Developmental Biology 433 (2018) 448–460

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Original research article

Organ specific gene expression in the regenerating tail of *Macrostomum lignano*

Birgit Lengerer[a], Julia Wunderer[a], Robert Pjeta[a], Giada Carta[c], Damian Kao[b], Aziz Aboobaker[b], Christian Beisel[d], Eugene Berezikov[e], Willi Salvenmoser[a], Peter Ladurner[a,*]

[a] Institute of Zoology and Center of Molecular Bioscience Innsbruck, University of Innsbruck, Technikerstr. 25, A-6020 Innsbruck, Austria
[b] Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, United Kingdom
[c] Division of Physiology, Medical University of Innsbruck, Schöpfstraße 41/EG, A-6020 Innsbruck, Austria
[d] Department of Biosystems Science and Engineering, ETH Zürich, Mattenstrasse 26, 4058 Basel, Switzerland
[e] European Research Institute for the Biology of Ageing, University of Groningen, University Medical Center Groningen, A. Deusinglaan 1, NL-9713 AV Groningen, The Netherlands

ARTICLE INFO

Keywords:
Differentiation
in situ hybridization
Stylet
Male copulatory apparatus

ABSTRACT

Temporal and spatial characterization of gene expression is a prerequisite for the understanding of cell-, tissue-, and organ-differentiation. In a multifaceted approach to investigate gene expression in the tail plate of the free-living marine flatworm *Macrostomum lignano,* we performed a posterior-region-specific *in situ* hybridization screen, RNA sequencing (RNA-seq) of regenerating animals, and functional analyses of selected tail-specific genes. The *in situ* screen revealed transcripts expressed in the antrum, cement glands, adhesive organs, prostate glands, rhabdite glands, and other tissues. Next we used RNA-seq to characterize temporal expression in the regenerating tail plate revealing a time restricted onset of both adhesive organs and copulatory apparatus regeneration. In addition, we identified three novel previously unannotated genes solely expressed in the regenerating stylet. RNA interference showed that these genes are required for the formation of not only the stylet but the whole male copulatory apparatus. RNAi treated animals lacked the stylet, vesicula granulorum, seminal vesicle, false seminal vesicle, and prostate glands, while the other tissues of the tail plate, such as adhesive organs regenerated normally. In summary, our findings provide a large resource of expression data during homeostasis and regeneration of the morphologically complex tail regeneration and pave the way for a better understanding of organogenesis in *M. lignano*.

1. Introduction

Regeneration and organ formation rely on restricted spatial and temporal gene expression. In recent years, several studies were aimed at the characterization of post-embryonic organogenesis in Platyhelminthes. Members of this phylum are known for their astonishing regeneration abilities, with some species being able to regrow complete animals from small tissue pieces (Morgan, 1901; Reddien and Sanchez Alvarado, 2004). Most regeneration studies were performed in the asexual strains of the freshwater species *Schmidtea mediterranea* and *Dugesia japonica* (reviewed in (Aboobaker, 2011; Adler and Sanchez Alvarado, 2015; Reddien, 2013; Rink, 2013)). Therefore, the molecular program required for the regeneration of most organs of these animals is relatively well-characterized (reviewed in (Roberts-Galbraith and Newmark, 2015)). A common approach to identifying regulatory genes for organ regeneration is to screen annotated transcription factors and genes involved in signaling pathways. In *S. mediterranea*, for example, the epidermal growth factor (EGF) receptor pathway was found to regulate regeneration and homeostasis in the pharynx and eye pigment cells (Fraguas et al., 2011), the gut (Barberan et al., 2016a), and the protonephridia (Barberan et al., 2016b; Rink et al., 2011). Although this approach is often successful, it may lead to a biased selection of candidate genes. Another, unbiased strategy is to characterize the expression profile of defined tissues and to functionally test upregulated transcripts. One elegant way to obtain tissue-specific expression is to purify organs, which has been successfully done with the intestines (Forsthoefel et al., 2012),

* Corresponding author.
E-mail addresses: birgit.lengerer@uibk.ac.at (B. Lengerer), julia.wunderer@uibk.ac.at (J. Wunderer), robert.pjeta@uibk.ac.at (R. Pjeta), giada.carta@i-med.ac.at (G. Carta), damian.kao@gmail.com (D. Kao), aziz.aboobaker@zoo.ox.ac.uk (A. Aboobaker), christian.beisel@bsse.ethz.ch (C. Beisel), e.berezikov@umcg.nl (E. Berezikov), willi.salvenmoser@uibk.ac.at (W. Salvenmoser), peter.ladurner@uibk.ac.at (P. Ladurner).

http://dx.doi.org/10.1016/j.ydbio.2017.07.021
Received 28 April 2017; Received in revised form 21 July 2017; Accepted 27 July 2017
Available online 28 July 2017

cephalic ganglia (Wang et al., 2016), and eyes (Lapan and Reddien, 2012) of *S. mediterranea*. Another practicable method is to amputate the area of interest and to characterize the expression of the regenerating tissue (Adler et al., 2014; Roberts-Galbraith et al., 2016).

In contrast to triclads, few molecular studies have been performed in other taxa of Platyhelminthes. Over recent years, the marine, obligatorily cross-fertilizing hermaphrodite *Macrostomum lignano* has been successfully developed as a model organism (Ladurner et al., 2005b). *M. lignano* belongs to the Macrostomorpha, the most basal group of Rhabditophora (Egger et al., 2015; Laumer et al., 2015). Its small size of around one millimetre and fast generation time of three weeks enables easy culturing within laboratory conditions (Ladurner et al., 2005b). Several methods, including *in situ* hybridization (ISH) (Pfister et al., 2007), RNA interference (Pfister et al., 2008), BrdU (Ladurner et al., 2000), antibody staining (Ladurner et al., 2005a), and transgenesis (Wudarski et al., 2017) have been established. *M. lignano* is able to regenerate its anterior-most region (Egger et al., 2006; Verdoodt et al., 2012) as well as any tissue posterior to the pharynx (Egger et al., 2006). A study focused on the regenerating tail plate demonstrated that the posterior blastema is an accumulation of proliferating neoblasts (Egger et al., 2009). After amputation of the tail plate adhesive organs can be observed in squeeze preparations and stained specimens after just 48 h of regeneration (Egger et al., 2009; Lengerer et al., 2016). Three days after amputation the male copulatory apparatus has begun to rebuild, and the vesicula granulorum and a small stylet are visible. Within the next two to three days the stylet grows to full size and the male copulatory apparatus regains functionality (Egger et al., 2009). Depending on the individual animal, a full set of adhesive organs are regenerated between six to ten days, marking the completion of tail plate regeneration (Egger et al., 2009; Lengerer et al., 2016).

Recently, literature on transcriptome and genome assemblies of *M. lignano* (Grudniewska et al., 2016; Wasik et al., 2015) has been published. A positional RNA sequencing (RNA-seq) analysis was performed by Arbore et al., to identify transcripts specifically expressed in the head-, testis-, ovary-, and tail region. Thereby, a collection of 366 tail-region-specific transcripts were identified (Arbore et al., 2015). Interestingly, a recent study by Ramm et al. has shown that 150 of these transcripts exhibited plasticity of mRNA expression levels depending on their social environment. As part of this study animals were kept alone or in groups of eight. A differential gene expression analysis revealed transcripts up- or downregulated in the larger group size (Ramm et al.). Based on the data of these studies we aimed to identify transcripts involved in regeneration of the tail region.

Here, we present a region-specific *in situ* hybridization screen of 111 transcripts predominately expressed in the posterior region of *Macrostomum lignano*. Using RNA-seq we characterized temporal expression in the regenerating tail plate. The expression of selected transcripts in the regenerating tissues were confirmed with *in situ* hybridization and analysed with RNA interference. Three novel genes were found to be expressed in the regenerating stylet, and their knock-down resulted in animals specifically lacking the male copulatory apparatus.

2. Material and methods

2.1. Animal culture

Macrostomum lignano (Ladurner et al., 2005b) cultures of the inbred line DV1 (Janicke et al., 2013) were kept in petri dishes with nutrient enriched artificial seawater (Guillard's f/2 medium) (Anderson, 2005) and were fed *ad libitum* with the diatom *Nitzschia curvilineata*. Animals were maintained in a climate chamber with 20 °C, 60% humidity and a 14:10 day-night cycle.

2.2. Whole mount in situ hybridization

Whole mount *in situ* hybridization was performed as previously described (Lengerer et al., 2014). Briefly, primers were designed with Primer3 (Untergasser et al., 2012) and a T7 promoter region was added at the 5`end of the reverse primers. Primer sequences are listed in Suppl. Table 1. Template DNA was produced using standard PCR reactions. To synthesize single stranded digoxigenin-labelled RNA probes, T7 polymerase (Promega or Thermo Scientific) and DIG labelling mix (Roche) were used. Anti-digoxigenin-AP Fab fragments (Roche) were diluted 1:2000 and the signal was developed using the NBT/BCIP system (Roche) at 37 °C. Specimen were mounted in Mowiol® 4–88 (Roth, Germany), prepared according to the manufacturer's protocol and images were taken using a Leica DM5000 microscope.

2.3. Sample preparation for RNA-seq

For RNA-seq, tissue samples were collected from 60 worms per sample. The “A” sample included whole regenerating animals (including the regenerating tail). In the “B” sample the regenerating tail was amputated and only the anterior part of the animals was collected for RNA isolation. For the first amputation, 120 adult worms were cut behind developing eggs at the level of cement glands, using a razor blade under a binocular microscope. Afterwards, the worms were transferred to petri dishes containing culture medium and algea. To avoid algea contamination in the RNA samples, animals were starved 16 h prior to the second amputation and fixation. In case of 12 h of regeneration, the amputated animals were not fed after the first amputation. After the given times of regeneration, 60 of the 120 worms were transferred to TRI reagent® (“A”) and 60 were amputated a second time to remove the regenerating tail. The anterior fragments of the twice amputated worms (“B”) were immediately transferred to TRI reagent® (Sigma) and the regenerating tails were discarded. The tissue samples was stored at −80 °C until total RNA extraction, done by TRI reagent/Chloroform extraction. All regeneration experiments were repeated in three replicates.

2.4. Differential gene expression

For the identification of differentially expressed transcripts, six TruSeq Stranded mRNA libraries (Illumina) for every regeneration time point (three biological replicates each of RNA-seq “A” and “B”) were generated. The libraries were sequenced with 50 bp Illumina single reads. However, the quality of the reads of one biological replicate from the timepoints 12 h until day four could not be used for the analysis. The reads of the regeneration time course were mapped to the reference transcriptome (version MLRNA131024, http://www.macgenome.org/download/MLRNA131024/) with Bowtie2 (Langmead and Salzberg, 2012). The data have been deposited with links to BioProject accession number PRJNA381865 in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/). Differentially expressed transcripts were identified using DESeq. 2 (Love et al., 2014). We defined transcripts as differentially expressed between RNA-seq “A” and “B”, if they show a 2-fold difference in the number of mapped reads and a cut off p-value of < 0.01. The list of the transcripts and the corresponding fold change throughout regeneration time course are provided in (Suppl. Table 2).

2.5. RNA interference

RNAi was performed as previously described (Kuales et al., 2011). Briefly, a double-stranded RNA (dsRNA) probe was generated by an *in vitro* transcription system using primer pairs with Sp6 and T7 promoter regions (T7 and SP6 Ribomax™ large scale RNA kit, Promega). DsRNA was diluted in artificial sea water (ASW) to a final