSp-1-positive cells. Larger testes contain more MSp-1-positive cells, and although the intercept, *a*, is not statistically significant there appears to be a minimal testis size before there are any MSp-1-positive cells

was shown to occur in the cytoplasm of spermatids (Fig. 8G). Roughly 25 clusters were present in standard animals. Three-week-starved adults or juvenile animals did not possess stained cells. A slight unspecific staining of some prostate glands in the tail plate and a weak overall background labelling of the testis was seen in all preparations.

MSp-1 number as a measure of male investment to gametes

MSp-1 can be used as marker to quantify male investment. Here we show a correlation between testis size and the number of MSp-1-positive cells (Fig. 9B).

The repeatability of the MSp-1 counts was very high and highly significant (intraclass correlation coefficient, $F_{(28,29)}=21.2$, $r_1=0.91$, $P<0.001$), suggesting that the MSp-1 signal is very clear and highly quantifiable. Worms on average had 48.9 (SE=4.5, $n=29$) MSp-1-positive cells and, as expected, there was a strong relationship between the testis size and the number of MSp-1-positive cells (see Fig. 9B for statistics).

Discussion

The promise of this approach—immunisation with cell suspensions of the whole organism—lies in the potential to identify new molecules specific for cells, tissues or organs. In *Macrostomum* sp. mABs are a valuable tool to study cell differentiation of multi- or totipotent stem cells during development and regeneration. In addition, other research fields, such as studies on reproductive allocation of this hermaphroditic species (Schärer and Ladurner 2003) and detailed insight into functions of different organs, will benefit particularly by the availability of specific markers.

Monoclonal antibodies in developmental studies and pattern formation

Monoclonal antibodies are extremely important markers for specific cell types and have a great variety of applications in biology and medicine (Milstein 1999). For Platyhelminthes only a limited number of studies concentrated on that method (Romero et al. 1991; Shirakawa et al. 1991; Shinozawa et al. 1995; Bueno et al. 1997; Orii et al. 2002). In *Dictyostelium*, the formation of glycoprotein was studied using mABs (Crandall and Newell 1989). In *Hydra*, mABs were applied to study the differentiation of neurons and pattern formation (Dunne et al. 1985; Littlefield et al. 1985; Yu et al. 1985, 1986; Javois et al. 1986, 1988; Yaross et al. 1986; Javois 1990). Monoclonal antibodies are available for all developmental model organisms such as, for example, *C. elegans* (Goetinck and Waterston 1994), *Drosophila* (Patel 1994), *Xenopus* (Itoh et al. 1994) or mouse (Kubota et al. 1996). Antibody libraries were raised to dissect, for example, nuclear structure in animals (Saumweber 1991) and plants (Mazzolini et al. 1989).

Monoclonal antibodies against muscle, gland, gut, nerve, epidermal and spermatogonial cells

Muscle-specific mABs

In flatworms, the muscle system has recently become an important character for studies of development, regeneration and evolution. In several flatworm species the organisation of the muscle system during embryonic development and regeneration was studied using fluorescent conjugated phalloidin (Rieger et al. 1994; Reiter et al. 1996; Hooge and Tyler 1999; Ladurner and Rieger 2000; Mair et al. 2000; Salvenmoser et al. 2001; Hooge 2001), mABs (Bueno et al. 1997; Cebria et al. 1997, 1999; Cebria and Romero 2001) or in situ hybridisation (Ko-

bayashi et al. 1999, 2000). It is important to note that phalloidin staining of musculature is not possible in combination with in situ hybridisation or BrdU staining, therefore, an antibody is necessary for further investigations in that field. Immunocytochemical analyses of the *Dugesia japonica* muscle system were performed (Orri et al. 2002) using polyclonal antibodies that were made against two myosin heavy chain peptides previously isolated from *D. japonica* (Kobayashi et al. 1998).

All muscle-specific mABs generated in this study are especially suited to address specific questions during embryonic development and regeneration of the muscle system:

1. Embryonic origin of longitudinal and circular muscle cells and pattern formation. Few data are available on the embryonic origin of the muscle grid of *Macrostomum hystricinum marinum* and the polyclade *Hoplodiplana inquilina* (Reiter et al. 1996) and two acoel species (Henry et al. 2000; Ladurner and Rieger 2000). The mAB MMu-1 specific for longitudinal muscles can be used to study early muscle differentiation and muscle fibre orientation at the onset of myogenesis in the *Macrostomum* embryo after 50% developmental time after egg laying.
2. Mesoderm evolution. An independent evolutionary origin of longitudinal and circular muscle fibres in flatworms from ecto- and gastrodermal muscle layers of coelenterate-like ancestor might be possible. Therefore, analyses of the embryonic origin of longitudinal and circular muscle fibres in flatworms can contribute to a better understanding of the transition between diploblastic and triploblastic organisation and the origin of mesoderm (Ladurner and Rieger 2000; Rieger and Ladurner 2003).
3. Regeneration of the muscle system was studied in acoel, macrostomid and planarian species (Cebria et al. 1997, 1999; Akesson et al. 2001; Salvenmoser et al. 2001; Gschwentner et al. 2003). During early steps of regeneration longitudinal muscle was shown to provide guidelines by outgrowth and forking (Salvenmoser et al. 2001). The availability of a specific marker for longitudinal muscles allows focusing on the longitudinal compartment during regeneration facilitating the complexity of observed muscle structures during the regeneration process.
4. Taxonomy. A comparison of muscle patterns was used for taxonomy of Acoela (Tyler and Hyra 1998; Hooge and Tyler 1999; Hooge 2001). The availability of various mABs provides new morphological characters for taxonomic studies.

Gland cell-specific mABs

A variety of gland cells are present in *Macrostomum* sp. serving different functions. Gland cells probably contain highly antigenic components. More than 20.5% (74/360) gland cell-positive supernatants were found. Specific

stainings of gland cells were identified around the pharynx, in the rostrum, at the female opening, rhabdites, rhamites, granular gut gland cells and prostate gland cells. About 45% of all mABs produced for *Dugesia* (*Girardia*) *tigrina* (Bueno et al. 1997) stained acidophilic (2.9%) or cyanophilic (29.3%) secretory cells, goblet cells (4.0%) and other unclassified secretory cells (9%).

In *Macrostomum* sp. the obtained gland cell-specific mABs will be applied to study gastrodermal cellular composition, the embryonic formation and regeneration of the gut (MDr-1 and MDr-2). The prostate gland-specific antibody (MPr-1) is used to analyse the formation of the male reproductive system during postembryonic development and regeneration. After prolonged starvation periods tissues and cells were abolished and cell proliferation was highly reduced (Nimeth et al. 2004). After animals are exposed to food, the reconstitution of prostate glands can be monitored. For reproductive allocation studies (Schärer and Ladurner 2003) quantitative measurements of the prostate cell number are possible to study this part of male allocation under different levels of reproductive competition.

Gut-specific mABs

A recent study of the embryonic development of *Macrostomum* sp. (Morris et al. 2004) has found the formation of the gut primordium at stages 4–5 (48–76 h, i.e. 40–63% time after egg laying). Gastrodermal cells and the gut precursor were identified based on the posterior-central location in the embryo and the high content of yolk. The availability of a specific marker for all gastrodermal cells allows a much more precise histological allocation of gut cells in the *Macrostomum* embryo and a detailed analysis of gut formation during embryogenesis and regeneration.

Epidermal-specific mABs

The formation of the epidermis during embryonic development (Morris et al. 2004) of *Macrostomum* sp. takes place during stage 5 (60–76 h, 50–63% time after egg laying). During these early stages independent patches of epidermal cells form and unite with progressing development to cover the whole embryo with epidermal cells. These findings were based on reconstructions of histological serial sections of different embryonic stages. A whole-mount approach using the epidermal cell-specific mAB MEp-1 will facilitate investigations on epidermis formation. In addition, double labelling of *Macrostomum* embryos of epidermis with muscle or nervous system could reveal potential developmental interactions of these tissues. As proposed (Tyler 2003) the evolutionary mechanisms of epithelial cell specification appear to be recapitulated during embryonic development. The application of MEp-1 will allow a better understanding of the formation of the epidermis during embryonic development of a basal flatworm and, therefore, might also pro-

vide clues on epithelial evolution. During regeneration epidermal cells flatten to cover the wound (Baguña et al. 1994). Epidermal reorganisation can be followed during regeneration using MEp-1.

Spermatid-specific mABs

Spermatogenesis in *Macrostomum* sp. occurs within a gonad that is delimited by a cellular and ECM layer. Spermatogonia, primary and secondary spermatocytes as well as spermatids can be distinguished by morphological characters. Spermatids occur in groups that develop synchronously and seem to be interconnected by cytoplasmic bridges. We found clusters with four or multiples of four cells stained by the mAB MSp-1 at the outer margin of the gonads. MSp-1 was tested positive in western blotting (Fig. 9A) and can be used for screening of a *Macrostomum* sp. cDNA library to identify the encoding gene and the respective protein.

MSp-1 number as a measure of male investment to gametes

The MSp-1 antibody represents a powerful tool to quantify male reproductive investment towards gamete production (Fig. 9B). In combination with BrdU pulse chase experiments the throughput can be calculated (see Schärer et al. 2004a). It will be possible to further characterise mating behaviour of *Macrostomum* sp. (Schärer et al. 2004b). For reproductive allocation studies this is a highly useful number that is very difficult to determine in any other organism. The MSp-1 antibody can be used as marker to study postembryonic development of gonads, for regeneration and to follow the rebuilding of gonads after long starvation periods (Nimeth et al. 2004).

Nervous system-specific mABs

The nervous system of macrostomid species was studied using commercially available polyclonal antibodies against serotonin (Ladurner et al. 1997) and FMRF-amide (Ladurner unpublished). According to stainings using these two antibodies the ventral nerve cord seemed to arise close to or from the postpharyngeal commissure. In contrast stainings with MNv-1 clearly showed that axons of the main nerve cord proceeded through the neuropile. Serotonergic cells are concentrated in two clusters at the postpharyngeal commissure and two cells posterior to the eyes, but serotonergic cells are lacking posterior to the postpharyngeal commissure (Ladurner et al. 1997). FMRF-amide cells and fibres are more abundant and can also be bound in the posterior half of the animal. Two pairs of small FMRF-amide cells can be located along the posterior part of the main lateral nerve cords. However, for regeneration studies the reorganisation of serotonin and FMRF-amide cells and fibres is not easy to follow.

The mAB MNv-1 stains the ventral nerve cord and large clusters of cells along the ventral nerve cord. Those cells can be easily surveyed using MNv-1 and can be used as landmarks to follow the regeneration of the nervous system.

Conclusions

The availability of specific mABs for *Macrostomum* sp. provides means to address problems within various research fields. *Macrostomum* sp. is currently used as a model organism to study: (1) the extraordinary stem cell system including neoblast proliferation, migration and differentiation, (2) the development and evolution of muscle and nervous system, (2) the evolution of mesoderm, (3) the origin and evolution of the body axis, (4) regeneration, (5) reproductive allocation and (6) embryonic development. Current efforts to obtain molecular markers by EST sequencing of a *Macrostomum* sp. cDNA library will supply additional resources. As for now the antigens of the mABs for *Macrostomum* sp. are unknown. With the availability of expression libraries antibody screening will lead to the isolation of DNA coding for the antigen as was shown for planarians (Vispo et al. 1996).

Further production of mABs using, for example, enriched cell populations or individual proteins will make available a monoclonal library for *Macrostomum* sp. The mABs demonstrated here will be made available at the Developmental Studies Hybridoma Bank (University of Iowa).

Note added in proof Ladurner P, Schärer L, Salvenmoser W, Rieger RM. A new model organism among the lower Bilateria and the use of digital microscopy in taxonomy of meiobenthic Platyhelminthes: *Macrostomum lignano*, n. sp. Rhabditophora, Macrostromorpha. J Zool Sys Evol Res, accepted.

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