Establishing a flatworm ageing model

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Ageing is a gradual and inherently complex process, with almost all aspects of physiology and phenotype undergoing steady modifications (1). For studying the different aspects and possible causes of ageing, diverse methodologies and model organisms are used. In recent research, increased attention is drawn to the role of stem cells and tissue homeostasis. Several studies have already demonstrated that ageing is invariably accompanied by a diminished capacity to adequately maintain tissue homeostasis or to repair tissues after injury (1-3). This declining capacity can, however, be caused by several plausible mechanisms, such as age-related changes in the stem cells themselves, in the local environment (niche) in which the stem cells reside, in the systemic milieu of the organism (e.g. the nervous system), or in any combination of these (2). To unravel these distinct contributions to the aged phenotype, more data on these processes is needed. Studying these processes in the existing model organisms is, however, difficult because of the relative inaccessibility of the stem cell population in the vertebrate models on one side, and because of the lack of or limited number of stem cells in adults of models such as *Caenorhabditis elegans* and *Drosophila melanogaster* on the other side (3,4). Therefore, new model organisms should be developed to obtain more data and gain a better insight in this matter.

Free-living flatworms are highly promising organisms in which to unravel such unanswered questions, mostly due to their experimentally-accessible population of likely totipotent stem cells, known as neoblasts (5-7). Furthermore, in several flatworm species, a lifespan extension induced by starvation or repeated regeneration is observed, and several authors even suggest that there is an actual rejuvenation (8-10). This makes it possible to study, not only ageing, but also rejuvenation and the interaction between younger and older tissues. Therefore, flatworms have the potential to become one of the new ageing models we are looking for.

The first step in developing a flatworm ageing model is choosing an appropriate species. An important criterion for making this choice is being able to maintain standardised cultures. This is a necessity for setting up ageing cultures and obtaining enough individuals of a desired age to perform experiments. After trying to culture several flatworm species, we made an initial selection of three species: *Schmidtea mediterranea*, *Schmidtea polychroa* and *Macrostomum lignano*. The next aims were to further standardise the cultures and test the potential of these three species for studying different aspects of ageing. Each species has it strengths and weaknesses, but our experience suggested that *M. lignano* (Macrostomorpha) is the most appropriate for ageing research. Some examples of the advantages of this worm compared to triclads are: 1) a transparent body and well-described anatomy, which makes the in vivo study of cells and organ systems possible during ageing and regeneration, 2) neoblasts and other cell types can be easily labelled by soaking the whole animals in medium containing antibodies, 3) a limited number of cells, 25,000 in total, which makes it possible to quantify neoblasts and other cell types (7,11). Consequently, we chose *M. lignano* to develop it further as a new ageing model. We will therefore focus on this species here.

Culturing *M. lignano* in the laboratory is straightforward and standardised. Individuals are incubated at 20°C and a 13h:11h light: dark cycle (12) and are maintained in f/2, a nutrient-enriched artificial seawater medium at a salinity of 32‰ (13). They are fed ad libitum with the diatom *Nitzschia curvilineata*, which is grown under identical conditions as the worms. Mature worms, which are simultaneous hermaphrodites, generally lay one egg per day and individuals have a short embryonic development of 5 days and a generation time of about 2 – 3 weeks (14).

The next step in initiating ageing research in a new model organism is to obtain a demographic data set, which is essential for designing experiments. These survival data indicate at what age individuals can be considered old and what proportion of the initial cohort is alive at a certain age. This allows one to choose age groups and calculate how big initial ageing cultures should be, in order to give experiments enough statistical power. Besides survival data, knowledge on the age-related changes in mortality rate provides a basic measure for the rate of senescence (15), and can be used to study whether experimental manipulations can alter the rate of ageing or even induce rejuvenation. We have previously followed the survival of *M. lignano* in three replicate cultures consisting of 100 individuals each (16). This experiment has demonstrated that in this species the average (± standard deviation) median lifespan is 205 ± 13 days and

90th percentile lifespan is 373 ± 32 days (16). The maximum lifespan currently observed is 861 days (2.4 years). Further analysis of the survival data allowed us to calculate the mortality rate doubling time (MRDT), which is held to be a fundamental measure of senescence (15,17). *M. lignano* has a MRDT of 0.20 ± 0.02 years and is thus, just as humans, a gradually ageing species according to Finch's (15) classification of senescence patterns. Remarkably, the mammalian models *Mus musculus* and *Rattus norvegicus* have a similar MRDT of 0.27 and 0.30 years respectively (15,17). Despite this, *M. lignano* has a shorter lifespan than these rodent models (maximum lifespan of 4.5 and 5.5 years respectively), which is a clear advantage for experimental work with this species (17). In contrast, other frequently-used invertebrate models such as *C. elegans* and *D. melanogaster* show rapid senescence, with a MRDT of 0.02-0.04 years and a short maximum lifespan of 0.16 and 0.30 years respectively (15). The survival curve itself and a detailed discussion of the above-mentioned demographic data set of *M. lignano* are presented in Mouton et al. (16).

As a next step, we are characterising the morphology as a function of age in *M. lignano*. Characteristic for ageing individuals is the appearance of body deformities, such as a slightly notched epidermis, the presence of grooves in the head region (called urn-shaped invaginations in Ladurner et al. (18)), liquid-filled cysts that tend to be present in all body regions and a disintegration of the gonads in some aged individuals (16,19). These deformities can be an indication of failing cell renewal and tissue homeostasis with advancing age.

To confirm this hypothesis, we conducted preliminary experiments with two concentrations of 5-fluorodeoxyuridine (FUdR): 0.2mM and 0.6mM. FUdR inhibits the cell cycle during DNA replication, which results in the loss of neoblast-functionality and an inhibited tissue homeostasis. Treatment was performed by culturing 1-month old individuals as described above, but with FUdR dissolved in the f/2-medium. Treatment of these young individuals initially resulted in similar deformities as described above, but malformations quickly became more severe and extreme during the progress of the experiment. In the first week of treatment, the epidermis became slightly notched. Later on, the notching of the epidermis became much more extreme. From the second week of treatment, lesions in the epidermis could be observed. Furthermore, organs such as the gonads and the gut started to disintegrate. After the third week, several individuals broke into pieces and died (Fig. 1.). In general, the observed malformations strongly resemble the phenotypes that became apparent following *macpiwi* RNA interference and irradiation of *M. lignano*, which also inhibit neoblast functionality and tissue homeostasis (20,21). An effect of the dosage of FUdR could not be observed by studying the morphology. As this experiment was preliminary, more research is needed to study more FUdR concentrations, recovery after stopping treatment and survival.

Currently we are also studying the number of stem cells and the rate of tissue homeostasis as functions of age. This is possible by labelling S-phase and M-phase neoblasts with the thymidine analog 5'-bromo-2'-deoxyuridine (BrdU) and anti-phosho-histone H3, respectively (7,22).

With the experiments described in this manuscript, we started characterising the phenotypic effects of the ageing process of *M. lignano*. Much more research is needed, but because of several strengths (ease of culturing, accessible stem cell population, available experimental toolbox), this species has the potential to play a crucial role in gaining better insight into the role of stem cell biology and tissue homeostasis for ageing and rejuvenation.

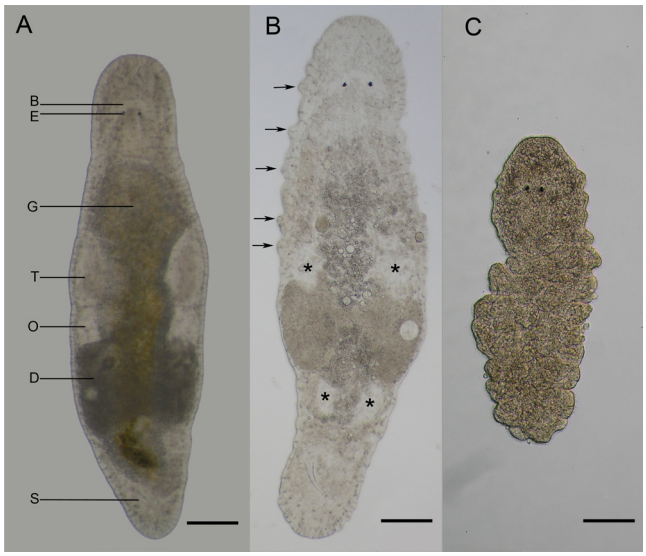

Fig. 1. – Morphology during FUdR-treatment. (A) Morphology of young adults of *M. lignano* in normal culture conditions. (B) Individual during the second week of FUdR-treatment. Several malformations can be observed such as a notched epidermis (bulges indicated with arrows), lesions in the epidermis (*) and a disintegration of the gonads. (C) Individual during the third week of FUdR-treatment. Organs such as the gonads and the gut are completely disintegrated and the epidermis is extremely notched. In all figures, anterior is at the top. A and B are ventral views, C a dorsal view. B: brain, E: eye, G: gut, T: right testis, O: right ovarium, D: developing egg, S: stylet. Scalebars: 100 µm.

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