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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Planarians activate their regenerative power to circumvent carcinogene sis: an in vivo and in vitro approach to investigate the role of stem cell potency

Promotor :
Prof. dr. Karen SMEETS

Charlotte Cosemans
Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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LIST OF ABBREVIATIONS

ASCs	Adult stem cells
API 20E	Analytical Profile Index 20E
BER	Base excision repair
b-act	Beta-actin
C. elegans	*Caenorhabditis elegans*
cys	Cystatin
DPA	Days post amputation
DDR	DNA-damage response
DSB	DNA double strand breaks
dsRNA	Double stranded Ribonucleic Acid
EM	Electron microscope
fgfr1	Fibroblast Growth Factor Receptor 1
fgfr4	Fibroblast Growth Factor Receptor 4
FISH	Fluorescent *in situ* hybridization
gapdh	Glyceraldehyde 3-phosphate dehydrogenase
gm2ap	GM2 Ganglioside Activator Pseudogene
HR	Homologous recombination
IPM	Isotonic planarian medium
ku80	80-kilodalton subunit of Ku complex
MMS	Methyl methane sulfonate
MCE	Mixed cellulose esters
NHEJ	Non-homologous end-joining
nlk1	Nemo-Like Kinase
p53	Tumor suppressor p53
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline – triton
pbx	Pre-B-Cell Leukemia Homeobox
pcna	Proliferating cell nuclear antigen
ppia	Peptidylprolyl Isomerase A (Cyclophilin A)
qPCR	Quantitative Polymerase Chain Reaction
rad51	rad51 recombinase
RNAi	RNA interference
rpl13	Ribosomal protein L13
S. mediterranea	*Schmidtea mediterranea*
SCs	Stem cells
SEM	Standard error of the mean
Smedwi-1	General stem cell marker
Smed-NB.21.11e	*Schmidtea mediterranea* novel neoblast gene
soxb1	Soxb1
soxp1	Soxp1
soxp2	Soxp2
soxp3	Soxp3
zfp1	Zinc finger protein

FOREWORD

This thesis is a part of my three years of studying Biomedical Sciences at Hasselt University. I would like to show my gratitude to my promotor prof. dr. Karen Smeets, who made it possible for me to start this project in the zoology group at the Centre for Environmental Sciences. I would like to thank her for all the tips and knowledge that she shared with me, as well as for the proofreading of my thesis. I had the benefit of having a daily supervisor, Annelies Wouters, and I would like to show my appreciation for her help during the experimental work and for reading my thesis, providing tips to improve it.

In addition, I would like to thank everyone of the zoology group for always helping me when I had questions or when I was in need of a helping hand. From day one, I always felt welcome and I had a great time during my internship. As for my fellow student Katrien, thank you for always being there if I needed someone to talk to and to motivate me during the past eight months. Due to our busy schedule, it was hard to have lunch together, but we always kept the fun by passing each other in the lab.

Last, but definitely not least, I would like to thank my parents who gave me the chance to study for the past several years and who supported me along this bumpy road. Also a word of gratitude to my friends and family, and especially my boyfriend Gillès, for keeping up with me during stressful moments and to always support me.

SUMMARY

Introduction: The highly regenerative planarian *Schmidtea mediterranea* does not develop tumors after carcinogenic exposure. Its regeneration is due to adult pluripotent stem cells (SCs) and it is likely that these SCs have a role in the evasion of tumor formation as well. We hypothesize that during carcinogenic exposure, pluripotent SCs activate specific damage responses to provoke the circumvention of carcinogenesis. Focusing on differentiation processes and DNA repair, it is hypothesized that SCs with high potency have better DNA repair mechanisms, explaining the effective response to carcinogens.

Material & methods: Regenerating planarians were exposed to 50 µM methyl methanesulfonate (MMS) for 3 (short-term), 7 (intermediate-term), and 17 days (long-term). The effect of MMS was analyzed phenotypically and SC reactions were monitored by *in situ* hybridization and immunohistochemistry. DNA repair was investigated on intact animals using gene silencing of the DNA repair gene *pcna* with RNA interference technology and MMS exposure for 10 days. Gene expression profiles were examined by qPCR.

Results: Phenotypical evaluation after 17 days of MMS exposure revealed a spotted pigmentation and a white blastema in treated animals, while untreated animals were completely regenerated with a uniform color. No tumors were observed after MMS exposure. *In situ* hybridization for *smedwi-1*, a general SC marker, revealed a decrease in the amount of SCs after short- and intermediate-term exposure, while the opposite was observed after long-term exposure. Since the general amount of SCs increased after long-term exposure, without the formation of tumors, the DNA damage response (DDR) outcome differentiation was investigated by comparing *smedwi-1 in situ* hybridizations and SMEDWI-1 immunohistochemistry stains. No significant differences were demonstrated after short-, intermediate-, and long-term exposure. Next, the DDR outcome DNA repair in SC subtypes was investigated by silencing the repair gene *pcna*. Sigma associated genes were strongly downregulated ($p<0.001$) after MMS exposure, while there was no significant effect of *pcna* knockdown. On the other hand, zeta associated genes were strongly downregulated ($p<0.001$) after both MMS exposure and *pcna* knockdown. Since SCs possibly play an important role in the circumvention of cancer, cell culture conditions were optimized.

Discussion & conclusions: The white blastema, seen in MMS treated animals, suggest an incomplete or delayed regeneration, or an altered differentiation process. The spotted pigmentation in exposed animals was consistent in all experiments. Literature demonstrated a similar depigmentation after light exposure, but less is known about the effect after carcinogenic exposure. The demonstrated effect of MMS exposure on the general amount of SCs corresponds with preliminary data suggesting an inhibition of SC proliferation after short-term exposure, while long-term exposure induced hyperproliferation, also without tumor formation. The inhibition of proliferation or hyperproliferation causes respectively a decrease or an increase in the amount of SCs. The equal amounts of SMEDWI-1 proteins between treated and untreated animals can be explained by the fact that when neoblasts differentiate, they no longer express *smedwi-1*, but the protein will remain present in the early progeny. The effect of MMS exposure on a second DDR outcome, DNA repair, was investigated next. The results suggest that the zeta-class is more affected by *pcna* knockdown and that *pcna* associated DNA repair is more important in zeta-neoblasts than in sigma-neoblasts. Due to irreversible DNA damage, zeta-neoblast can react by apoptosis, explaining the downregulation of zeta associated genes. It is also a possibility that less sigma-neoblasts convert to zeta-neoblasts, since a subset of the sigma-class gives rise to the zeta-class. Although these data indicated the importance of SCs in the circumvention of carcinogenesis, it still needs to be unraveled further how SCs cope with this DNA damage and thereby avoid tumor formations.

SAMENVATTING

Introductie: De sterk regeneratieve platworm *Schmidtea mediterranea* ontwikkelt geen tumoren tijdens blootstelling aan kankerverwekkende stoffen. Hun regeneratie is te wijten aan volwassen pluripotente stamcellen (SCn) en het is waarschijnlijk dat deze SCn een rol spelen in de omzeiling van kanker. Onze hypothese is dat deze SCn specifieke reacties activeren tijdens blootstelling aan kankerverwekkende stoffen, die de omzeiling van kanker veroorzaken. Met de focus op het differentiatieproces en DNA-herstel is onze onderliggende hypothese dat SCn met een hoge potentie betere DNA-herstel mechanismen hebben waardoor de effectieve respons tegen kankerverwekkende stoffen verklaard wordt.

Materiaal & methoden: Regenererende platwormen werden blootgesteld aan 50 µM methylmethaansulfonaat (MMS) gedurende korte (3 dagen), middellange (7 dagen) en lange termijn (17 dagen). Het effect van MMS werd fenotypisch geëvalueerd en SC-reacties werden gemonitord met *in situ* hybridisatie en immunohistochemie. DNA-herstel werd onderzocht op intacte dieren door het uitschakelen van het DNA-herstel gen *pcna* met behulp van RNA-interferentie en blootstelling aan MMS gedurende 10 dagen. Genexpressies werden onderzocht met qPCR.

Resultaten: De fenotypische evaluatie na 17 dagen MMS-blootstelling vertoonde een gespikkelde pigmentatie en een wit blastema in behandelde dieren, terwijl onbehandelde dieren volledig geregenereerd waren met een egale kleur. Er werden geen tumoren geobserveerd na MMS-blootstelling. *In situ* hybridisatie voor *smedwi-1* (algemene stamcelmerker) vertoonde een daling in het aantal SCn na korte en middellange termijn blootstelling, terwijl het tegenovergestelde geobserveerd werd na lange termijn blootstelling. Doordat het totaal aantal SCn verhoogde na lange termijn blootstelling, zonder de vorming van tumoren, werden mogelijke pistes van de DNA-schade responsen onderzocht. Eerst werd het differentiatieproces onderzocht door het vergelijken van *smedwi-1 in situ* hybridisaties en SMEDWI-1 immunohistochemische kleuringen. Er werd geen significant verschil aangetoond na korte, middellange en lange termijn blootstelling. Als tweede werd DNA-herstel onderzocht in SC-subtypen door het uitschakelen van *pcna*. Zowel sigma als zeta geassocieerde genen waren sterk neerwaarts gereguleerd na MMS-blootstelling, maar enkel de zeta-klasse ook na *pcna* uitschakeling. Omdat SCn waarschijnlijk een rol spelen in het omzeilen van kanker, werden de condities van de celcultuur geoptimaliseerd.

Discussie & conclusie: Het witte blastema, gezien in blootgestelde dieren, suggereert een onvoltooide of vertraagde regeneratie of een veranderd differentiatieproces. De gespikkelde pigmentatie was consistent in alle experimenten. In literatuur werd een gelijkaardige pigmentatie beschreven na blootstelling aan licht, maar er is weinig geweten over het effect van kankerverwekkende stoffen. Het effect van MMS op het algemeen aantal SCn komt overeen met preliminaire data waar een inhibitie van SC-proliferatie na korte termijn en een hyperproliferatie na lange termijn blootstelling werd aangetoond, zonder tumorvorming. De inhibitie en hyperproliferatie komen respectievelijk overeen met de daling en stijging in aantal SCn. De gelijke hoeveelheden SMEDWI-1 proteïnen tussen blootgestelde en controledieren kan verklaard worden door het feit dat wanneer neoblasten differentiëren, *smedwi-1* niet meer tot expressie komt maar het proteïne blijft wel aanwezig in vroege nakomelingen. Het effect van MMS werd vervolgens bekeken op een tweede DNA-schade response, namelijk DNA-herstel. De resultaten suggereren dat de zeta-klasse meer beïnvloed wordt door het uitschakelen van *pcna* en dat *pcna* geassocieerd DNA-herstel belangrijker is in deze klasse dan in de sigma-klasse. Door onherstelbare schade kunnen zeta-neoblasten in apoptose gaan, wat de neerwaartse regulatie van zeta geassocieerde genen verklaard. Het is ook mogelijk dat minder sigma-neoblasten converteren naar de zeta-klasse omdat deze voortkomen uit de sigma-klasse. Hoewel deze data het belang van SCn aantonen in de omzeiling van kanker, moet het verder onderzocht worden hoe deze SCn omgaan met DNA-schade en zo tumorontwikkeling vermijden.

1. INTRODUCTION

1.1. STEM CELLS

Stem cells (SCs) are found in almost all multicellular organisms that can divide and differentiate into diverse specialized cell types and can self-renew to produce more SCs. When a SC divides, each new cell has the potential to remain a SC or to become a specialized cell, such as a muscle cell or a brain cell. This is called an asymmetric cell division, which produces two daughter cells with different cellular fates: one copy of the original SC and one programmed to differentiate into a non-SC fate (1). The capacity to differentiate into specialized cell types and being able to give rise to any mature cell type is referred to as potency. Totipotent SCs can differentiate into embryonic and extraembryonic cell types and can construct a complete, viable organism. Pluripotent SCs are the descendants of totipotent cells and can differentiate into all cell types of the adult organism. Multipotent SCs can differentiate into a limited number of cells, e.g. neural SCs give rise to brain cells (2). Thus as development proceeds, SC potency gradually diminishes and lineage-restricted SCs are produced. These tissue-specific SCs replenish dying cells and maintain the physiological function of our organs in a process called homeostasis (3).

Adult stem cells (ASCs) produce descendants that differentiate to replace cells lost to physiological turnover, age, disease, and injury. They are normally lineage-restricted and generate only the cell types found in the tissues in which they reside. In case of tissue regeneration, ASC-fate specification is likely to have an additional level of complexity, as some ASCs may be specified toward a distinct lineage only after an injury instead of contributing to physiological turnover (4). Normal development and tissue homeostasis depend on a balance between cell loss and renewal. To maintain this balance, SCs are necessary for self-renewal as well as differentiation in a controlled system of gene activation and silencing. They perform these processes during both tissue homeostasis and repair (5). Stem cells are not only important during homeostasis, but are also essential in a process called regeneration, which is the ability of an organism to repair and regrow lost or damaged tissues without the formation of scar tissue (6).

1.2. STEM CELLS IN REGENERATION AND CANCER

Stem cells are essential in both regeneration and cancer. Although tumors are populated by a heterogeneous group of cells, only a small subset of cells has the ability to initiate and maintain cancerous growth. This subset of cells is called cancer SCs and shares properties of self-renewal and chemo- and radioresistance with normal SCs. Cancer SCs do not have the capacity to terminate proliferation, but regenerative tissue is able to control and end proliferation (5, 7, 8). There is accumulating evidence that loss of control over normal tissue repair or renewal mechanisms may lead to malignant transformation (5).

Tissue regeneration and tumorigenesis are complex, adaptive processes controlled by signals from the host and from the tissue microenvironment. Both tissue regeneration and carcinogenesis involve cell proliferation, survival, and migration that are controlled by growth factors and cytokines as well as inflammatory and angiogenic signals. These signals derive from multiple cellular and extracellular sources in the microenvironment of wounds and cancer. Therefore, wounds and cancers share a number of phenotypic similarities in cellular behavior, signaling molecules, and gene expression (9). Regeneration might in fact both contribute to the source of abnormal growth and also provide a means to prevent and correct growth abnormalities, since highly-regenerative organism are resistant to tumors (10). Regeneration and carcinogenesis can be linked through different mechanisms (**Figure 1**). A connection is observed when cell proliferation during regeneration proceeds in an uncontrolled way. The process of

regeneration can be repeated without causing malignant transformation, while in cancer the regenerative process is incomplete such that chronic injury and inflammation leads to continuous proliferation (11).

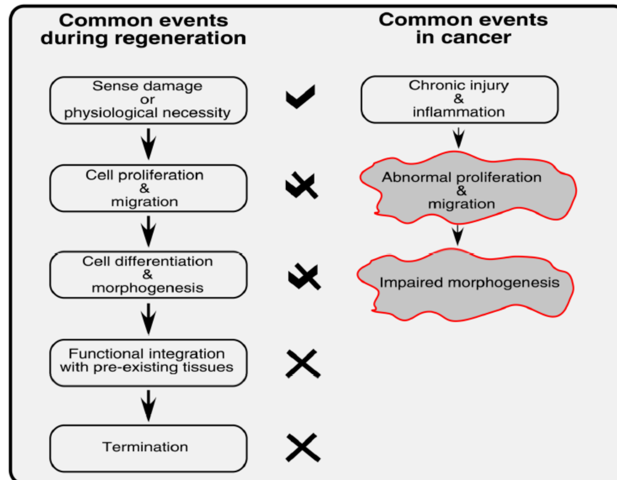

Figure 1: Links and differences between regeneration and cancer. Adopted from Oviedo, 2009 (11).

A major limitation in the study of SCs lies in the difficulty of accessing and studying these cells *in vivo*. Furthermore, *in vitro* culture systems are unable to mimic the microenvironments in which SCs reside and which are known to provide regulatory signals for their proliferation and differentiation (12). Given the complexity of vertebrate embryonic and adult SC populations and their relative inaccessibility to *in vivo* molecular analyses, the study of SCs could benefit from analyzing their counterparts in simpler model organisms. In the past, SCs in *Drosophila* and *Caenorhabditis elegans* (*C. elegans*) have already been studied. Stem cells in these organisms are mostly restricted to the gonads and neither *Drosophila*, nor *C. elegans* are capable of regenerating body parts lost to injury. Therefore, the planarian *Schmidtea mediterranea* (*S. mediterranea*) is used as a model to study SCs *in vivo*. *S. mediterranea* has regenerative properties driven by a SC population capable of producing all cell types found in this organism (12).

1.3. PLANARIANS

Planarians are non-parasitic flatworms that display remarkable regenerative capacities for all of their tissues. Planarians are small, easy and relatively inexpensive to rear in great numbers in the lab, allowing for genome-wide functional studies of regeneration and SC biology (13). The freshwater planarian *S. mediterranea* has emerged as an experimental invertebrate model system that provides a unique window into major aspects of *in vivo* SC biology, including regeneration, fate determination and homeostatic plasticity (13-15). *S. mediterranea* can regenerate any body part after amputation, as a result of pluripotent SCs called neoblasts, that are dispersed throughout the body and which generate a constant supply of progeny to sustain the high rate of physiological somatic cell turnover (4). Despite their simple outward appearance, planarian anatomy is rather elaborate, consisting of outgrowths of all three germ layers (**Figure 2**). This complex anatomy allows the identification of tissue-specific markers and thus defines and visualizes all organ systems (3, 13). There are several other reasons why *S. mediterranea* is used as a model for regeneration. First, this species is a stable diploid possessing four pairs of chromosomes. Second, it has a relatively small genome, which makes it easy to sequence. Finally, *S.*

mediterranea exists in two biotypes, one sexual and the other asexual, allowing the comparison of their reproduction, embryogenesis and regeneration (13).

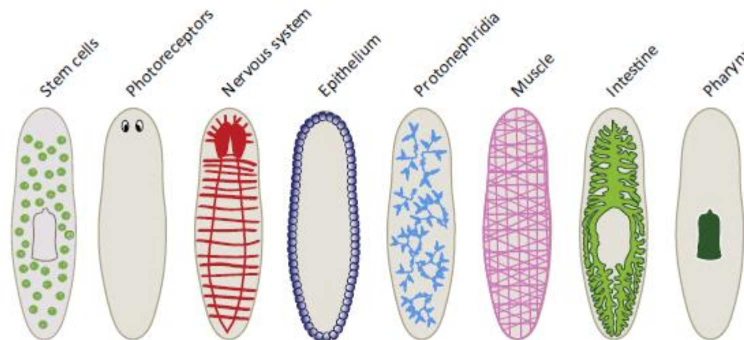

Figure 2: Various organs in asexual flatworms. Adopted from Adler, 2015 (3).

After injury, the initial reaction in planarians is wound healing, which is necessary to prevent disruption of body fluid balance and to reduce the invasion of pathogens. Next, neoblasts proliferate to form the blastema, which is an accumulation of cells with undifferentiated morphology. Blastema cells eventually differentiate and regenerate the missing body parts (16). As with any SC, neoblast proliferation needs to be tightly regulated. Blocking the capacity of neoblasts to proliferate or depleting their population leads to the loss of regenerative capacity. In contrast, an excess of their proliferation can lead to the formation of overgrowths or tumors. Elucidating how this regulation is achieved is essential in order to not only understand the cellular basis of planarian regeneration, but also the role of SCs in processes such as tumorigenesis (15).

1.4. NEOBLASTS

Neoblasts are pluripotent, somatic SCs that are widely distributed across the mesenchyme of the animal, yet are absent in the pharynx and the region anterior to the photoreceptors (4). In asexual animals, neoblasts are the only cells capable of undergoing cell division. Neoblasts are small cells (approx. 5 µm in diameter) and share the characteristic of other SCs in having a large nucleus containing highly decondensed chromatin (13).

Eisenhoffer *et al.* (4) identified and characterized a cohort of genes specifically expressed in neoblasts and their descendants. They categorized different irradiation-sensitive genes expressed in planarians using whole mount *in situ* hybridization. They demonstrated four distinct categories. Category 1 genes were absent anterior to the photoreceptors and from the pharynx, but detected throughout the animal. This pattern is indistinguishable from that of *smedwi-1* and suggests that genes in Category 1 define planarian ASCs. Category 2 genes contained *Smed-NB.21.11e* and *Smed-NB.32.1g*, which are expressed in cells that are present slightly anterior to the photoreceptors. Category 3 genes are expressed in cells closer to the animal's outer side than cells labeled by Category 2 genes. Category 4 genes are absent from the pharynx but expressed throughout the whole animal. Because of their broad expression pattern, these genes are unlikely to be specific markers for neoblasts. The cells labeled by Categories 2 and 3 genes have an

increased peripheral expression, and because the progeny of neoblasts are known to migrate through these areas, they may represent cells in distinct stages of differentiation (**Figure 3**) (4, 15).

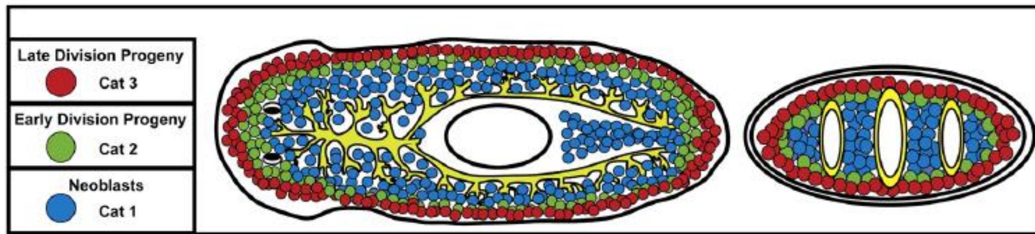

Figure 3: Distribution of expression patterns observed for Categories 1, 2, and 3 genes in cells along the anteroposterior (left) and dorsoventral (right) axes. Adopted from Eisenhoffer, 2008 (4).

Currently, more evidence is found that neoblasts are a heterogeneous population. Reddien (17) focused on two possible models for the way in which neoblasts produce replacement parts: a naïve and a specialized neoblast model (**Figure 4**). In the naïve neoblast model, neoblasts produce non-dividing, multipotent blastema cells. These cells differentiate according to signals received. In the specialized neoblast model, neoblasts produce different lineage-committed and non-dividing blastema cells. He concluded that data mostly supported the specialized neoblast hypothesis, as well as Scimone *et al.*, who also provided data supporting this hypothesis (17, 18).

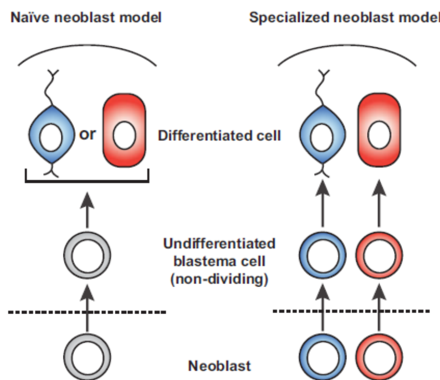

Figure 4: Two models for cell fate specification in planarian regeneration. In the naïve neoblast model, neoblasts at wound sites produce undifferentiated blastema cells that are non-dividing and multipotent. These blastema cells differentiate according to signals received. In the specialized neoblast model, the fate of individual neoblasts at wound sites is specified. Undifferentiated blastema cells that are neoblast progeny and non-dividing are therefore specified to adopt particular differentiated cell identities. Adopted from Reddien, 2013 (17).

Recently, Van Wolfswinkel *et al.* (19) confirmed that SCs are a heterogeneous cell population by single-cell analyses. They identified two prominent neoblast classes, named zeta and sigma. Zeta-neoblasts encompass specified cells that give rise to an abundant postmitotic lineage, including epidermal cells, and are not required for regeneration. In contrast, sigma-neoblasts proliferate in response to injury, possess broad lineage capacity, and can give rise to zeta-neoblasts. Their findings indicated that planarian neoblasts comprise at least two major and functionally distinct cellular compartments (**Figure 5**) (15, 19).

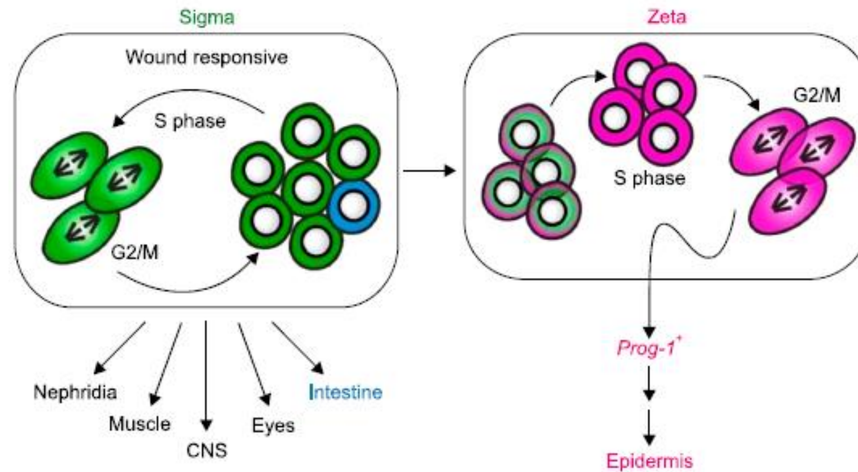

Figure 5: Model of the neoblast population. Two prominent classes of neoblasts are identified, namely sigma- and zeta-neoblasts. Sigma-neoblasts are able to self-renew and give rise to a wide range of tissue types. A subset of these neoblasts give rise to the zeta-neoblasts. These cells give rise to the *prog-1*-related lineages and to epidermal cells. Adopted from Karami, 2015 (15).

1.5. STEM CELL REACTIONS AFTER CARCINOGENIC STRESS

Since tumors are a result of uncontrolled proliferation, it is expected that malignant transformations derive from mitotically active cells in planarians, namely their neoblasts. Despite their proliferative responses, cell division in neoblasts seems to be held in check most of the time and only rarely forms the basis of tumor-like structures, which suggest a presence of efficient protection mechanisms (8, 11). Fujimori *et al.* (20) investigated the effect of carcinogenic stress on mouse embryonic SCs. They revealed that differentiating embryonic SCs were subject to carcinogenic stress, resulting in genome instability and the appearance of cancerous SCs. Since neoblasts rarely gave rise to cancerous growths, investigating their protection mechanisms after carcinogenic exposure can provide promising information.

A previous study demonstrated DNA damage in the SC population after carcinogenic exposure (An-Sofie Stevens, submitted data). DNA damage is rapidly sensed and activates signaling pathways known as DNA-damage response (DDR). These pathways include damage sensors, which transmit signals via transducers, leading to different cellular outcomes: repair, apoptosis, senescence/cell cycle arrest or differentiation. The overall goal of the DDR pathway is optimal repair of DNA damage to enable cell survival and function while disabling genomic instability. Both single- and double-strand breaks can arise after genotoxic exposure which require different repair pathways. DNA damage response, particularly the repair of DNA double strand breaks (DSB), serves as an early anticancer barrier (21-24).

1.6. OBJECTIVES

The primary aim of this project was to unravel the mechanisms underlying the evasion of carcinogenesis in the planarian *S. mediterranea*. We hypothesized that **during carcinogenic exposure** in *S. mediterranea*, **pluripotent SCs activate specific damage responses** to provoke the circumvention of carcinogenesis. Focusing on **differentiation processes and DNA repair**, it could be that SCs with **high potency** have **better DNA repair mechanisms**, explaining the **effective response** to carcinogens.

To study *in vivo* SC reactions after carcinogenic exposure, regenerating animals were exposed to the genotoxic compound MMS. Short- (3 days), intermediate- (7 days) and long-term (17 days) exposure were assessed. In this study, the differentiation process and DNA repair were investigated, because after DNA damage SCs can react, among other things, by altering their differentiation process or by DNA repair. First, animals were evaluated phenotypically to determine whether tumors were formed after carcinogenic exposure. Next, the general amount of SCs was investigated using *in situ* hybridization, since preliminary data indicated an increase of SC proliferation after long-term exposure. Finally, it was defined if the differentiation process was altered after carcinogenic exposure using immunohistochemistry.

For the second part of the hypothesis, we investigated if SCs with high potency have better DNA repair mechanisms. RNA interference (RNAi) is used to silence a DNA repair gene (*pcna*). The animals were exposed to MMS after which phenotypical analysis and gene expression profiles for the two major subtypes of SCs were determined using qPCR.

To support the advantages of *in vivo* research and to acquire more detailed information, an *in vitro* culture is necessary. This allows the individual investigation of SCs as well as a clearer view of the linkage between SC subtypes and several processes (e.g. the differentiation process or DNA repair mechanisms). In the past several attempts to keep planarian SCs in culture have been made (25, 26). As a primary neoblast cell culture is not yet available, the third goal of this study is to optimize culture conditions. The culture medium used is based on the conditions as stated by Schürmann *et al.* (25) and neoblast isolation is performed with a papain-based dissociation (27).

2. MATERIALS AND METHODS

2.1. PLANARIAN CULTIVATION

Asexual strains of the freshwater planarian *S. mediterranea* were cultivated in Milli-Q water containing 1.6 mM NaCl, 1.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.1 mM $MgCl_2$, 0.1 mM KCl, and 1.2 mM $NaHCO_3$. The animals were kept in the dark at a temperature of 20°C and were fed once a week with calf liver. Prior to *in vivo* experiments, the worms were starved for at least 7 days.

2.2. EXPERIMENTAL SET-UP

Experiments were performed on regenerating head, trunk, and tail fragments. Half an hour post amputation, animals were exposed to 0 µM and 50 µM MMS (Sigma-Aldrich, Diegem, Belgium) in 6-well plates containing 4 ml/well. Based on preliminary data, animals were exposed for 3, 7, and 17 days. RNAi experiments were performed on intact animals, which were exposed for 10 days to 0 µM and 50 µM MMS (Sigma-Aldrich, Diegem, Belgium) in 6-well plates containing 4 ml/well.

2.3. WHOLE MOUNT *IN SITU* HYBRIDIZATION

Probe synthesis

To generate cDNA template material, an RNA extraction of *S. mediterranea* was performed using the NucleoSpin RNA XS kit following manufacturer's instructions (Macherey-Nagel). Next, cDNA was obtained using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Thermo Fisher Scientific). Using this cDNA as template, PCR fragments were produced under standard PCR conditions using *Taq* polymerase (35 cycles and 55°C annealing temperature). Reverse primers were provided with a T7 recognition site to allow binding with the T7 RNA polymerase during the probe synthesis. Primers were designed with Primer3 and primer sequences are shown in **Table 1**, whereby the T7-promotor sequence is underlined.

Table 1: Primer sequence sets used for *in situ* hybridization.

	GenBank #	Primer sequences (5' → 3')	Probe length (base pairs)
smedwi-1	DQ186985.1	F: GTGACGCAGAGAAACGGAAG R-T7: GGATCCTAATACGACTCACTATAGGGTTGGATTAGCCCCATCTTTG	543
smed-NB.21.11e	JX122762.1	F: GTGATTGCGTTCGCGTATATT R-T7: GGATCCTAATACGACTCACTATAGGGATTTATCCAGCGCGTCATATTC	574

F = forward, R = reverse

PCR fragments were gel-purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific). Next, these purified PCR products were used as template to produce RNA probes using the DIG RNA labeling kit (SP6/T7) (Sigma-Aldrich, Diegem, Belgium). 10x NTP labeling mix, 10x transcription buffer, RNase inhibitor, and RNA polymerase T7 were added to the PCR product and incubated at 37°C for 2 hours. Afterwards, DNase I was added and samples were incubated at 37°C for 30 minutes. Next, EDTA (0.2 M, pH 8), 4 M LiCl, glycogen and 100% ice cold ethanol were added and samples were kept at -20°C for 30 minutes. After centrifugation (maximum speed, 4°C, 30 minutes), supernatant was removed and 70% ice cold ethanol was added. Another centrifugation step (maximum speed, 4°C, 30 minutes) was performed before the pellet was resuspended in resuspension buffer. Samples were incubated at 65°C for 10 minutes before stored at -20°C. RNA concentrations were assessed with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

Whole mount *in situ* hybridization

Regenerating animals were treated with 2% hydrogen chloride (HCl)/PBS for 5 minutes on ice to remove the mucus layer. The samples were incubated in Carnoy's fixative (60% ethanol, 30% chloroform, 10% acetic acid) for 2 hours and were rinsed in 100% methanol during 1 hour. After bleaching with 6% hydrogen peroxide overnight, samples were stored in 100% methanol at -20°C.

Next, samples were rehydrated by a series of ethanol washes and treated with 20 µg/ml proteinase K (Ambion)/PBST for 8 minutes at 37°C. The proteinase K/PBST was removed with two PBS washes and the animals were exposed to 4% paraformaldehyde (PFA)/PBS. Tissues were acetylated by incubation in 0.1 M TEA after which 2.5 µl/ml and 5 µl/ml of acetic anhydride was added. The animals were washed with PBS before being incubated in prehybridization buffer for 1 hour at 56°C. The hybridization with the DIG-labeled probe was performed for at least 18 hours at 56°C in hybridization buffer. Samples were washed through a series of posthybridization buffers and Buffer I (0.01 M maleic acid, 0.15 M NaCl, 0.15 M NaOH, and pH 7.5) and next blocked in Buffer II (Buffer I with 1% blocking solution). Samples were incubated at RT for 3 hours in 1:2000 anti-DIG/Buffer II. The antibody was removed by several washes with Buffer I. Next, the color development was carried out by incubation of the samples in 25 µl/ml NBT/BCIP at RT for 2 to 3 hours. Afterwards, the animals were washed with PBS and fixed in 4% PFA/PBS. A series of ethanol washes were performed to optimize the color development.

2.4. PHENOTYPICAL ANALYSIS

To investigate the phenotypical effect of MMS exposure on planarians, regenerating animals were observed during 17 days while exposed to 0 µM and 50 µM MMS. To investigate the phenotypical effect of 0 µM and 50 µM MMS exposure after *pcna* knockdown, intact animals were observed during 10 days. Differences in phenotypes were evaluated on day 3, day 7, day 10, day 14 and day 17. Pictures were taken on day 10 or 17 using a binocular microscope (Nikon SMZ800).

2.5. WHOLE MOUNT IMMUNOHISTOCHEMISTRY

To perform whole mount immunohistochemistry, animals were fixed in the same way as with the *in situ* hybridization protocol. Samples were rehydrated by a series of methanol washes and were blocked in 1% BSA/PBST (0.3% Triton X-100) for 3 hours. The primary antibody used was rabbit anti-SMEDWI-1 (1:1500; Guo *et al.*, 2006 (28); März *et al.*, 2013 (29)) and samples were incubated at 4°C for 18-20 hours. Next, samples were washed repeatedly in PBST (0.3% Triton X-100) and again blocked in 1% BSA/PBST (0.3% Triton X-100) for 7 hours. The secondary antibody used was Alexa 568-conjugated goat anti-rabbit (Millipore, 1:500) and samples were incubated at 4°C for 14-16 hours. Afterwards, pictures were taken with a confocal microscope (Zeiss LSM510 META, Carl Zeiss, Jena, Germany).

2.6. RNA INTERFERENCE

Probe synthesis

The effect of the DNA repair gene *pcna* was investigated using RNAi. To generate cDNA template material, an RNA extraction of *S. mediterranea* was performed using the NucleoSpin RNA XS kit following manufacturer's instructions (Macherey-Nagel). Next, cDNA was obtained using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Thermo Fisher Scientific). RNAi probes were produced using the T7 Ribomax™ Express RNAi System (Promega, Leiden, The Netherlands) following the manufacturer's

instructions. **Table 2** shows the primer sequences used to produce the RNAi probes, whereby the T7-promotor sequence is underlined.

Table 2: Primer sequences for the production of RNAi probes.

	SmedGD #	Primer sequences (5' → 3')	Probe length (base pairs)
pcna	mk4.000845.01	F: GCAACATTGGATTGCTCAGA R: TTGAACTCGGCATTTTCACA F-T7: GGATCCTAATACGACTCACTATAGGGGCAACATTGGATTGCTCAGA R-T7: GGATCCTAATACGACTCACTATAGGGTTGAACTCGGCATTTTCACA	352

F = forward, R = reverse, proliferating cell nuclear antigen (*pcna*)

RNA interference

Injections of double stranded RNA (dsRNA) of the *pcna* gene were performed for three consecutive days. Each worm received three times 22.2 nl containing 1 µg/µl dsRNA each day, which was injected in the gut in front of the pharynx using the Nanoject II (Drummond Scientific, Broomall, USA). As a control group, worms were injected with the same amount of water. One day after the last injection, animals were exposed to 0 µM and 50 µM MMS during 10 days. Medium was refreshed twice a week.

2.7. GENE EXPRESSION

After a papain based cell dissociation (see 2.8. Cell culture) of 6 worms per sample, total RNA was extracted from the cell suspension using a phenol:chloroform extraction. Cell pellets were resuspended in lysis buffer after which acidic phenol:chloroform was added. After centrifugation (13400 rpm, 4°C, 5 minutes), 3 washing steps with chloroform were performed. To precipitate nucleic acid, 3 M Na-Acetate and 100% ethanol were added. After incubation (-80°C, 30 minutes) and centrifugation (maximum speed, 4°C, 30 minutes), supernatant was removed. The pellet containing RNA was washed 4 times with ice cold 80% ethanol, after which the pellet was resuspended in RNase free water. RNA concentrations were assessed with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Genomic DNA was removed using the Turbo DNA free kit (Ambion, AM1907). Next, cDNA was obtained using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Thermo Fisher Scientific) following manufacturer's instructions.

Real-Time PCR was carried out in an optical 96-well plate using Applied Biosystems 7500 Real-Time PCR System under universal cycling conditions. SYBR Green (Applied Biosystems) chemistry-based real-time PCR was performed. The potential reference genes were based on Plusquin *et al.* (30) and Stevens A-S. (submitted data) and the most stable ones (*gapdh*, *b-act*, *ppia*, and *cys*) during MMS exposure and *pcna* knockdown were determined by GeNorm analysis. As mentioned, Van Wolfswinkel *et al.* (19) identified two major classes of neoblasts, namely the zeta-class and the sigma-class. The zeta-class expressed high levels of a discrete set of genes: *zfp1*, *fgfr1*, *p53*, *soxp3*, *egr1*, and *g6pd*, while the other class expressed low levels of those genes but had elevated expression of *soxp1*, *soxp2*, *soxb1*, *pbx*, *fgfr4*, *nlk1*, *smad6/7*, and *inx13*. Primers were designed with Primer3 and are presented in **Table 3**, whereby the T7-promotor sequence is underlined.

Table 3: mRNA primers for qPCR.

Gene name	Abbreviation	Primer sequences (5' → 3')	
Beta-actin	*b-act*	F: AGAACAGCTTCAGCCTCGTCA R: TGGAATAGTGCTTCTGGGCAT	Reference genes
Cystatin	*cys*	F: AACTCCATGGCTAGAACCGAA R: CCGTCGGGTAATCCAAGTACA	
Glyceraldehyde 3-phosphate dehydrogenase	*gapdh*	F: GCAAAACATTATTCCGGCTTC R: GCACTGGAACTCTAAAGGCCA	
GM2 Ganglioside Activator Pseudogene	*gm2ap*	F: CCGTCAGATTAAAGCTCGGTT R: TTTCGGACATTCGTTACCCAT	
Peptidylprolyl Isomerase A (Cyclophilin A)	*ppia*	F: GCAAATGCAGGTCCAAATACA R: ATGCCTTCAGCAACTTCTCC	
Proliferating Cell Nuclear Antigen	*pcna*	F: TCTTCTCAAGTATCTCTGTCGTTG R: CTCGTCGTCTTCGATTTTAGG	
Ribosomal protein L13	*rpl13*	F: AGGTGTCCCAGCTCCTTATGA R: GGCCCAATTGACAGAATTTTC	
Fibroblast Growth Factor Receptor 1	*fgfr1*	F: GGCACTGTTTTCCTTGGTTC R: AACAGCATGTCTTGTCCCTGT	Zeta-class
Tumor suppressor p53	*p53*	F: CCAATTATTTACCAAACCTCATCCT R: GGATCCCCAAAAACTGGAAC	
Soxp3	*soxp3*	F: ACCTGTGCAGACAATTCGAAGA R: TGTGGTTGGAATTTTTCACTGATTTCT	
Zinc finger protein 1	*zfp1*	F: CCGTGCCTGAACAATTTGAC R: CTTTGAGTGAAGCTGGTGTTG	
Fibroblast Growth Factor Receptor 4	*fgfr4*	F: CACTCTCAACTGCTCAACCAA R: GGGGCTTGTCCTCAAATCTA	Sigma-class
Nemo-Like Kinase	*nlk1*	F: ACCGGGTAATATGCTGGTCA R: CAACCTCTTGGGTCAAAGGA	
Pre-B-Cell Leukemia Homeobox	*pbx*	F: GAGTTATGGCGGTCATTCTG R: CTGGTTCGTCTTCTCTCATGC	
Soxb1	*soxb1*	F: CGTCTCAGTCCAATATCACGAGC R: GCCATTCTTCGACGCTGACC	
Soxp1	*soxp1*	F: ACCCATTACCTCCTGAATGGCT R: TGGTTGGACGTCGCCTTCTT	
Soxp2	*soxp2*	F: TCAGTGGATGAGTGATTTGG R: CCTTGGAGTGATATGCTTCTTG	

2.8. CELL CULTURE

Cell dissociation was performed under a laminar flow with a papain-based method as described by Moritz *et al.* (27). Planarians were incubated in 2% L-cysteine hydrochloride (Sigma-Aldrich, Diegem, Belgium) (5M NaOH was added to obtain a pH of 7.0) for 2 minutes and washed with CMFH (2.5 mM $NaH_2PO_4.2H_2O$; 14.3 mM NaCl; 10.2 mM KCl; 9.4 mM $NaHCO_3$; 15 mM Hepes; 0.1% BSA; 0.5% Glucose; pH 7.2). Next, the animals were cut into small pieces on a glass slide. The pieces were transferred with wide borehole 1000G tips (Art-tips, MβP, USA) into 1.5 ml reaction tubes using 250 µl CMFH. Then 250 µl of 2x papain solution

(30 U/ml Papain (Sigma-Aldrich, Diegem, Belgium); 2 mM L-Cysteine in CMFH) was added and the reaction was incubated for 1 hour at 26°C. After adding 250 µl of 3x STOP solution (30 mg/ml Trypsin inhibitor from chicken egg white (Sigma-Aldrich, Diegem, Belgium); 60 µg/ml RQ1 DNase I (Promega, USA) in CMFH) the samples were gently pulverized by pipetting. Finally, the cell suspension was filtered (35 µm pore size) and the cells were collected through centrifugation (4 x 5 minutes at 400 x g) after which they were resuspended in isotonic planarian medium (IPM, as described by Schürmann *et al.* (25) with slight modifications, **Table 4**). This composition forms the base from which the cell culture is optimized. The optimization process is further discussed in the result section. Cells were cultured at a density of 800 000 cells/ml in a petri dish with a total volume of 3 ml and medium was replaced every 3 days.

Table 4: Components Isotonic Planarian Medium.

IPM medium			
mOsmol/l	126		
pH	7.40		
Inorganic ions (mM/l)			
Na^+	42.39	HCO_3^-	9.63
K^+	0.89	$H_2PO_4^-$ + HPO_4^-	0.51
Mg^{2+}	0.38	SO_4^-	0.37
Ca^{2+}	1.03	Cl^-	20.34
Buffers (mM/l)			
Hepes, anion + free acid	43.75		
Nutrients (mM/l)			
Pyruvate	1.36	D-Glucose	1.83
L-Glutamine	0.34	D-Trehalose	0.15
Amino acids	+	Antibiotics	Cefotaxime
Vitamins	+	BSA	15 g/l

IPM = Isotonic Planarian Medium

Identification bacteria

To identify the bacterial contamination, a Gram staining was performed. A colony was picked from an isolation plate and suspended in miliQ water. A droplet of this suspension was fixed on a microscopic glass. First, samples were incubated with crystal violet for 1 minute. After washing with water, samples were incubated with lugol/iodine for 1 minute. Another wash step was performed and samples were decolorized with alcohol for 30 seconds. Finally, samples were incubated with safranin for 1 minute, washed with water, and air-dried. Gram negative bacteria have a pink-red color while Gram positive bacteria have a blue-purple color.

Next, a further identification of the bacteria was performed using an Analytical Profile Index (API) 20E. The API 20E fast identification system allows the identification of a limited number of Gram negative *Enterobacteriaceae* or non-*Enterobacteriaceae*. The test systems are stored in 20 small reaction tubes, which include the substrates (**Figure 6**). One colony from an isolation plate was resuspended in 5 ml distilled water. The API 20E strip was prepared in a humid atmosphere and inoculated by distributing the bacterial suspension into the reaction tubes. The strip was incubated at 36°C for 24 hours. Afterwards, the strip was evaluated and a 7-digit profile number was obtained, which is specifically for a bacteria species.

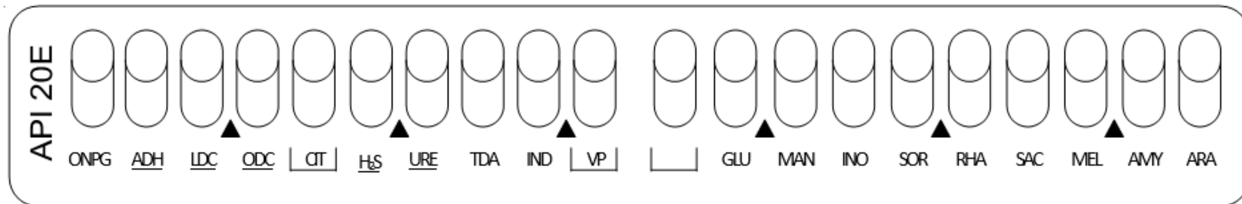

Figure 6: Schematic overview Analytical Profile Index 20E (API 20E).

2.9. ELECTRON MICROSCOPY

Cells of the cell culture were pelleted and fixed in 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer without saccharose (pH 7.3, 425 mOsm). A secondary fixation was performed with 2% osmiumtetroxide in 0.05 M Na-cacodylate buffer without saccharose (pH 7.3, 425 mOsm). Next, samples were dehydrated with increasing acetone concentrations (50%, 70%, 90%, and 100%). Samples were infiltrated and embedded in individual rubber molds filled with Spurr resin. After hardening at 70°C, slides were cut using the Leica ultracut UCT. Slices of 70 nm were captured on copper grids coated with Pioloform and contrasted using uranyl acetate (4% in 50% ethanol) for 15 minutes. Lastly, samples were incubated in lead citrate (3% in water) for 4 minutes. Slides were analyzed with the transmission electron microscope EM 208S (Philips).

2.10. STATISTICAL ANALYSIS

To quantify the fluorescent signal after immunohistochemistry, the integrated density of the pictures was measured using ImageJ and statistical analyses were performed with GraphPad Prism 5.01. Normality of the data was tested using the Shapiro-Wilk test. When the data were normally distributed, an unpaired t-test was performed. Otherwise, a Mann-Whitney test was carried out. A *p*-value less than 0.05 was considered as significant.

The statistical analyses of the RNAi experiment were performed with R. Statistical Software (31). Normality was assessed using the Shapiro-Wilk test and homoscedasticity was assessed using the Bartlett's test. When the data were normally distributed and homoscedastic, a two-way ANOVA was performed. When normality was not met, data was transformed (Log, Square root, 1/x or e^x) or a Kruskal-Wallis test was performed. A *p*-value less than 0.05 was considered as significant.

3. RESULTS

To study *in vivo* SC reactions after carcinogenic exposure, regenerating animals were exposed to MMS. Short- (3 days), intermediate- (7 days), and long-term (17 days) exposure were assessed. First, the general amount of SCs was investigated using *in situ* hybridization. Next, it was defined if the differentiation process was altered after carcinogenic exposure using immunohistochemistry. A second focus was to investigate whether DNA repair is an important factor during carcinogenic stress, more specifically whether SCs with high potency have better DNA repair mechanisms. RNA interference technology was used to silence a DNA repair gene (*pcna*). The animals were exposed to MMS after which gene expression profiles for different subtypes of SCs were determined using qPCR on a SC-enriched suspension.

3.1. GLOBAL AMOUNT OF STEM CELLS

To evaluate whether long-term exposure to MMS leads to the formation of tumors, regenerating planarians were examined phenotypically until 17 days after exposure (**Figure 7**). Untreated worms were completely regenerated and larger (data not shown) compared with the exposed group. On the contrary, the blastema of MMS treated worms were still white and the pigmentation of the worms was more spotted compared with the control group. However, no tumors were observed.

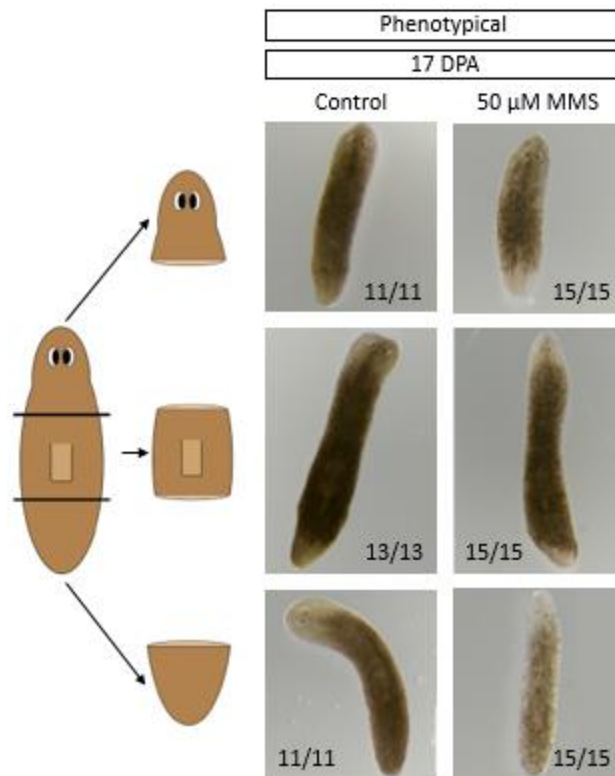

Figure 7: Phenotypical evaluation of exposure to 0 µM and 50 µM MMS after 17 days. Control worms (0 µM) were completely regenerated, while the blastema cells of the treated worms were still white. The pigmentation of the treated worms was more spotted compared to the control group. No tumors were observed in both groups. For each time point, at least 11 biological replicates were used as indicated on the figures. DPA, days post amputation; MMS, methyl methanesulfonate

Preliminary data demonstrated an increase of SC proliferation after long-term exposure. To determine whether the amount of SCs is altered after exposure to a carcinogen, regenerating animals were exposed to MMS for 3, 7, and 17 days. Stem cell reactions were monitored using *in situ* hybridization for *smedwi-1*. *Smedwi-1* is a PIWI-like protein that regulates planarian SCs and is used as a general SC marker (32). Short-term exposure (3 days post amputation (DPA), **Figure 8**), as well as intermediate-term exposure (7 DPA, **Figure 8**) revealed a decrease in the amount of SCs, while long-term exposure (17 DPA, **Figure 8**) showed an equal amount of SCs compared to the non-exposed control group. Since the general amount of SCs increased after long-term exposure, without the formation of tumors, it was investigated if the differentiation process was altered by comparing *smedwi-1 in situ* hybridizations and SMEDWI-1 immunohistochemistry stains.

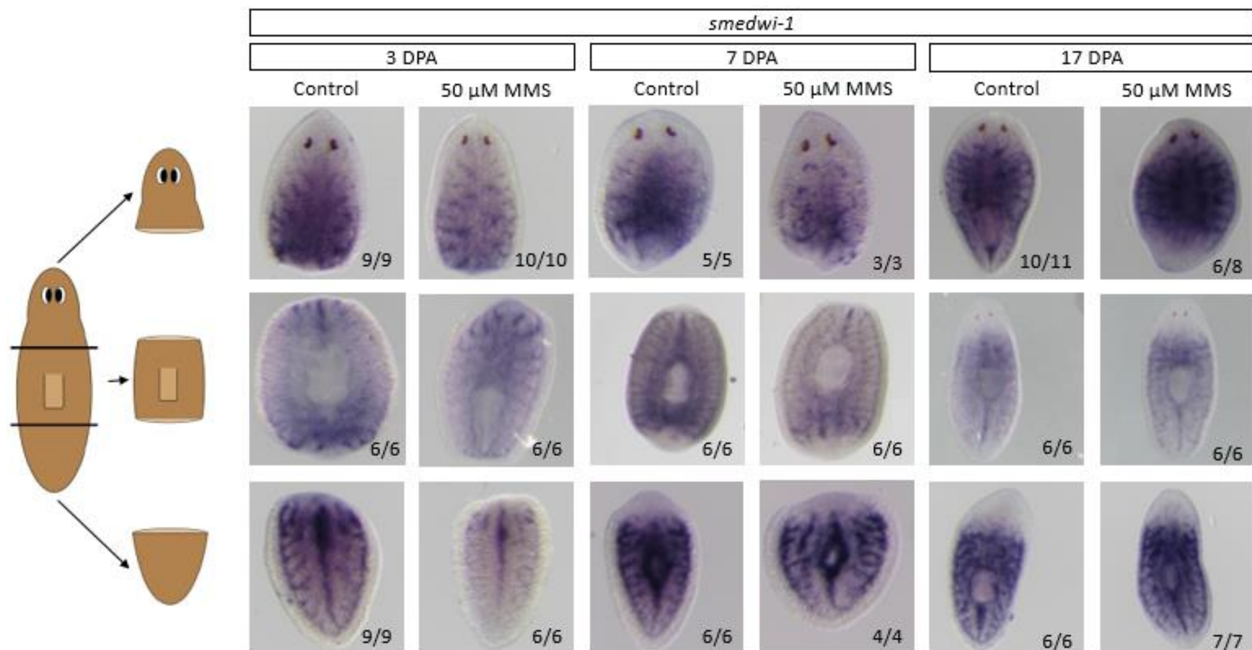

Figure 8: Whole mount *in situ* hybridization for *smedwi-1*. Regenerating animals (head – trunk – tail) were exposed to 0 µM (control) or 50 µM MMS for 3, 7, and 17 days. Short-term exposure (3 DPA), as well as intermediate-term exposure (7 DPA) revealed a decrease in the amount of SCs compared to the control group, while long-term exposure (17 DPA) showed an equal amount of SCs. For each time point, at least 3 biological replicates were used as indicated on the figures. DPA, days post amputation; MMS, methyl methanesulfonate

3.2. ALTERATIONS IN THE DIFFERENTIATION PROCESS

To determine if the differentiation process is altered, *in situ* hybridization of *smedwi-1* is compared with immunohistochemistry staining of SMEDWI-1. As neoblasts differentiate, they no longer express *smedwi-1*. However, early neoblast progeny will have the SMEDWI-1 protein which will eventually degrade in the differentiated cells. If SMEDWI-1 positive cells in exposed animals are more abundant in comparison with the control animal, without an increase in the general amount of SCs, it suggests that those neoblasts fail to fully differentiate (33).

Regenerating animals were exposed to MMS for 3, 7, and 17 days. Short-, intermediate-, and long-term exposure showed no differences in the SMEDWI-1 staining pattern between control and exposed animals (**Figure 9**). After quantification of the fluorescent signal, no significant changes ($p>0.05$) were observed, except for the trunk ($p<0.1$) and tail fragments ($p<0.05$) 7 DPA and the head ($p<0.05$) and tail fragments ($p<0.05$) 17 DPA (**Supplemental Figure 1**).

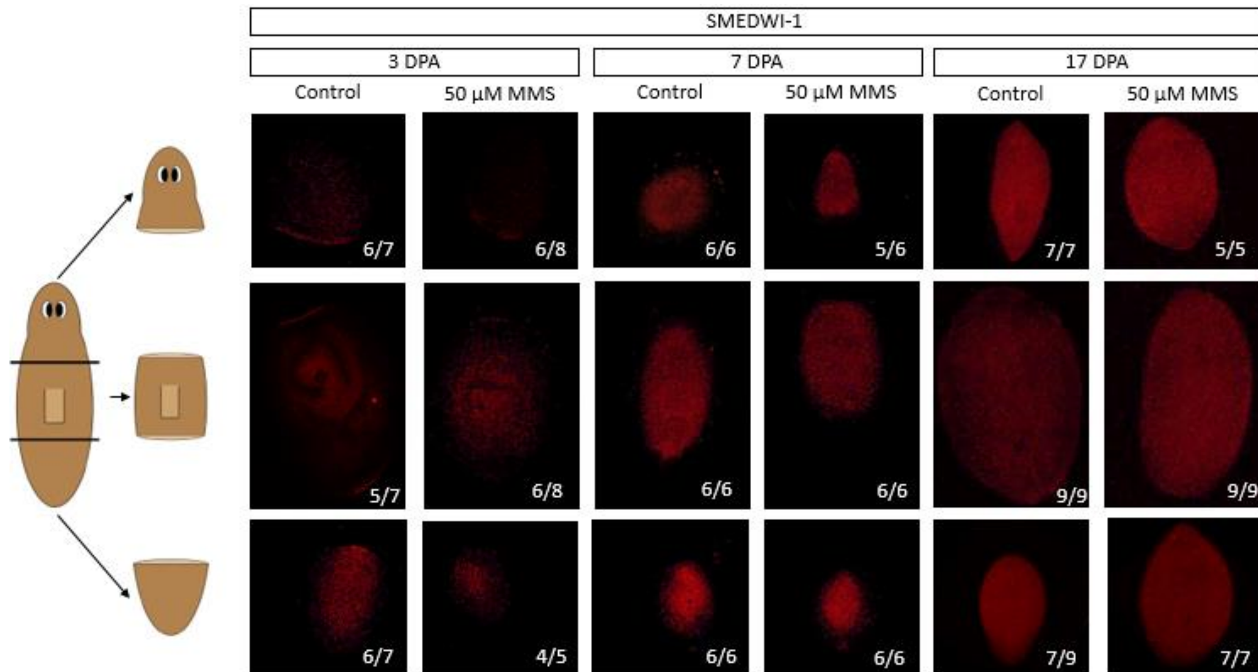

Figure 9: Whole mount immunohistochemistry for SMEDWI-1. Regenerating animals (head – trunk – tail) were exposed to 0 µM (control) or 50 µM MMS for 3, 7, and 17 days. No differences were observed in the MMS treated group compared to the control group. For each time point, at least 5 biological replicates were used as indicated on the figures. DPA, days post amputation; MMS, methyl methanesulfonate

Since no differences were demonstrated after SMEDWI-1 immunohistochemistry, but the general amount of SCs was altered, it was investigated if *Smed-NB.21.11e* (Category 2, early progeny marker (4)) positive cells were affected after MMS exposure. Since Category 2 genes have an increased peripheral expression, they may represent cells in a distinct stage of differentiation. Short-term exposure (3 DPA), as well as intermediate-term exposure (7 DPA) (**Figure 10**), revealed a decrease in the MMS treated group compared to the control group. 17 DPA showed no differences between both groups, except for the tail fragments where exposed animals showed a decrease compared with the control group.

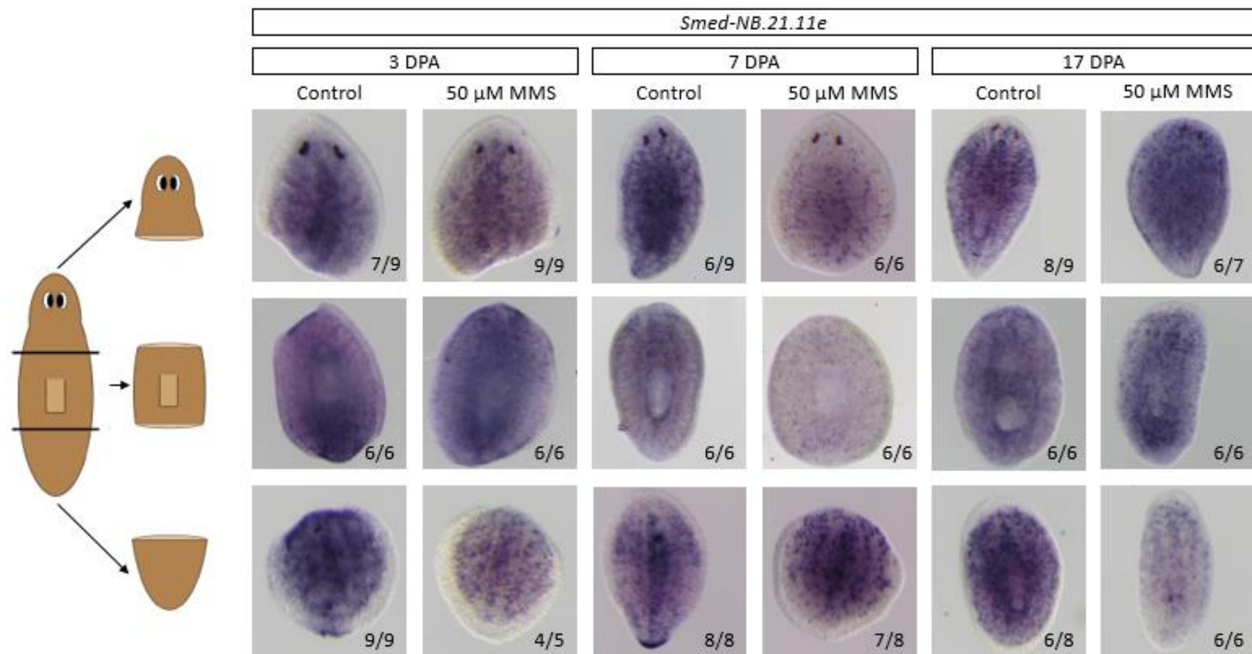

Figure 10: Whole mount *in situ* hybridization for *Smed-NB.21.11e*. Regenerating animals (head – trunk – tail) were exposed to 0 µM (control) or 50 µM MMS for 3, 7, and 17 days. Short-term exposure (3 DPA), as well as intermediate-term exposure (7 DPA), revealed a decrease of *Smed-NB.21.11e* positive cells in the MMS treated group compared to the control group. 17 DPA showed no differences between both groups. For each time point, at least 5 biological replicates were used as indicated on the figures. DPA, days post amputation; MMS, methyl methanesulfonate

3.3. DNA REPAIR IN STEM CELL SUBTYPES

To investigate whether DNA repair is an important factor during carcinogenic stress, it is investigated if SCs with high potency have better DNA repair mechanisms. Van Wolfswinkel *et al.* (19) identified two major classes of neoblasts, namely the zeta-class and the sigma-class. They demonstrated that the zeta-class expressed high levels of a discrete set of genes: *zfp1*, *fgfr1*, *p53*, *soxp3*, *egr1*, and *g6pd*, while the other class expressed low levels of those genes but had elevated expression of *soxp1*, *soxp2*, *soxb1*, *pbx*, *fgfr4*, *nlk1*, *smad6/7*, and *inx13*.

Proliferating cell nuclear antigen (*pcna*), which plays a role in both DNA replication and repair, is essential for single-strand repair and associated with base excision repair (BER). The effect of *pcna* knockdown in combination with MMS exposure was investigated both phenotypically (**Figure 11**) and on the different SC subtypes, since MMS can induce single-strand breaks. After *pcna* knockdown, intact animals were exposed to MMS for 10 days. Phenotypical evaluation revealed a slightly spotted pigmentation after MMS exposure in *pcna* knockdown and control animals, compared with the non-exposed group. Afterwards, gene expression profiles of *pcna* and the above mentioned genes were determined via real-time qPCR on a SC-enriched suspension. To determine if the *pcna* knockdown was efficient, the relative gene expression of *pcna* was measured and proved that *pcna* was strongly downregulated ($p<0.001$, **Figure 12**). **Figure 12** shows an upregulation ($p=0.0783$) of *pcna* expression in MMS treated animals injected with H_2O compared to the control group.

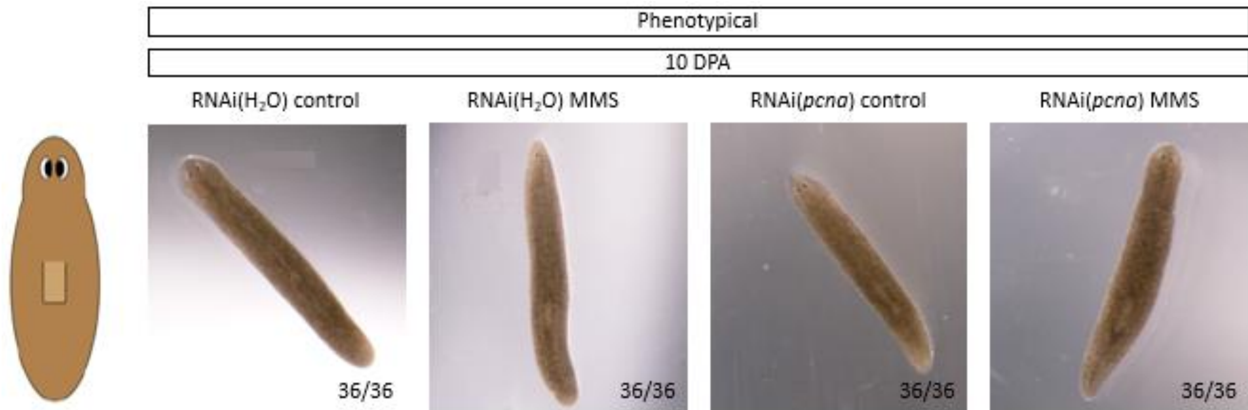

Figure 11: Phenotypical evaluation of *pcna* knockdown after exposure to 0 µM (control) and 50 µM MMS for 10 days. Animals exposed to MMS showed a slightly spotted pigmentation compared with the non-exposed group. No tumors were observed in the different groups. For each time point, 36 biological replicates were used as indicated on the figures. DPA, days post amputation; MMS, methyl methanesulfonate; *pcna*, proliferating cell nuclear antigen

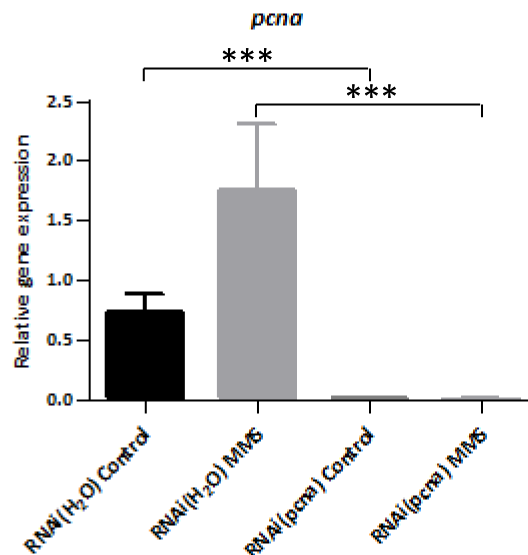

Figure 12*:* Relative gene expression of *pcna* after RNAi(*pcna*) knockdown. The expression of *pcna* was investigated in RNAi(H_2O) (control) and RNAi(*pcna*) animals after 10 days exposure to 0 µM (control) and 50 µM MMS. The expression of *pcna* is strongly downregulated after *pcna* knockdown after 10 days of MMS exposure. For each group, 5 replicates were used. Error bars represent the standard error of the mean (SEM). ***$p<0.001$

Next, the effect of *pcna* knockdown on the SC subtypes was investigated using the genes stated by Van Wolfswinkel *et al.* (19). Sigma associated genes were strongly downregulated ($p<0.001$) when exposed to MMS, while there was no significant effect ($p=0.2489$) of *pcna* knockdown (**Figure 13A**). For zeta-

associated genes on the other hand, both MMS ($p<0.001$) and *pcna* knockdown ($p<0.05$) induced a strong downregulation (**Figure 13B**). Gene expression profiles of the individual genes showed the same pattern, except for *pbx* and *fgfr4*, as demonstrated in the average gene expression profiles (**Supplemental Figure 2**).

These results demonstrated differences on a population level. Since the gene *zfp1* is the only neoblast gene specific for zeta-neoblasts (19), the expression profile of *zfp1* gives a strong indication of the effect of *pcna* knockdown and MMS exposure on the zeta-class. The expression of *zfp1* was strongly downregulated ($p<0.001$) after *pcna* knockdown, as well as after 10 days of MMS exposure (**Figure 13C**).

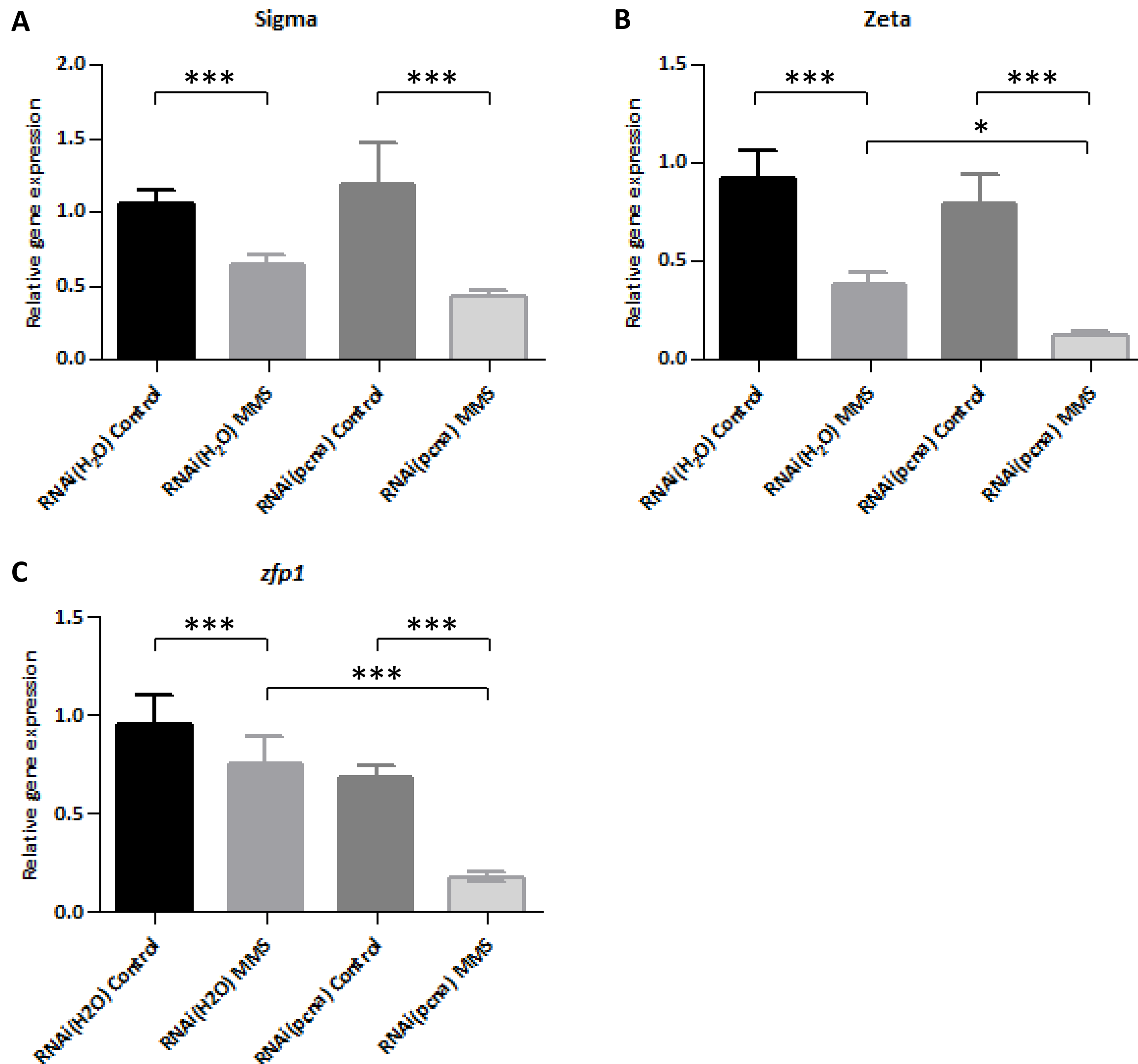

Figure 13: The relative gene expression of genes of interest. The expression of sigma and zeta associated genes was investigated in RNAi(H_2O) (control) and RNAi(*pcna*) animals after 10 days exposure to 0 µM (control) and 50 µM MMS. **A.** The average relative gene expression of genes indicating sigma-neoblasts were strongly downregulated ($p<0.001$) when exposed to MMS. **B.** The average relative gene expression of genes indicating zeta-neoblasts, where both MMS ($p<0.001$) and *pcna* knockdown ($p<0.05$) induced a strong downregulation. **C.** The relative gene expression of *zfp1* was strongly downregulated ($p<0.001$) after *pcna* knockdown, as well as after 10 days of MMS exposure. Error bars represent the standard error of the mean (SEM). ***$p<0.001$; *$p<0.05$

3.4. OPTIMIZATION CELL CULTURE

The use of an *in vitro* neoblast culture provides the possibility of studying neoblast in detail. Since there is currently no primary cell culture available, the starting point for this optimization was the review by Schürmann *et al.* (25), which proposed isotonic planarian medium to culture planarian neoblast (**Table 4**). During the optimization process several alterations were made in order to improve the cell culture, mainly focusing on bacterial and/or fungal infections (**Table 5**).

Table 5: Optimization steps for cell culture.

<table>
<tr><td rowspan="8">Isolation</td><td colspan="2">Fed vs. starved animals</td></tr>
<tr><td colspan="2">Additional centrifugation/washing steps</td></tr>
<tr><td colspan="2">Additional Swinnex flush-back filtration</td></tr>
<tr><td colspan="2">Regenerating animals vs. intact animals</td></tr>
<tr><td colspan="2">Only tail fragments of animals</td></tr>
<tr><td rowspan="3">Addition of antibiotics in advance</td><td>0.2 mg/ml Neomycin Sulfate</td></tr>
<tr><td>10 μg/ml Ciprofloxacin</td></tr>
<tr><td>37.5 mg/l Cefotaxime Sodium Salt</td></tr>
<tr><td rowspan="13">Medium composition</td><td rowspan="3">Addition of Neomycin Sulfate (Life Technologies, Carlsbad, USA)</td><td>0.2 mg/ml</td></tr>
<tr><td>0.2 mg/ml + Streptomycin (0.1 mg/ml)</td></tr>
<tr><td>0.2 mg/ml + Gentamicin (50 mg/l)</td></tr>
<tr><td rowspan="5">Addition of Ciprofloxacin (Sigma-Aldrich, Diegem, Belgium)</td><td>10 μg/ml</td></tr>
<tr><td>12 μg/ml</td></tr>
<tr><td>15 μg/ml</td></tr>
<tr><td>25 μg/ml</td></tr>
<tr><td>Boost 16 μg/ml</td></tr>
<tr><td rowspan="5">Addition of Cefotaxime Sodium Salt (Duchefa, Haarlem, The Netherlands)</td><td>37.5 mg/l</td></tr>
<tr><td>37.5 mg/l + Amphotericin B (2.5 μg/ml)</td></tr>
<tr><td>75 mg/l</td></tr>
<tr><td>75 mg/l + Amphotericin B (1.25 μg/ml)</td></tr>
<tr><td>75 mg/l + Amphotericin B (3.75 μg/ml)</td></tr>
</table>

Stem cells were successfully isolated using the papain-based method and cell cultures were evaluated every 3 days. In most cases a persistent bacterial infection was present by day 3, latest by day 6 (**Figure 14A**). To confirm the presence of neoblasts in the cell culture, EM photographs were made. The neoblast contained a large nucleus with highly decondensed chromatin, as well as rough endoplasmic reticulum (**Figure 14B**). Since the addition of Neomycin Sulfate (Life Technologies, Carlsbad, USA) (0.2 mg/ml), as described by Schürmann *et al.* (25), had no effect on the present infection, the bacteria were identified to allow a more targeted administration of antibiotics. First, a Gram staining was performed to determine if the infection consisted of Gram negative or Gram positive bacteria. **Figure 14C** shows a Gram staining of bacteria in the cell suspension after 6 days. The bacteria were colored red/pink, which indicated Gram negative bacteria. Next, a further identification of the bacteria was performed using an API 20E strip. **Figure 14D** shows two separate API 20E test strips of bacteria in the cell suspension after 3 days. Both strips differ from each other and the 7-digit profile numbers were not exclusively for a specific species. Therefore, several antibiotics against Gram negative bacteria were tested.

While Ciprofloxacin was unsuccessful, Cefotaxime Sodium Salt (Duchefa, Haarlem, The Netherlands) was able to remove the bacterial infection with a concentration of 75 mg/l. However, after the administration of this antibiotic, a fungal infection appeared. Therefore, the broad-spectrum antifungal drug Amphotericin B (Sigma-Aldrich, Diegem, Belgium) was added to the cell culture medium, yet proved ineffective as the infection persisted.

Since the infections could not be controlled, attempts were made to minimalize the possible source of the infection (i.e. the worm itself). On the one hand, there is a possibility that the infection comes from within the animal's intestine. Therefore, the effect of feeding was investigated by comparing dissociations of starved (at least 7 days) and fed animals (3 hours post feeding), and by using only tail fragments. On the other hand, additional centrifugation and wash steps were performed, as well as a flush-back filtration step using the Swinnex Filter Holder (Merck Millipore) with a 5 µM mixed cellulose esters (MCE) membrane (Merck Millipore). Nevertheless, in all conditions the infection remained present. The combination of 75 mg/l Cefotaxime Sodium Salt and the flush-back filtration demonstrated the best results. However, further optimization is necessary.

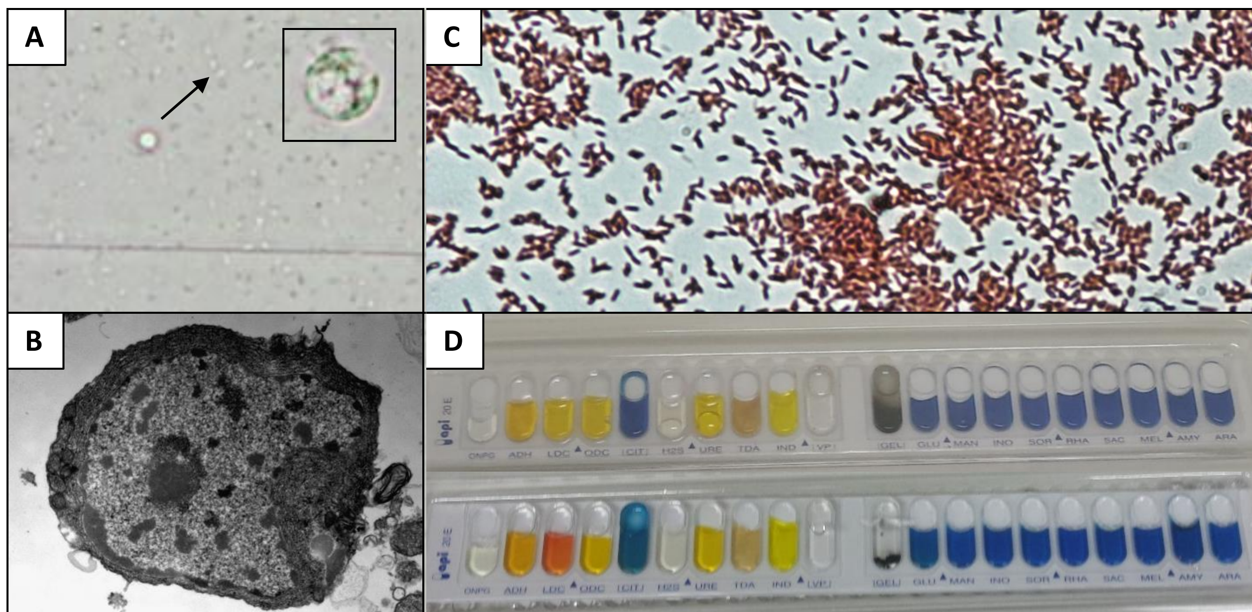

Figure 14: Identification neoblasts and bacterial infection in cell culture. A. Microscopic photograph (20x) of the cell culture at day 6 after addition of 10 µg/ml Ciprofloxacin. A stem cell is indicated in the black box; the arrow indicates bacteria. **B**. Electron microscopic photograph (7100x) of a neoblast in cell culture. The neoblast contains a large nucleus with highly decondensed chromatin, as well as rough endoplasmic reticulum. **C.** Gram staining (40x) of bacteria in the cell suspension after 6 days. **D**. API20E strip of two separate tests.

4. DISCUSSION

It has been long known that regeneration and cancer are closely related (10). Since tumors are a result of uncontrolled proliferation, it is expected that malignant transformations derive from mitotically active cells in planarians, namely their neoblasts. Despite their proliferative responses, cell division in neoblasts seems to be held in check most of the time and only rarely forms the basis of tumor-like structures, which suggest a presence of efficient protection mechanisms (8, 11). DNA damage activates DNA damage responses leading to different cellular outcomes: DNA repair, apoptosis, senescence or differentiation. Investigating which response SCs activate during carcinogenic exposure can give valuable information about their protection mechanisms. Van Wolfswinkel *et al.* (19) demonstrated the existence of two major neoblast subtypes. Unraveling the protection mechanisms in both subtypes can provide promising knowledge about how *S. mediterranea* circumvents carcinogenesis.

The goal of this study was to investigate whether pluripotent SCs activate specific damage responses after carcinogenic exposure in order to circumvent carcinogenesis. Genotoxic carcinogens, like MMS, interact directly with DNA, leading to various types of DNA damage (34). The methylating agent MMS has been used for decades as a DNA damaging agent in the field of cancer research (35-37). This agent has been utilized to uncover and explore pathways of DNA repair, DDR and mutagenesis. Methyl methanesulfonate is a highly toxic DNA-alkylating agent that methylates DNA bases and causes damage leading to strand breaks, chromosome breaks, micronucleus formation, and eventually cell death (35, 38). In this study, MMS was used to induce DNA damage since it is a strong genotoxic compound. A previous study demonstrated DNA damage in *S. mediterranea* and more specifically in SCs after MMS exposure. Despite this DNA damage, no tumors were observed (An-Sofie Stevens, submitted data). How they cope with this damage and avoid tumor formation still needs to be unraveled further.

Global amount of stem cells and their differentiation process

The phenotypical effect of MMS exposure was investigated to evaluate if long-term exposure leads to the formation of tumors. In a normal environment (i.e. without stress factors), animals are completely regenerated 7 DPA. The white blastema, seen in MMS treated animals, suggested an incomplete or delayed regeneration, or an altered differentiation process. The spotted pigmentation in exposed animals was consistent in all experiments using regenerating animals and less intense using intact worms. Stubenhaus *et al.* (39) demonstrated light-induced depigmentation in *S. mediterranea*, but less is known about the depigmentation after carcinogenic exposure. Besides the phenotypical evaluation, the effect of MMS exposure was investigated on the total amount of SCs using *in situ* hybridization for *smedwi-1*. *Smedwi-1*, expressed in neoblasts, encodes a PIWI-like protein that regulates the adult somatic SCs of planarians and therefore is a general SC marker. *Smedwi-1*-expressing cells reside in the mesenchymal tissue excluded from the nervous system, pharynx, and gastro-vascular system (32). Short-term, as well as intermediate-term exposure demonstrated a decrease in the amount of SCs in all body parts, while exposure for up to 17 days showed an increase in SC numbers. These results correspond with preliminary data suggesting an inhibition of SC proliferation after short-term exposure, while long-term exposure induced hyperproliferation, also without tumor formation (An-Sofie Stevens, submitted data). The inhibition of proliferation (short-term) and hyperproliferation (long-term) causes respectively a decrease or an increase in the amount of SCs. The increase in SC numbers after long-term exposure did not lead to the formation of tumors, which corresponds with literature, where it is stated that highly-regenerative organisms are resistant to tumors (10).

After DNA damage, cells can react, among other things, by altering their differentiation process. Cells usually differentiate without malignant transformation, although genetic alterations that arrest differentiation are frequently observed in cancer. The DDR-enforced differentiation of SCs could help preserve the genome integrity of a cell type, tissue, organisms, or species (24). The effect of MMS exposure on the differentiation process was investigated by comparing *in situ* hybridization for *smedwi-1* and immunohistochemistry for SMEDWI-1. In general, no significant changes were observed after short-, intermediate- and long-term exposure between 0 µM and 50 µM MMS. As neoblasts differentiate, they no longer express *smedwi-1*, but the protein will remain present in the early neoblast progeny and will eventually degrade in the differentiated cells. This explains the equal amounts of proteins between treated and untreated animals, despite the decrease in the general amount of SCs after short- and intermediate-term exposure. Compared with short-term exposure, long-term exposure showed an increase in SMEDWI-1 positive cells which can be explained by the increase of the general amount of SCs. If SMEDWI-1 positive cells in exposed animals are more abundant in comparison with the control animals, without an increase in the general amount of SCs, it suggests that those neoblasts fail to fully differentiate (33). Thus these data indicated no alterations in the differentiation process of SCs after carcinogenic exposure.

The differentiation process was further investigated using *in situ* hybridization for *Smed-NB.21.11e* (Category 2, early progeny marker (4)). Category 2 genes have an increased peripheral expression so they may represent cells in a distinct stage of differentiation. Both short- (3 DPA) and intermediate-term (7 DPA) exposure revealed a decrease of the Category 2 gene *Smed-NB.21.11e*, while long-term (17 DPA) exposure showed no differences between both groups. A possible explanation for this decrease is that it is proportional with the decrease of the general amount of SCs after MMS exposure.

Taken together, these data indicated different SC responses after short-, intermediate-, and long-term carcinogenic exposure. In future experiments, the phenotypical effect of MMS exposure can be investigated over a longer period than 17 days to determine if the depigmentation gets worse or actually improves and if tumors are present. When it improves, it is a possibility that the differentiation process slows down after carcinogenic exposure. It can also be investigated if Category 3 genes (late progeny) are affected by MMS exposure. However, this research focused on the initial SC reactions after carcinogenic exposure, making investigating late progeny genes less interesting. When a primary neoblast culture is available, the differentiation process can be investigated in the major SC subtypes (19) to give a more detailed view on their possible different responses.

DNA repair in stem cell subtypes

To further unravel the DDR outcomes after MMS exposure, it is investigated if SCs with high potency have better DNA repair mechanisms, explaining the effective response against carcinogens. Recently, Van Wolfswinkel *et al.* (19) demonstrated that SCs are a heterogeneous cell population. Therefore, DNA repair was investigated for different SC subtypes in a SC-enriched population. Stevens A-S. (submitted data) demonstrated the importance of *pcna* in MMS-induced damage responses. RNA interference technology was used to silence the DNA repair gene *pcna*, which encodes a protein that plays a role in both DNA replication and repair. It is essential for several forms of DNA repair, including BER, the major pathway by which cells remove DNA damage introduced by a variety of chemical carcinogens (40, 41).

The downregulated expression of *pcna* ($p<0.001$) after *pcna* knockdown confirmed the efficiency of the gene silencing. Exposed RNAi(H_2O) animals showed an upregulated ($p=0.0783$) expression of *pcna* compared with untreated RNAi(H_2O) animals. Xu et al. (42) stated that the expression of *pcna* was

upregulated after genotoxic insult, confirming the upregulation after MMS exposure. Next, the gene expression profiles of the sigma and zeta associated genes stated by Van Wolfswinkel *et al.* (19) were determined after *pcna* knockdown. Both sigma and zeta associated genes were strongly downregulated ($p<0.001$) after MMS exposure, but the downregulation of zeta associated genes ($p<0.05$) after *pcna* knockdown in combination with MMS exposure was stronger than in sigma associated genes ($p=0.2489$). The expression of *zfp1* after *pcna* knockdown and MMS exposure was strongly downregulated ($p<0.001$), which gave a strong indication of the effect on zeta-neoblasts, since *zfp1* is the only neoblast gene specific for the zeta-class (19). These data suggested that the zeta-class is more affected by *pcna* knockdown and that *pcna* associated DNA repair is more important in zeta-neoblasts than in sigma-neoblasts. Due to irreversible DNA damage, zeta-neoblasts can react by apoptosis, explaining the downregulation of zeta associated genes. It is also a possibility that less sigma-neoblasts convert to zeta-neoblasts, since a subset of the sigma-class give rise to the zeta-class (15).

Since *pcna* also plays a role in proliferation (40, 43), these data can suggest an increase of proliferation after MMS exposure for 10 days, which matches the hyperproliferation seen by Stevens A-S. (submitted data) after 17 days exposure. However, the gene expression of both sigma and zeta associated genes was downregulated after MMS exposure. This suggested that the hyperproliferation had not been initiated yet after 10 days exposure and confirmed the upregulated expression of *pcna* after genotoxic insult (42).

These data give a first indication of the effect of *pcna* knockdown in both SC classes. Single-Cell RT-PCR is necessary to specifically investigate the gene expression in individual cells and thereby evaluating specific subtypes. Double fluorescent *in situ* hybridization (FISH) can also be performed with a double labeling for the specific class and a DNA repair gene. This experiment can be repeated for other DNA repair genes using different repair pathways. For instance *rad51*, a component of homologous recombination (HR), or *ku80*, a component of non-homologous end-joining (NHEJ) can be used to investigate double strand repair (21, 23, 36). If a primary neoblast culture is available, the experiments can be performed using a suspension containing only neoblasts. In this case, results would give a more specific outcome compared with using a SC-enriched suspension. Therefore, culture conditions for neoblasts were optimized.

Optimization cell culture

A cell culture is often used in SC research, but despite several attempts to establish a primary neoblast culture, it has not been successful up until now (25, 26). Nevertheless, to support the advantages of *in vivo* research and to acquire more detailed information, an *in vitro* culture is necessary. This allows the individual investigation of SCs as well as a clearer view of the linkage between SC subtypes and several processes (e.g. the differentiation process and DNA repair mechanisms).

The starting point for the optimization was the review by Schürmann *et al.* (25), which proposed isotonic planarian medium to culture planarian neoblasts. During the optimization process several alterations were made in order to improve the cell culture. Electron microscopic photographs of the cell culture revealed cells containing a large nucleus with highly decondensed chromatin, confirming the presence of neoblasts. Some neoblasts contained rough endoplasmic reticulum, which indicated the entrance into cytoplasmic differentiation (44, 45).

The most important obstacle of the cell culture was a severe bacterial infection. Schürmann *et al.* (25) used Neomycin Sulfate (0.2 mg/ml) to control bacterial infections, but for our culture bacteria were resistant to this antibiotic. Therefore, the bacteria were identified to allow a more targeted administration. A Gram

staining indicated that Gram negative rods were present. Cefotaxime Sodium Salt, which is highly active against Gram negative bacteria, was successful in removing the bacterial infection without affecting the cells using the optimal concentration of 75 mg/l. The administration of this antibiotic led to the appearance of a fungal infection wherefore the broad-spectrum antifungal drug Amphotericin B was added. This infection could not be controlled yet.

It was investigated if the source of the infection came from the environment, despite aseptic techniques. These results suggested that the source of the infection was the worm itself. Additional centrifugation and washing steps, as well as a flush-back filtration step, reduced the initial amount of bacteria present instantly after cell dissociations. There was a possibility that the infection came from within the animal's intestine. Therefore, the effect of feeding was investigated by comparing dissociations of starved animals (at least 7 days) and fed animals (3 hours post feeding), and by using only tail fragments. Nevertheless, in all conditions the infection remained present. The combination of 75 mg/l Cefotaxime Sodium Salt and the flush-back filtration demonstrated the best results, but further optimization is necessary.

Another point of improvement was the induction of SC proliferation, which was a common problem for previous attempts to create a primary cell culture. Baguna (46) demonstrated an increase in proliferation *in vivo* 3-4 hours after the end of feeding. Therefore, dissociations were performed using both starved and fed animals. Preliminary data demonstrated an inhibition of proliferation in the presence of manganese, because it can become toxic for living organisms when applied in higher concentrations (47). Therefore, manganese was not added to the culture medium. The effect of feeding revealed no differences and cell counting showed a decrease in the amount of cells in the cell culture. This can be explained by the presence of the infection. Bacteria and fungi affect the SCs, causing them to die. Therefore, it is necessary to first control the contamination before other improvements can be made.

These results demonstrated the difficulty of culturing neoblasts. American Type Culture Collection (ATCC) stated that there are several challenges associated with the development of a primary cell culture, including contaminations introduced from the host tissue (48). Since the most important obstacle was the presence of an infection, future experiments must focus on the removal of the contamination first. Using both Cefotaxime Sodium Salt and a flush-back filtration step, the bacterial infection was controlled. The indicated fungal infection could not be controlled, thus other antifungal drugs that remove this infection without affecting the cells must be found. Since it is possible that the source of the infection is the worm itself, using planarians from other research institutes can give valuable information concerning this topic. It can also be investigated if the infection can be controlled by cultivating the animals under sterile conditions (e.g. CO_2-incubator). There is a possibility that the source of the infection comes from the food they receive, so autoclaving the calf liver might reduce the infection. When the infection is removed, the proliferation problem can be investigated by adding growth factors that promote cell proliferation. The experiment using starved and fed animals can be repeated to investigate if the proliferation actually increases 3-4 hours post feeding.

5. GENERAL CONCLUSION

The goal of this study was to investigate whether pluripotent SCs activate specific damage responses after carcinogenic exposure in order to circumvent carcinogenesis. Following DNA damage, SCs can react by activating specific DNA damage responses, like altering their differentiation process or DNA repair. Results demonstrated an effect of MMS exposure on the global amount of SCs, as well as on the differentiation process and the *pcna* associated DNA repair mechanisms. It is also demonstrated that SC subtypes react differently after DNA damage induced by *pcna* knockdown and carcinogenic exposure. To acquire a more detailed view of the repair mechanisms involved in both subtypes, more DNA repair genes must be investigated. Despite DNA damage, no tumors were observed in *S. mediterranea*. Although these data indicated the importance of SCs in the circumvention of carcinogenesis, it still needs to be unraveled further how SCs cope with this DNA damage and thereby avoid tumor formations.

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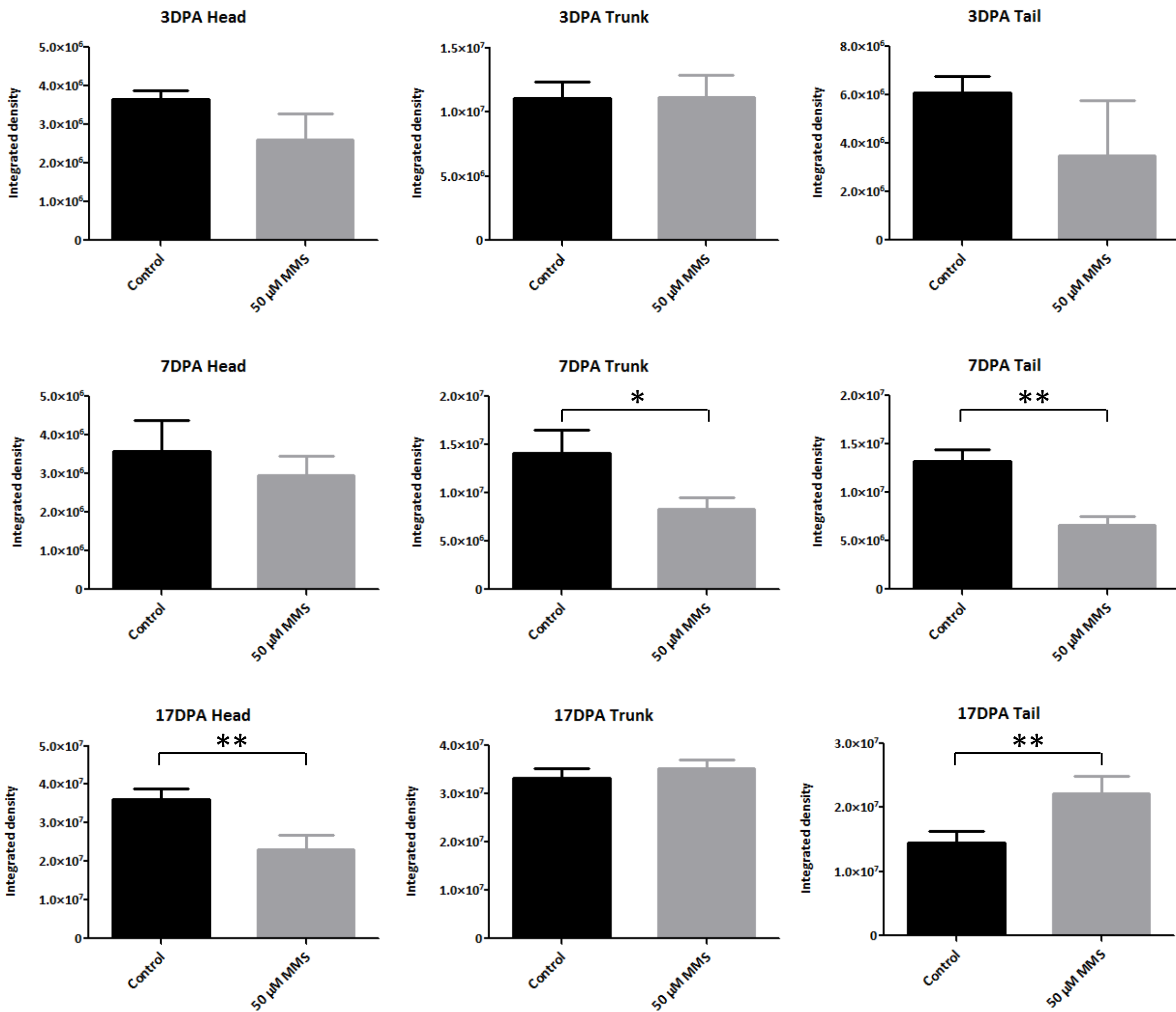
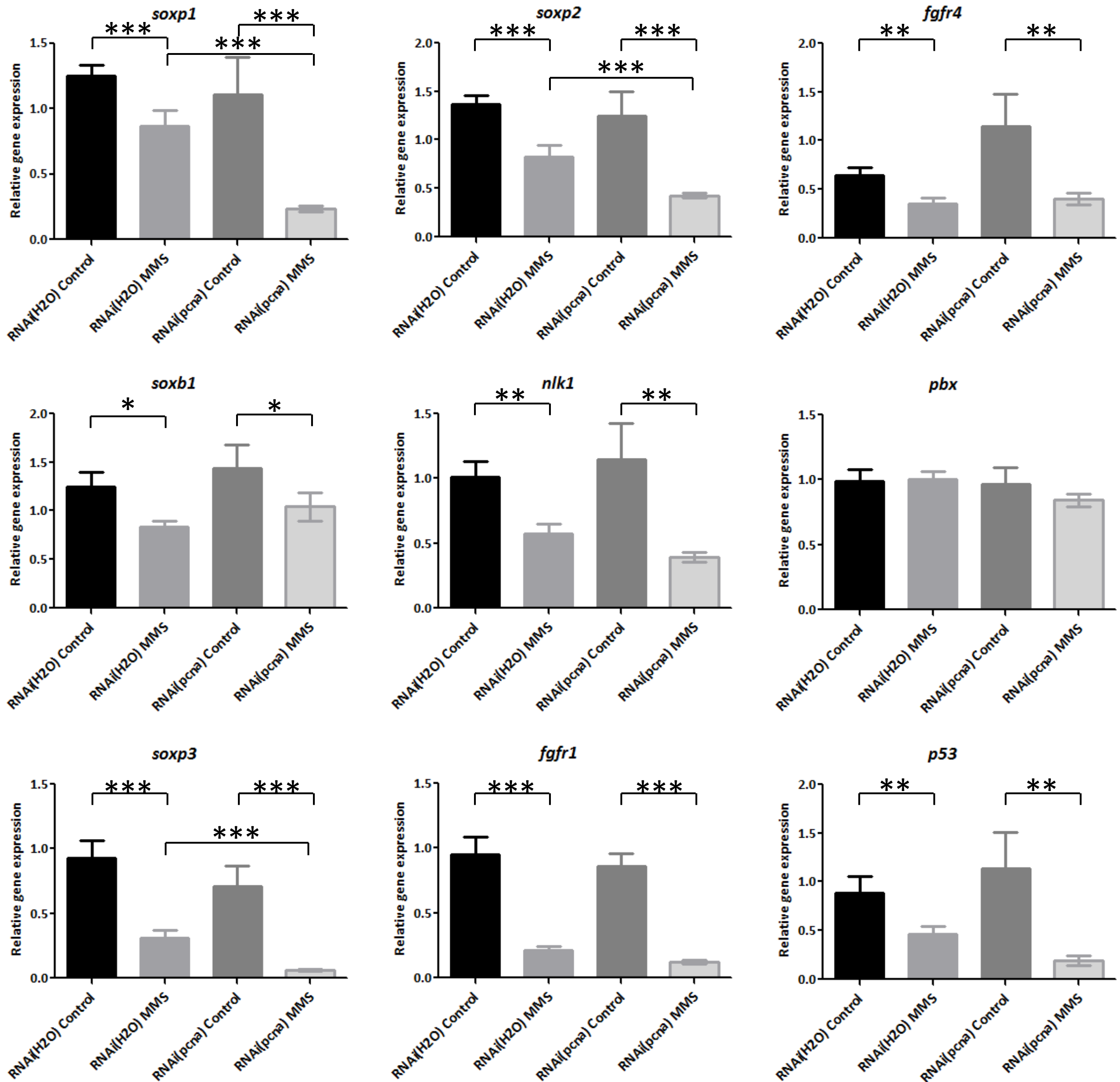
7. SUPPLEMENTAL INFORMATION



Supplemental Figure 1: Quantification immunohistochemistry for SMEDWI-1. The fluorescent signal was quantified by comparing the integrated density of both control and MMS treated animals. No significant changes were observed, except for the trunk and tail fragments 7DPA and the head and tail fragments 17DPA. Error bars represent the standard error of the mean (SEM). * p<0.1; ** p<0.05



Gene	General significant effects	
pcna	RNAi effect ***	
soxp1	MMS effect ***, RNAi effect ***, Interaction effect **	Sigma associated genes
soxp2	MMS effect ***, RNAi effect ***, Interaction effect **	
fgfr4	MMS effect **	
soxb1	MMS effect *	
nlk1	MMS effect **	
pbx	/	
soxp3	MMS effect ***, RNAi effect ***, Interaction effect **	Zeta associated genes
fgfr1	MMS effect ***	
p53	MMS effect **	
zfp1	MMS effect ***, RNAi effect ***, Interaction effect *	

Supplemental Figure 2: Gene expression profiles of individual genes. Gene expression profiles of individual genes showed the same pattern, except for *pbx* and *fgfr4*, as demonstrated in the average gene expression profiles. General significant effects of all used genes are represented in the table. Error bars represent the standard error of the mean (SEM). ***p<0.001; **p<0.01; *p<0.05

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Richting: **master in de biomedische wetenschappen-klinische moleculaire wetenschappen**
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