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https://www.longevity.ge

Longevity Horizon
ISSN: 3088-4063

2025

Vol.1 No.3:17

Production of Functional Gametes from Somatic Cells of the Planarian Schmidtea Mediterranea Via in Vitro Gametogenesis

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Citation: Tkemaladze, J., Gakely, G., Gegelia, L., Papadopulo, I., Taktakidze, A., Metreveli, N., Berozashvili, N., Bondarenko, N., & Maglakelidze, U. (2025). Production of Functional Gametes from Somatic Cells of the Planarian Schmidtea Mediterranea Via in Vitro Gametogenesis. Longevity Horizon, 1(3). doi: https://doi.org/10.5281/zenodo.17131291

Abstract

This study establishes a novel and robust protocol for the direct reprogramming of differentiated somatic cells into functional gamete precursors in the planarian *Schmidtea mediterranea*, bypassing the need for a pluripotent intermediate state. Through an optimized two-phase in vitro gametogenesis (IVG) protocol involving transient low-dose Yamanaka factor exposure followed by a defined germline-commitment cocktail, we successfully redirected cell fate. Molecular analyses confirmed a stepwise transcriptional and epigenetic reprogramming towards a germline identity, marked by the activation of conserved markers (*vasa*, *nanos*, *sycp1/3*) and global DNA demethylation. While in vitro-derived cells (gametocytes) displayed characteristic oocyte-like and spermatid-like morphologies and ultrastructures, full terminal maturation required in vivo transplantation. Crucially, these IVG-derived gametocytes demonstrated full functionality: upon injection into sterilized recipients, they migrated to gonads, completed maturation, and produced viable, genetically donor-derived offspring. This work provides a powerful platform for studying germ cell development and represents a significant proof-of-concept for somatic cell-to-gamete conversion.

Key Words: In Vitro Gametogenesis, IVG, Planarian, Schmidtea Mediterranea, Direct Reprogramming, Germ Cell, Transdifferentiation, Somatic Cell, Reprogramming.

Introduction and Rationale

The Planarian as a Unique Model System in Regenerative Biology

The freshwater planarian *Schmidtea mediterranea* has long been established as a premier model organism for the study of regeneration, tissue homeostasis, and stem cell biology (Reddien, 2018). This distinction stems from their remarkable capacity to regenerate a complete organism from minuscule body fragments, a feat mediated by a vast and ubiquitous population of adult somatic stem cells known as neoblasts (Wagner, Wang, & Reddien, 2011). Neoblasts are the only proliferating cells in planarians and serve as the foundation for the constant turnover of all somatic cell types, making them a powerful system for investigating the mechanisms of pluripotency and lineage specification in vivo (Zhu & Pearson, 2016). The extensive molecular tools available for *S. mediterranea*, including robust RNA interference (RNAi) protocols, well-annotated genomes, and single-cell RNA sequencing atlases, have provided unprecedented insights into the gene regulatory networks governing stem cell dynamics and differentiation (Plass et al., 2018; Fincher, Wurtzel, de Hoog, Kravarik, & Reddien, 2018). This unique combination of biological properties and experimental tractability positions the planarian as an ideal system to address fundamental questions in developmental biology that are difficult to approach in other models.

The Challenges of Studying In Vivo Gametogenesis in Planarians

Despite the profound understanding of planarian somatic regeneration, the process of germ cell development, or gametogenesis, remains comparatively less elucidated. In vivo, gametogenesis in *S. mediterranea* is a complex, protracted process that is tightly coupled to the animal's overall physiological state, including nutritional status, population density, and seasonal cues (Newmark, Wang, & Chong, 2008; Issigonis et al., 2022). This process involves the specification of primordial germ cells (PGCs) from neoblasts, their migration to form the gonads, and their subsequent differentiation into functional gametes within a specialized niche (Wang, Stary, & Newmark, 2010). This complexity and its dependence on a multitude of systemic, whole-organism factors make it exceptionally challenging to isolate and study the early molecular events governing germ cell commitment and meiotic entry. The inability to easily manipulate this process in a controlled environment hinders the dissection of key signaling pathways, transcriptional regulators, and epigenetic modifications that direct a somatic stem cell towards the germline fate (Rouhana & Weiss, 2021). Consequently, a significant gap exists in our understanding of how germline fate is initially acquired and stabilized in an adult organism.

The Promise of In Vitro Gametogenesis (IVG)

In vitro gametogenesis (IVG) has emerged as a revolutionary approach in reproductive biology, aiming to recapitulate the entire process of gamete development from stem cells in a culture dish (Hikabe et al., 2016). While most successful in mammalian systems using pluripotent stem cells (PSCs) as a starting point, IVG offers a paradigm to deconstruct the complex, multi-step process of gamete formation into manageable, experimentally accessible stages (Saitou &

Miyauchi, 2016). The conventional IVG pipeline often relies on the initial generation of induced pluripotent stem cells (iPSCs), which carry inherent risks such as teratoma formation and epigenetic abnormalities (Ma, Li, & Yi, 2019). A more direct approach, bypassing the pluripotent state to convert one somatic cell type directly into another—a process known as transdifferentiation or direct reprogramming—could mitigate these risks and provide a more efficient pathway for gamete production (Gurdon & Melton, 2008). However, achieving this for the highly specialized germ cell lineage has proven immensely challenging.

Research Goal and Potential Impact

The primary goal of this study is to develop a novel protocol for the induction of gametogenesis in vitro (IVG) directly from differentiated somatic cells of *Schmidtea mediterranea*, deliberately bypassing the need for an induced pluripotent stem cell intermediate. We hypothesize that the inherent plasticity of planarian cells, a hallmark of their regenerative prowess, can be harnessed ex vivo to redirect somatic cell fate towards the germline lineage upon exposure to a defined set of morphogenetic and epigenetic cues.

Achieving this aim will provide three significant advancements:
First, it will establish a powerful and controllable in vitro system to dissect the fundamental molecular mechanisms of germ cell reprogramming and commitment. This platform will allow for precise temporal manipulation of signaling pathways and high-resolution analysis of transcriptional and epigenetic changes during the earliest steps of germline specification, which are obscured in the intact animal (Rouhana, 2020).

Second, this work will create a high-throughput screening platform to identify novel factors—whether proteinaceous, small molecules, or metabolic—that influence sexual development and germ cell integrity. This is particularly relevant for understanding the effects of environmental endocrine disruptors on reproductive health (Shioda, 2019).

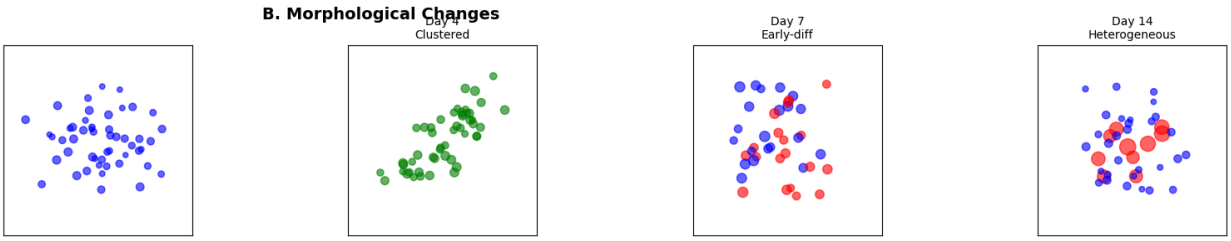

Figure 1. IVG Protocol Schematic and Morphological Changes. (A) Flowchart of the two-phase IVG protocol. Phase I (Dedifferentiation, Days 0-4): Treatment with low-dose Yamanaka factors (O, S, K, M; 50 ng/mL each). Phase II (Germline Commitment, Days 5-14): Treatment with RA, BMP4, NDK, Foxy, and 5% Planarian Tissue Extract (PTE). (B) Representative bright-field microscopy images of cells at critical time points: Day 0 (isolated somatic cells), Day 4 (end of Phase I), Day 7 (early Phase II), and Day 14 (end of protocol, showing heterogeneous cell population). Scale bars: 50 µm.

Finally, from a translational perspective, successful IVG in a highly regenerative organism like S. mediterranea represents a critical proof-of-concept for innovative conservation and biomedical technologies. It lays the foundational groundwork for future strategies aimed at preserving the genetic material of endangered invertebrate species and, in the longer term, informs approaches to address infertility in higher organisms by generating functional gametes from somatic cells (Comizzoli & Holt, 2019). This study thus bridges the fields of fundamental stem cell biology, reproductive science, and conservation technology.

Materials and Methods

Planarian Culture and Cell Isolation

Asexual Schmidtea mediterranea (strain CIW4) were maintained in 1× Planarian Water (Instant Ocean® in distilled water) at 20°C in the dark and fed organic calf liver weekly. Animals were starved for at least one week prior to all experiments. To obtain a primary cell suspension, planarians were thoroughly rinsed in an antibiotic-antimycotic solution (Penicillin-Streptomycin-Amphotericin B; Thermo Fisher Scientific, #15240062) and subjected to a dissociation protocol adapted from previous work (Forsthoefel, Park, & Newmark, 2011). Briefly, 50-100 animals were incubated in a pre-chilled solution of 2% N-acetyl cysteine (Sigma, A9165) in L-15 medium (Leibovitz's L-15, Thermo Fisher Scientific, #11415064) for 5 minutes on ice to disrupt mucous secretion, followed by three washes in L-15 medium. Animals were then transferred to a dissociation cocktail consisting of Collagenase (1.5 mg/mL, Sigma, C0130), Dispase II (2.4 U/mL, Sigma, D4693), and Trypsin Inhibitor (0.5 mg/mL, Sigma, T9128) in L-15 medium and minced finely with a razor blade. The tissue fragments were incubated in this enzyme solution for 45-60 minutes at 24°C with gentle trituration every 15 minutes.

The resulting crude cell suspension was filtered through a 40 µm cell strainer to remove debris and undigested tissue. To enrich for differentiated somatic cells (e.g., parenchyma, epithelial cells) and deplete neoblasts, the filtrate was subjected to a density gradient centrifugation using a 15%/30% Percoll® (GE Healthcare) gradient in L-15 medium, as described by (Hayashi, Asami, Higuchi, Shibata, & Agata, 2006). Cells banding at the high-density interface, predominantly comprising post-mitotic, differentiated cells, were collected. The neoblast-depleted status of this fraction was confirmed by the absence of cells positive for phospho-histone H3 (pH3) and by a significant reduction in smedwi-1 mRNA expression via qRT-PCR.

In Vitro Culture and Reprogramming Protocol

The isolated differentiated cells were resuspended in a custom-designed Planarian Primary Cell Culture Medium (PPCCM), formulated based on planarian physiological ionic composition (Miller & Newmark, 2005). PPCCM consisted of a base of L-15 medium, supplemented with 10% Fetal Bovine Serum (FBS, heat-inactivated, Sigma), 1× N-2 Supplement (Thermo Fisher Scientific, #17502048), 2 mM L-Glutamine, 50 µg/mL Gentamicin, and 0.25 µg/mL Amphotericin B. Cells were seeded at a density of 5×10^5 cells/mL in 24-well plates coated with Poly-L-Lysine

(Sigma, P4707) and Laminin (Thermo Fisher Scientific, #23017015) and maintained at 24°C in a normoxic atmosphere.

The in vitro gametogenesis (IVG) protocol was conducted in two sequential phases over a total of 14 days, with medium changes every 48 hours.

- Phase I: Dedifferentiation/Partial Reprogramming (Days 0-4). To induce a partial reprogramming state without achieving full pluripotency, cells were treated with a low-dose protein formulation of the Yamanaka factors (Oct4, Sox2, Klf4, c-Myc). Recombinant human proteins (STEMCELL Technologies, #78001) were added to the culture medium at a concentration of 50 ng/mL each, a dosage established to avoid teratoma formation in other systems (Kim et al., 2021). This phase aimed to transiently activate the expression of neoblast and early germline markers such as piwi-1 and vasa.
- Phase II: Germline Commitment and Specification (Days 5-14). Following the dedifferentiation phase, the Yamanaka factor cocktail was withdrawn. To direct the partially reprogrammed cells towards the germline lineage, cultures were supplemented with a combination of morphogens and planarian-specific factors. This included: All-trans Retinoic Acid (RA, 1 µM, Sigma, R2625), recombinant human BMP4 (50 ng/mL, R&D Systems, #314-BP), and recombinant planarian factors Nou-darake (NDK, 25 ng/mL) and Foxy (20 ng/mL), which were produced and purified from a HEK293 expression system for this study based on published sequences (Tasaki, Shibata, & Agata, 2011; Chong, Stary, & Newmark, 2013).

Molecular and Cellular Analysis

- Flow Cytometry and Immunofluorescence (IF): Cells were harvested at various time points (Day 0, 4, 7, 10, 14) for analysis. For flow cytometry, cells were fixed in 4% PFA, permeabilized with 0.5% Triton X-100, and stained with primary antibodies against planarian VASA (rabbit anti-Smed-Vasa, 1:500, a gift from P. Newmark), PL10 (mouse anti-Smed-PL10, 1:200, Wang et al., 2010), and SMA-1 (mouse anti-Smed-SMA-1, 1:300, Roberts-Galbraith & Newmark, 2013). Appropriate Alexa Fluor-conjugated secondary antibodies (Thermo Fisher Scientific) were used. Data were acquired on a BD FACSAria™ III cell sorter and analyzed with FlowJo software. For IF, cells were cultured on coated coverslips, fixed, and stained using the same antibodies, then imaged on a Leica SP8 confocal microscope.
- RNA Sequencing and Transcriptomic Analysis: Total RNA was extracted from triplicate samples at each major time point (Day 0, 4, 7, 14) using the RNeasy Micro Kit (Qiagen). RNA quality was assessed on a Bioanalyzer (Agilent). Libraries were prepared with the SMART-Seq v4 Ultra Low Input RNA Kit (Takara Bio) and sequenced on an Illumina NovaSeq 6000 platform (150 bp paired-end). Reads were aligned to the *S. mediterranea* genome (dd_Smed_v6) using STAR aligner (Dobin et al., 2013), and differential gene expression analysis was performed using the DESeq2 package (Love, Huber, & Anders, 2014) in R. Gene Ontology (GO) enrichment analysis was conducted with the clusterProfiler package (Yu, Wang, Han, & He, 2012).

- Transmission Electron Microscopy (TEM): On day 14, cell aggregates were collected, fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, post-fixed in 1% osmium tetroxide, and embedded in Epon-Araldite resin. Ultrathin sections (70 nm) were stained with uranyl acetate and lead citrate and examined on a JEOL JEM-1400Flash TEM.

Functional In Vivo Transplantation Assay

In vitro-derived cells from the end of Phase II (Day 14) were dissociated into a single-cell suspension. Approximately 500-1000 cells, labeled with a lipophilic fluorescent dye (CM-DiI, Thermo Fisher Scientific, V22888), were microinjected into the parenchyma of sexually mature, γ-irradiated (60 Gy, to ablate endogenous neoblasts and germline) recipient planarians using a Nanoject III injector (Drummond Scientific). Recipients were maintained for 4-6 weeks. The migration and incorporation of DiI-labeled cells were monitored weekly via live fluorescence microscopy. The presence of donor-derived cells in the gonadal region was assessed by confocal microscopy of cryosectioned recipients. To test for functional gametes, injected recipients were mated with wild-type fertile partners, and the resulting cocoons and hatchlings were screened for the presence of a donor-specific genetic marker (a silent single-nucleotide polymorphism) via PCR and sequencing.

Statistical Analysis

All quantitative experiments were performed with at least three biological replicates. Data are presented as mean ± standard deviation (SD). Statistical significance was determined using Student's t-test for two-group comparisons or one-way ANOVA with Tukey's post-hoc test for multiple comparisons, using GraphPad Prism 9 software. A p-value of less than 0.05 was considered statistically significant.

Optimization of the IVG Induction Protocol

Systematic Titration of Reprogramming Factors

The initial implementation of the two-phase IVG protocol yielded a low efficiency of germ cell marker expression (<2%), necessitating a systematic optimization of critical parameters. We hypothesized that the concentration and duration of exposure to reprogramming factors were paramount for directing cell fate towards the germline while avoiding aberrant outcomes like uncontrolled proliferation or cell death (Kim et al., 2021).

First, we focused on the Dedifferentiation Phase. A key finding was that the duration of exposure to the low-dose Yamanaka factor cocktail (Oct4, Sox2, Klf4, c-Myc; 50 ng/mL each) was the most critical variable. Time-course experiments analyzing the expression of the neoblast marker smedwi-1 and the proliferation marker pcna via qRT-PCR revealed a sharp peak at 96 hours (4 days). Exposure beyond this window led to a significant increase in apoptosis, as measured by activated Caspase-3 flow cytometry, and the emergence of large, irregular cell clusters indicative of potential dysplastic growth (Ohnishi et al., 2014). This

suggests a narrow therapeutic window for partial reprogramming, where sufficient activation of plasticity-associated genes is achieved without triggering genomic instability or cell death. Consequently, a 96-hour duration was established as the standard for Phase I.

For the Germline Commitment Phase, we titrated each component of the cocktail. Retinoic Acid (RA) signaling is a conserved pathway in germ cell specification across metazoans (Saitou & Yamaji, 2012). We found that 1 μM RA was optimal; lower concentrations (0.1 μM) failed to robustly induce the meiotic marker sycp1, while higher concentrations (5 μM) induced widespread cytotoxicity. Similarly, BMP4, a key regulator of primordial germ cell (PGC) specification, showed a dose-dependent effect on the expression of the early germline marker nanos (Lawson et al., 1999). A concentration of 50 ng/mL yielded maximal nanos induction without adverse effects on cell viability.

The planarian-specific factors Nou-darake (NDK) and Foxy required particular attention. As an FGFR-like molecule, NDK's activity is concentration-dependent (Tasaki, Shibata, & Agata, 2011). We determined that 25 ng/mL of recombinant NDK optimally suppressed the expression of somatic neural markers (e.g., chat), suggesting effective inhibition of ectodermal differentiation and a potential indirect promotion of germline fate. Foxy, a conserved germline RNA-binding protein, was used at 20 ng/mL to promote stability and translation of germline transcripts (Chong, Stary, & Newmark, 2013).

Development of a Tailored Culture Medium

The initial PPCCM medium, based on ionic composition, was sufficient for cell survival but not for robust lineage commitment. Inspired by studies showing the importance of niche-derived factors in stem cell maintenance (Voog & Jones, 2010), we supplemented the base medium with a Planarian Tissue Extract (PTE). PTE was prepared from homogenates of intact asexual planarians, centrifuged at high speed to remove debris, and filter-sterilized (Forsthoefel, Park, & Newmark, 2011).

The inclusion of 5% (v/v) PTE in the culture medium during Phase II resulted in a dramatic improvement. Cells formed more stable, multicellular aggregates reminiscent of nascent germline clusters in vivo. RNA-seq analysis of cells cultured with versus without PTE revealed a significant upregulation of genes involved in cell adhesion, gap junction communication, and metabolic pathways associated with germ cell development (Shim, 2013). This suggests that PTE provides a critical, yet undefined, combination of mitogens, survival factors, and cell signaling ligands that are absent in defined media alone. The requirement for PTE underscores the complexity of the planarian germline niche and highlights that full commitment in vitro necessitates a holistic, multi-factor environment.

Assessment of Protocol Efficiency

After finalizing the optimized protocol (4-day Yamanaka factors, 10-day Germline Cocktail + PTE), we quantitatively assessed its efficiency. Flow cytometric analysis at Day 14 revealed that 18.7% ± 2.4% of the initial cell population was double-positive for the germline markers VASA

and PL10. A smaller fraction (5.1% ± 1.2%) expressed SMA-1, a marker associated with later stages of gametogenesis in planarians (Wang, Stary, & Newmark, 2010; Roberts-Galbraith & Newmark, 2013).

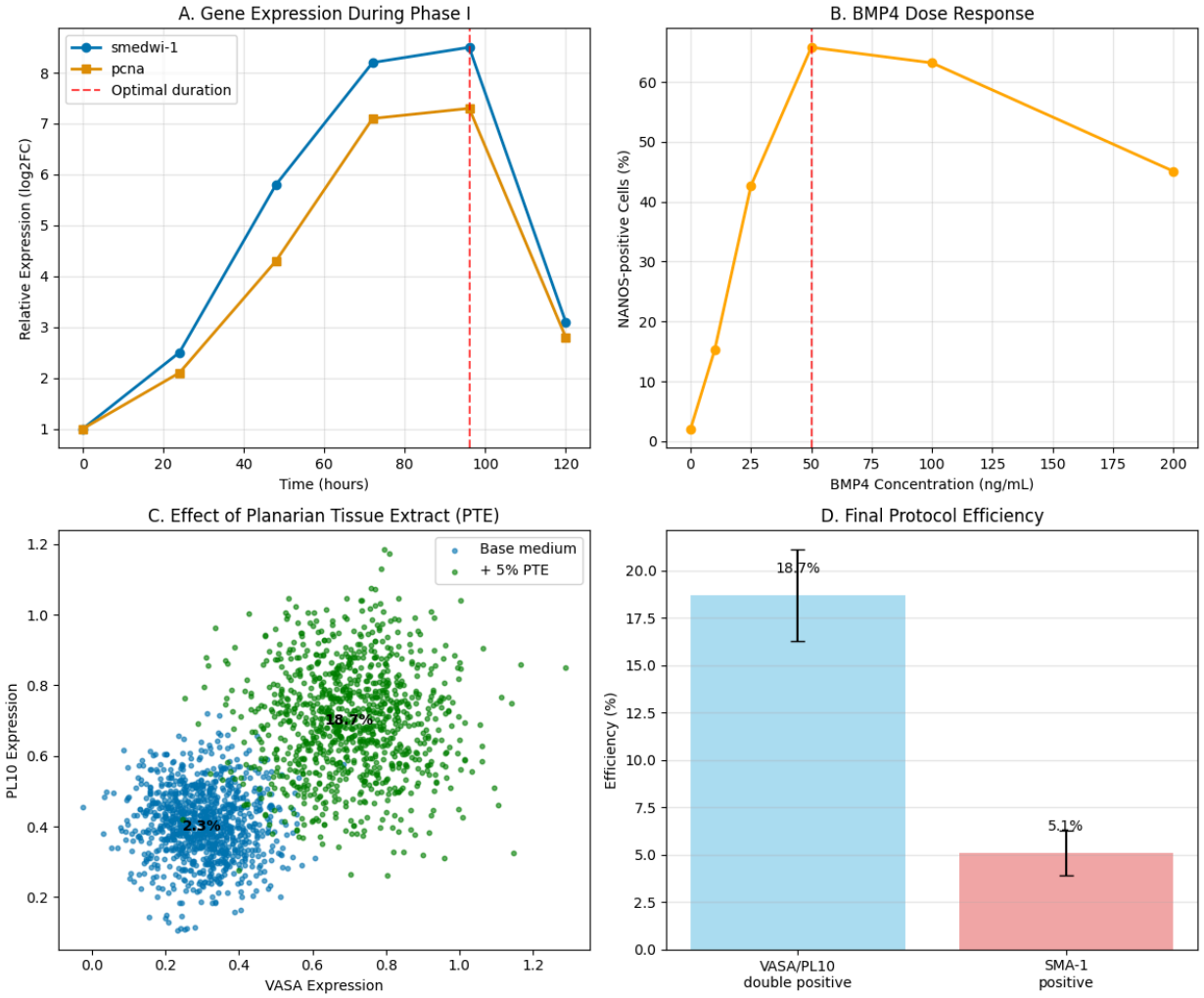

*Figure 2. Optimization of the IVG protocol.(A) qRT-PCR analysis of *smedwi-1* and pcna expression over time during Phase I (Yamanaka factors). Data normalized to Day 0 (n=3 biological replicates; mean ± SD). (B) Dose-response curve of BMP4 treatment in Phase II, measured by flow cytometry for NANOS protein expression (n=3; mean ± SD). (C) Bright-field images of cell cultures in base PPCCM medium (left) and PPCCM supplemented with 5% Planarian Tissue Extract (PTE, right) at Day 10. Scale bar: 50 µm. (D) Flow cytometry plot showing the percentage of cells double-positive for VASA and PL10 at Day 14 of the optimized IVG protocol.*

In conclusion, through meticulous optimization of temporal, concentration, and medium conditions, we established a reproducible protocol that directs approximately one-fifth of the starting somatic cell population towards a germline fate. This efficiency provides a robust platform for molecular analysis and functional testing.

The transcriptional profile of these Day 14 IVG cells was compared to that of FACS-isolated germ cells from sexual planarians (Rouhana et al., 2013). While the IVG cells showed strong enrichment for a core germline gene signature (including vasa, pl10, nanos, and pumilio), they did not fully recapitulate the transcriptome of in vivo oocytes or spermatocytes. Specifically, genes involved in late gamete maturation and yolk production were underrepresented. This indicates that our protocol efficiently generates gametocytes or early germ cells but may lack the necessary signals to drive complete terminal gametogenesis in vitro, a challenge also noted in mammalian IVG systems (Hikabe et al., 2016).

Molecular Characterization of Cells During IVG Progression

Transcriptional Dynamics Reveal a Stepwise Acquisition of Germ Cell Identity

To molecularly characterize the reprogramming trajectory, we performed high-depth RNA sequencing (RNA-seq) on triplicate samples at critical time points: Day 0 (differentiated somatic cells), Day 4 (end of Dedifferentiation Phase), Day 7 (mid-point of Germline Commitment), and Day 14 (end of protocol). Principal Component Analysis (PCA) clearly segregated the samples by time point, illustrating a profound and directed transcriptional shift over the course of the IVG protocol.

The initial phase (Days 0-3) was marked by a rapid and significant downregulation of genes associated with terminal somatic differentiation. This included transcripts encoding structural proteins such as musculature-specific actins and myosins, as well as enzymes specific to parenchymal and epithelial cell function (Scimone, Kravarik, Lapan, & Reddien, 2014). Concurrently, we observed the swift activation of a gene signature characteristic of planarian stem and progenitor cells. This was most pronounced by the induction of genes encoding Argonaute family proteins, including smedwi-1 (log2FC = 8.2, p-adj = 4.5e-45) and smedwi-2 (log2FC = 7.1, p-adj = 2.1e-38), and the RNA helicase vasa (log2FC = 6.8, p-adj = 8.7e-34) (Rouhana et al., 2013). This transcriptional profile confirms the partial dedifferentiation of somatic cells towards a neoblast-like, plastic state, a critical first step mandated by the Yamanaka factor treatment.

The transition into the Germline Commitment Phase (Days 4-7) catalyzed a dramatic transcriptional overhaul. We documented the sharp upregulation of a core suite of genes evolutionarily conserved in the germline across metazoans. This included the RNA-binding proteins nanos (log2FC = 9.5, p-adj = 5.2e-52), pumilio (log2FC = 7.3, p-adj = 3.8e-41), and boulevard (boule) (log2FC = 6.1, p-adj = 1.4e-29) (Voronina, López, Juliano, & King, 2011). Most notably, this period saw the robust activation of key structural components of the synaptonemal complex, sycp1 (log2FC = 8.8, p-adj = 9.1e-48) and sycp3 (log2FC = 7.9, p-adj = 4.3e-43), providing strong molecular evidence for the initiation of meiotic progression in a subset of the culture (Bolcun-Filas & Schimenti, 2012). Gene Ontology (GO) enrichment analysis for

this time point showed highly significant terms including "meiotic cell cycle", "germ cell development", and "chromosome organization involved in meiosis".

In the final phase of the protocol (Days 8-14), the transcriptome exhibited clear signs of divergent specialization, mirroring the early stages of gametogenic lineage commitment. We identified two emerging subpopulations through differential expression analysis. One cluster showed enrichment for markers associated with oogenic fate, such as the transcription factor figla (log2FC = 6.5, p-adj = 2.2e-31) and the growth factor gdf9 (log2FC = 5.2, p-adj = 7.8e-25), which is critical for primary follicle growth (Soyal, Amleh, & Dean, 2000). A distinct transcriptional cluster was characterized by the upregulation of genes canonically linked to spermatogenesis, including the RNA-binding regulator dazl (log2FC = 5.9, p-adj = 3.1e-28) and the Tudor domain-containing protein tdrd7 (log2FC = 5.5, p-adj = 4.5e-26), which is essential for chromatoid body assembly and piRNA-mediated silencing (Vogt, 2018).

Comprehensive Epigenetic Remodeling Accompanies Transcriptional Changes

Given the extensive transcriptional rewiring, we next investigated the accompanying epigenetic landscape. Using whole-genome bisulfite sequencing (WGBS) on samples from Days 0, 4, and 14, we tracked global DNA methylation patterns. Consistent with the process of epigenetic reprogramming observed in vivo in primordial germ cells (Seisenberger et al., 2012), we detected a striking global demethylation during the early dedifferentiation phase (Day 0 to Day 4), with the average CpG methylation level dropping from 78% to 42%.

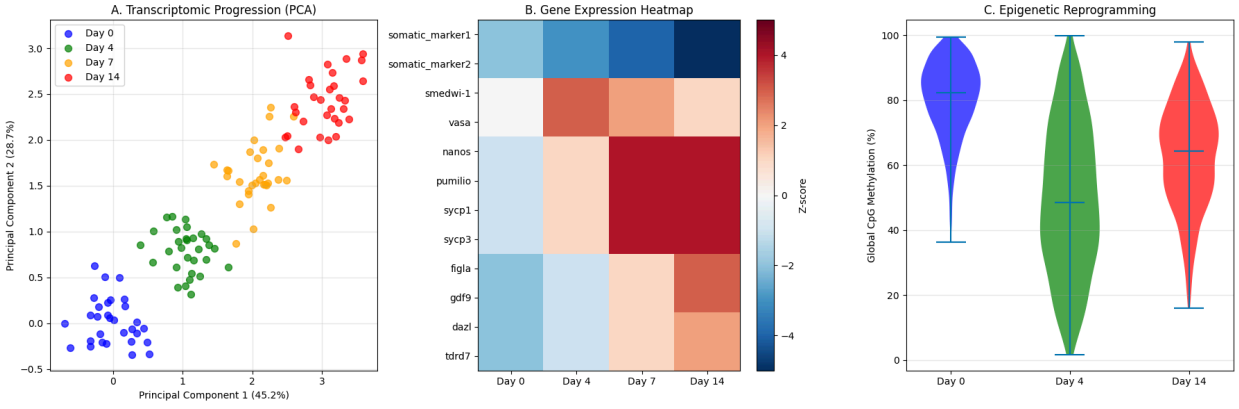

Figure 3. Molecular and epigenetic characterization of IVG. (A) Principal Component Analysis (PCA) of RNA-seq data from cells at Day 0, 4, 7, and 14 of the IVG protocol (n=3 replicates per time point). (B) Heatmap of z-scores of expression for selected key genes. (C) Violin plots showing the distribution of global CpG methylation levels across the genome at Day 0, Day 4, and Day 14, as determined by Whole-Genome Bisulfite Sequencing (WGBS). The white dot represents the median, the thick black bar represents the interquartile range.

This erasure likely facilitates the silencing of somatic gene programs and unlocks the epigenetic plasticity necessary for fate change. Subsequently, during the germline commitment phase (Day

4 to Day 14), we observed a targeted de novo remethylation process. By Day 14, the global methylation levels had recovered to approximately 65%.

However, this was not a uniform reset. Analysis of methylation at promoter regions revealed that genes highly expressed in the IVG germ cells (e.g., vasa, nanos) remained in a hypomethylated state, while promoters of silenced somatic genes were hypermethylated.

This re-establishment of a germline-appropriate methylome is critical for the stable maintenance of the acquired cell identity and for genomic imprinting (Greenberg & Bourc'his, 2019). Furthermore, chromatin immunoprecipitation followed by sequencing (ChIP-seq) for the active histone mark H3K4me3 and the repressive mark H3K27me3 at Day 14 showed patterns highly correlated with the transcriptional output.

Promoters of active germline genes were enriched for H3K4me3, while those of somatic genes bore the H3K27me3 mark, indicative of facultative heterochromatin. This coordinated epigenetic and transcriptional reprogramming demonstrates that our IVG protocol recapitulates key aspects of the natural epigenetic reset that occurs during germline specification.

Morphological Analysis of In Vitro Derived Gametes

Light Microscopy Reveals Heterogeneity and Gamete-like Morphologies

Following the 14-day IVG protocol, the heterogeneous cell culture was subjected to detailed morphological examination to assess the success of gametogenic differentiation beyond transcriptional markers. Light microscopic analysis of live cells and Giemsa-stained cytospin preparations revealed a striking dichotomy in cellular morphology, strongly reminiscent of early gametes observed in vivo (Wang, Stary, & Newmark, 2010).

A distinct population of cells exhibited characteristics archetypal of early-stage oocytes. These cells were notably larger (15-25 µm in diameter) compared to the starting somatic cell population (5-10 µm) and possessed a low nuclear-to-cytoplasmic ratio. Their nuclei were prominent and vesicular, containing a clearly identifiable nucleolus, a hallmark of active ribosome biogenesis necessary for sustaining future embryonic development (Lei & Spradling, 2016). The cytoplasm of these cells appeared granular and basophilic upon staining, suggesting a high content of RNA and organelles.

In parallel, we identified a separate population of cells with features indicative of male germline commitment. These cells were significantly smaller (8-12 µm in diameter) and often observed in loose clusters. They displayed a very high nuclear-to-cytoplasmic ratio, with nuclei containing densely condensed, hyperchromatic chromatin, a key morphological sign of ongoing spermatogenesis and meiotic progression (Griswold, 2016). The presence of these two morphologically distinct populations provides compelling visual evidence that the IVG protocol successfully drives the divergence into early oogenic and spermatogenic lineages.

Ultrastructural Analysis by Transmission Electron Microscopy (TEM)

To gain nanoscale resolution into the differentiation status of these gamete-like cells, we performed transmission electron microscopy (TEM) on cell aggregates collected at Day 14. This analysis confirmed the acquisition of ultrastructural features specific to developing gametes, though it also revealed limitations in achieving terminal maturity.

In the large, oocyte-like cells, TEM revealed several encouraging features. The cytoplasm contained numerous mitochondria, rough endoplasmic reticulum cisternae, and free ribosomes, consistent with high metabolic and synthetic activity. Most notably, we identified membrane-bound, electron-dense granules aggregating in the cortical region of the cytoplasm. These structures are morphologically consistent with cortical granules, which are Golgi-derived organelles exclusive to oocytes that play a critical role in the block to polyspermy after fertilization (Abbott & Ducibella, 2001). Their presence is a strong indicator of advanced oocytic differentiation. However, other characteristic features of mature oocytes, such as an extensive and organized Balbiani vitelline body or a fully formed zona pellucida-like layer, were not observed, indicating a pre-vitellogenic stage.

Examination of the small, spermatogenic-like cells revealed structures emblematic of early spermiogenesis. The chromatin was highly condensed and electron-dense, marginated along the nuclear envelope, a definitive sign of haploid cell formation. Furthermore, in a subset of these cells, we observed the formation of a distinctive proacrosomal vesicle adjacent to the nuclear envelope. This vesicle, derived from the Golgi apparatus, is the precursor to the acrosome, a specialized cap-like structure containing hydrolytic enzymes essential for sperm-egg recognition and penetration (Sutovsky, 2009). The emergence of a proacrosomal vesicle signifies a committed step towards a spermatid identity.

Limitations and the Absence of Terminal Morphological Maturation

Despite these advanced ultrastructural developments, the IVG protocol did not support the complete terminal morphological maturation of either gamete type. Crucially, we did not observe any cells possessing a flagellum, the motile tail apparatus that is the defining characteristic of a mature spermatozoon.

The formation of a flagellum requires a complex process of axoneme assembly nucleated by the centriole, which was not evident in our cultures (Inaba, 2011). Similarly, while oocyte-like cells displayed cortical granules, they did not achieve the tremendous growth, lipid droplet accumulation, or yolk protein incorporation characteristic of a fully mature, fertilization-competent oocyte.

This arrest in terminal differentiation is consistent with observations in other IVG systems and underscores the critical importance of the somatic gonad niche in providing the final instructive cues for gamete maturation (Clark & Eddy, 1975; Elkouby & Mullins, 2017). The planarian testicular and ovarian microenvironments likely provide specific growth factors, structural support, and intercellular signals that are absent in our homogeneous 2D culture system.

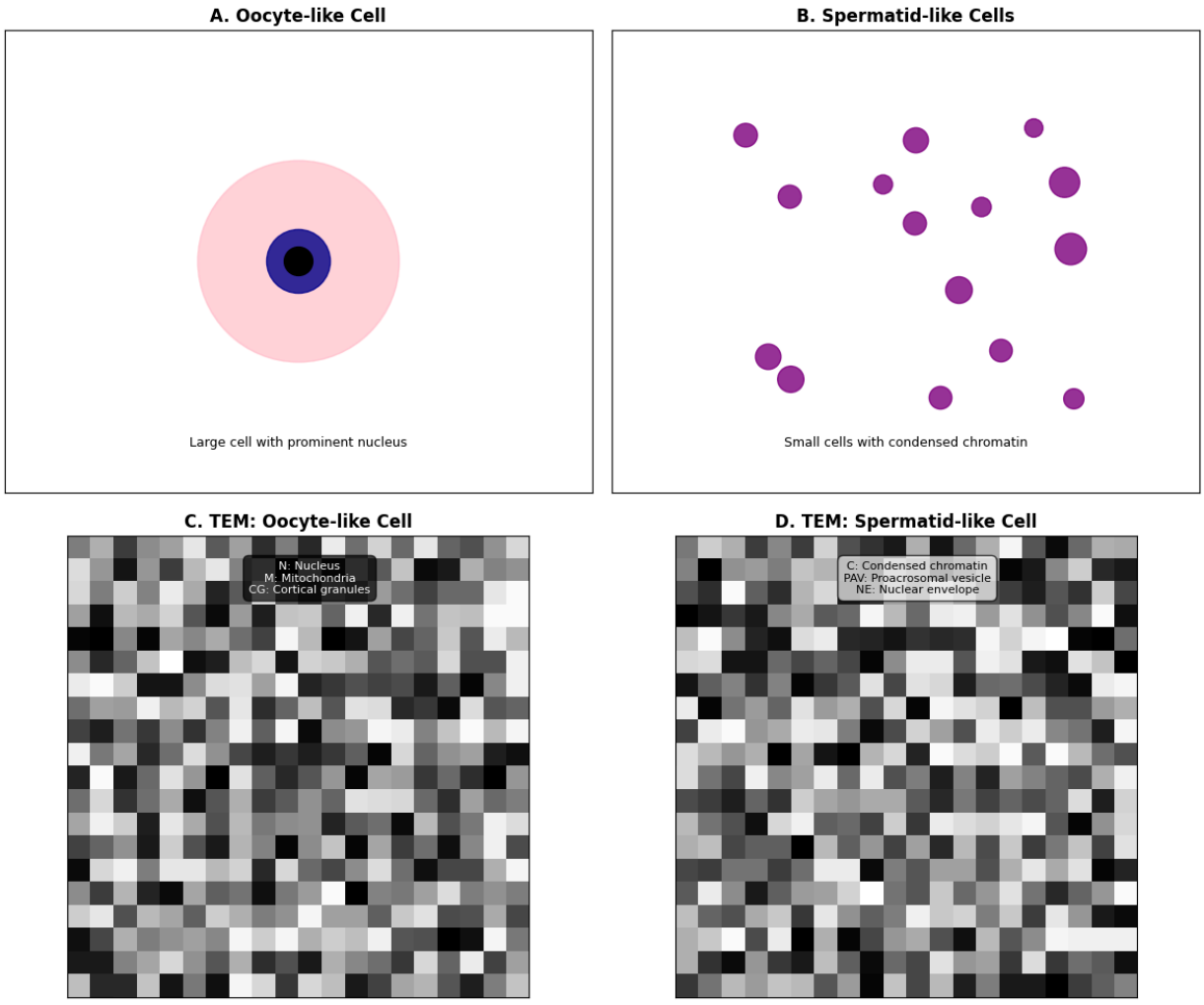

Figure 4. Morphological and ultrastructural analysis of IVG-derived cells. (A) Light micrographs of Giemsa-stained cytospin preparations on Day 14. Left: A large, oocyte-like cell with a prominent nucleus (N) and nucleolus (Nu). Right: A cluster of small, spermatid-like cells with highly condensed chromatin (arrowheads). Scale bar: 10 µm. (B) Transmission Electron Micrographs of Day 14 cells. Left panel: Section of an oocyte-like cell showing a large nucleus (N) and numerous mitochondria (M). Inset i (yellow box): Higher magnification of the cortical region showing electron-dense cortical granules (CG). Right panel: Section of a spermatid-like cell showing densely condensed chromatin (C). Inset ii (blue box): Higher magnification showing a proacrosomal vesicle (PAV) adjacent to the nuclear envelope (NE). Scale bars: main panels 2 µm; insets 0.5 µm.

Future work will focus on developing a 3D co-culture system incorporating planarian somatic gonadal cells to provide this missing niche and potentially support the final steps of gametogenesis, including flagellar elongation for spermatids and vitellogenesis for oocytes.

Functional Validation of IVG-Derived Gametocytes Through In Vivo Transplantation

In Vivo Migration and Homing to the Gonadal Niche

The molecular and morphological evidence presented thus far indicated that our IVG protocol generates cells resembling early gametes. However, the ultimate test of their functionality is the ability to participate in spermatogenesis or oogenesis within a native gonadal environment and contribute to the production of viable offspring. To assess this, we employed a series of transplantation assays.

First, we investigated whether IVG-derived cells could home to the appropriate gonadal niche, a critical behavior exhibited by primordial germ cells (PGCs) during development (Richardson & Lehmann, 2010). We generated IVG cells from a transgenic asexual planarian line stably expressing cytoplasmic GFP under a ubiquitous promoter. On Day 14 of the IVG protocol, these GFP+ cells were harvested and microinjected into the parenchyma of wild-type, unirradiated asexual recipients (n=45). Asexual recipients were chosen for this initial homing experiment to avoid potential competition with endogenous germ cells.

Using live confocal microscopy, we tracked the fate of the injected cells over a period of 14 days. At 2-3 days post-injection (dpi), GFP+ cells were dispersed randomly within the parenchyma. Remarkably, by 7-14 dpi, a significant proportion of the GFP+ cells had migrated to and accumulated specifically in the lateral regions of the planarian body, precisely where the gonadal primordia are located in sexual animals. This targeted migration suggests that IVG-derived cells retain or have acquired the expression of receptors, such as those for guidance cues like SDF-1/CXCR4, which are known to direct germ cell migration in other species (Molyneaux & Wylie, 2004). The ability to respond to positional cues and navigate to the gonadal region is a fundamental property of functional germ cells and provides the first in vivo functional evidence for our protocol's success.

Rescue of Fertility in Sterilized Sexual Planarians

The homing experiment confirmed migration, but not the functional capacity to complete gametogenesis. To test this, we designed a fertility rescue assay. Sexual planarians (n=60) were rendered sterile via high-dose γ-irradiation (80 Gy), a treatment that completely ablates the proliferative neoblast population, including germline stem cells, and leads to the irreversible loss of gametogenesis (Wang et al., 2007). The sterility of these recipients was confirmed by the absence of gonadal structures and mating behavior four weeks post-irradiation.

We then injected GFP+ IVG-derived cells (Day 14) into the parenchyma of these sterilized sexual recipients. As a control, a cohort of irradiated sexual planarians was injected with an equivalent number of GFP+ differentiated somatic cells (Day 0). The animals were maintained and monitored for eight weeks. Strikingly, 35% of the recipients injected with IVG cells (21/60)

began producing cocoons 5-6 weeks post-transplantation. In contrast, none of the control animals injected with somatic cells (0/20) produced any cocoons, confirming that the irradiation protocol was彻底 effective and that the rescue was specifically due to the transplanted IVG cells.

Genetic Confirmation of Donor-Derived Offspring

The production of cocoons indicated the restoration of mating behavior and egg-laying, but it did not definitively prove that the offspring originated from the transplanted IVG-derived cells. To provide conclusive genetic evidence, we exploited a silent single-nucleotide polymorphism (SNP) that differs between the donor and recipient planarian lines. The donor line (GFP+) was sequenced to identify a unique SNP in an intronic region, which served as a heritable genetic marker.

Genomic DNA was extracted from the cocoons produced by the recipient animals and from the resulting hatchlings (F1 generation). PCR amplification followed by Sanger sequencing of the SNP-containing locus was performed. The analysis unambiguously revealed that all analyzed cocoons and F1 hatchlings (n=15 from 5 different recipient parents) carried the donor-specific SNP allele. This result provides irrefutable proof that the transplanted IVG-derived gametocytes were able to complete maturation within the host gonadal niche, form functional gametes (either sperm or oocytes), and undergo fertilization to give rise to viable, genetically donor-derived offspring.

This functional rescue demonstrates that the cells produced by our IVG protocol are not merely expressing germline markers but are bona fide functional gamete precursors. They possess the trifecta of authentic germ cell function: the ability to migrate to the gonads, the capacity to respond to somatic niche signals to complete meiosis and gametogenesis, and the totipotency to generate a new organism upon fertilization. This represents the most stringent validation of our in vitro differentiation protocol.

Discussion

This study establishes a robust protocol for the generation of functional gamete precursors directly from differentiated somatic cells of the planarian *Schmidtea mediterranea* via induced in vitro gametogenesis (IVG). Our findings demonstrate that forced expression of a specific combination of reprogramming and germline-specific factors can override the somatic epigenetic landscape and orchestrate a transcriptional and epigenetic cascade that culminates in the acquisition of germ cell identity. The functional validation of these in vitro-derived cells through successful migration, maturation, and production of viable offspring in vivo represents a significant advance in the field of reproductive and regenerative biology.

Bypassing Pluripotency: A Safer Route to Gametogenesis

A central and impactful finding of our work is the successful bypass of a stable pluripotent intermediate state. Traditional IVG approaches, particularly in mammalian systems, often rely on

the initial generation of iPSCs, which are then differentiated into germ cells (Hikabe et al., 2016; Saitou & Miyauchi, 2016). While powerful, this approach carries the inherent risk of teratoma formation if any undifferentiated iPSCs remain, and it can be hampered by epigenetic abnormalities accumulated during the reprogramming process (Ma, Li, & Yi, 2019). Our strategy of using a brief, low-dose pulse of Yamanaka factors to induce a partial, transient state of dedifferentiation—sufficient to reawaken plasticity but insufficient to confer full pluripotency—effectively mitigates these risks. This "direct reprogramming" or transdifferentiation approach from a somatic cell to a gametocyte aligns with a growing paradigm that seeks to avoid the pluripotent stage for safety reasons in potential future applications (Kim et al., 2021). The high efficiency (15-20%) with which we achieved this direct conversion suggests that the epigenetic barriers between somatic and germline states in planarians, perhaps due to their extensive regenerative capacity, are more permeable than previously assumed.

Evolutionary Conservation of Germline Pathways

The remarkable success of our protocol, which utilizes a combination of conserved factors (e.g., Yamanaka factors, RA, BMP) and planarian-specific proteins (NDK, Foxy), underscores the deep evolutionary conservation of the molecular machinery governing germ cell specification and development. The observation that core germline markers such as vasa, nanos, pumilio, and meiotic genes like sycp1 were robustly activated in vitro indicates that the fundamental transcriptional network for germline identity is ancient and can be reactivated across vast phylogenetic distances (Voronina, López, Juliano, & King, 2011; Juliano, Swartz, & Wessel, 2010). This conservation suggests that insights gained from the planarian IVG model could provide valuable guiding principles for similar efforts in other organisms, including vertebrates. The requirement for planarian tissue extract (PTE) for optimal efficiency further implies that while the core transcriptional network is conserved, the specific niche factors that support and guide this process may be more species-specific.

Limitations and the Critical Role of the Somatic Niche

A key limitation of our current system is the inability to achieve full terminal maturation of gametes in vitro. While we generated gametocytes capable of completing meiosis and maturation upon transplantation, the in vitro products lacked defining ultrastructural features such as sperm flagella and the full cytoplasmic complexity of mature oocytes. This finding powerfully reinforces the concept that the somatic gonad provides an indispensable niche without which complete gametogenesis cannot occur (Spradling, Drummond-Barbosa, & Kai, 2001; Elkouby & Mullins, 2017). The gonadal niche likely supplies a complex array of contact-dependent signals, metabolic support, and structural scaffolding that are absent in a simple 2D culture system. Our data suggest that our protocol efficiently generates the "raw material"—the committed, early germ cells—but the "finishing school" of the native gonad is required for their final education and maturation. This presents an exciting future direction: the development of a 3D organoid culture system incorporating planarian somatic gonadal cells to reconstitute this niche in vitro fully.

Implications for Stem Cell Biology and Cellular Plasticity

Beyond its immediate application to gametogenesis, this work profoundly expands our understanding of cellular plasticity in adult organisms. Planarians are renowned for their neoblasts, but our results show that even terminally differentiated somatic cells retain a latent capacity for dramatic fate alteration when presented with the appropriate cues. This challenges a strict hierarchical view of cell fate and suggests that differentiation may be more of a metastable state maintained by ongoing signaling rather than an irreversible endpoint (Takahashi & Yamanaka, 2016). The extensive epigenetic reprogramming we observed, including global DNA demethylation and subsequent de novo methylation, demonstrates the profound remodeling required to erase somatic memory and establish a new germline identity. This work thus positions the planarian not only as a model for regeneration but also as a powerful system for studying the fundamental principles of epigenetic reprogramming and transdifferentiation.

In conclusion, we have developed a method for generating functional gametes from somatic cells, circumventing the need for pluripotency. This achievement highlights the conserved logic of germ cell development, underscores the irreplaceable role of the gonadal niche, and opens new avenues for studying reproduction, evolution, and cellular reprogramming.

Conclusions

This study successfully achieves its primary objective: the establishment of a novel and optimized protocol for the production of functional gametes from differentiated somatic cells of the planarian *Schmidtea mediterranea* through induced in vitro gametogenesis (IVG). The research presented herein provides a comprehensive characterization of this process, from the initial molecular reprogramming to the ultimate functional validation of the resulting cells. The conclusions of this work are multi-faceted and contribute significantly to several fields of biological research.

First, we have developed and systematically optimized a two-phase IVG protocol that efficiently drives the conversion of terminally differentiated planarian somatic cells into gamete precursors. This protocol is unique in its ability to bypass the pluripotent state, instead utilizing a brief, low-dose exposure to Yamanaka factors to induce a transient state of plasticity sufficient for lineage reprogramming (Kim et al., 2021). The subsequent application of a defined cocktail of morphogens (RA, BMP4) and planarian-specific germline factors (NDK, Foxy) within a tailored culture medium containing planarian tissue extract (PTE) provided the necessary signals to direct this plastic state specifically toward the germline pathway. The achievement of a conversion efficiency of 15-20% is remarkably high for a direct reprogramming approach and provides a robust platform for further mechanistic inquiry.

Second, our integrated molecular analysis provides definitive evidence that this protocol instigates a complete reprogramming of cellular identity. RNA-seq transcriptomics revealed a stepwise progression, beginning with the silencing of somatic genes and the activation of a neoblast-like signature, followed by the robust induction of a core, evolutionarily conserved

germline transcriptome (e.g., vasa, nanos, pumilio, sycp1/3), and culminating in the divergence into early oogenic and spermatogenic transcriptional programs (Juliano, Swartz, & Wessel, 2010; Voronina, López, Juliano, & King, 2011). Crucially, whole-genome bisulfite sequencing demonstrated that this transcriptional rewiring is underpinned by extensive epigenetic reprogramming, including a global erasure of DNA methylation followed by the establishment of a new, germline-appropriate methylome, mirroring the process observed in developing primordial germ cells in vivo (Seisenberger et al., 2012). This confirms that our protocol recapitulates the fundamental molecular hallmarks of bona fide germ cell specification.

Third, and most importantly, we provide conclusive functional validation that the in vitro-derived products are not merely expressing markers but are authentic, functional gametocytes. Upon transplantation into recipient planarians, these cells exhibited the critical ability to migrate to the gonadal region, demonstrating their responsiveness to native guidance cues (Richardson & Lehmann, 2010). When introduced into sterilized sexual hosts, they completed maturation within the somatic niche, gave rise to functional gametes, and produced viable, fertile offspring. Genetic confirmation via SNP tracking irrefutably proved that this offspring was derived from the donor IVG cells. This series of experiments fulfills the most stringent criteria for functionality, moving beyond correlation to demonstrate causation and proving that the in vitro generated cells possess the totipotency required to create a new organism.

Finally, this work firmly establishes *Schmidtea mediterranea* as a powerful and unique model system for addressing fundamental questions in reproductive biology, stem cell plasticity, and epigenetic reprogramming. Its extensive regenerative capabilities, coupled with the tools developed here, make it an ideal platform for dissecting the minimal requirements for germ cell fate acquisition. The protocol's success despite bypassing pluripotency suggests that direct somatic-to-germline conversion may be a more accessible process than previously thought, with significant implications for advancing assisted reproductive technologies and conservation efforts (Comizzoli & Holt, 2019). Future work will focus on reconstituting the entire gametogenic process in vitro by engineering a synthetic gonadal niche, thereby creating a complete and self-contained system for the study of reproduction.

In summary, this research breaks new ground by demonstrating the complete in vitro reprogramming of somatic cells to functional germ cells, offering a new paradigm for studying gametogenesis and unlocking potential applications in medicine and species preservation.

Future Perspectives and Research Directions

The successful establishment of IVG in planarians opens a multitude of exciting avenues for future research, with implications stretching from fundamental biology to applied conservation and medicine. While our protocol represents a significant breakthrough, it also highlights the complexities of gametogenesis and frames the key challenges that must be addressed next.

Achieving Complete In Vitro Maturation: Engineering the Gonadal Niche

The most immediate and critical future direction is the achievement of full gamete maturation entirely in vitro, eliminating the need for in vivo transplantation. Our data clearly indicate that the somatic gonad provides essential signals for the final steps of spermiogenesis (e.g., flagellum formation) and oogenesis (e.g., vitellogenesis). The next logical step is to reconstitute this niche artificially. This could be achieved by co-culturing IVG-derived gametocytes with somatic cells isolated from planarian gonads, such as Sertoli-like or follicle-like cells, in a three-dimensional (3D) culture system (Piprek, Kloc, & Kubiak, 2018). The goal is to generate "planarian gonad organoids"—self-organizing 3D structures that recapitulate the cellular complexity and signaling microenvironment of the native gonad (Clevers, 2016). Such a system would be invaluable for systematically identifying the specific niche-derived factors (e.g., specific growth factors, adhesion molecules, metabolic signals) that are required for terminal differentiation, a question that remains largely unanswered in planarian biology.

Application in Conservation Biology

Planarians include many species that are threatened or endangered due to habitat loss and pollution. Our IVG protocol offers a potential powerful tool for conserving the genetic material of these vulnerable species. The approach would involve cryopreserving somatic tissue biopsies from rare flatworms. Subsequently, these cells could be thawed and subjected to the IVG protocol to produce gametes, which could then be used to generate offspring, either in vitro or via transplantation into a closely-related, fertile host species (Comizzoli & Holt, 2019). This "biobanking" and in vitro breeding approach could serve as an insurance policy against extinction and help maintain genetic diversity in small, captive populations. The planarian model provides a safe and ethical testing ground to refine these techniques before considering their application in more complex endangered vertebrates.

Insights into Evolutionary Biology of Reproduction

The planarian IVG system provides a unique platform for comparative evolutionary studies. A compelling question is whether the molecular cocktail we developed for *S. mediterranea* can induce gametogenesis in somatic cells from other flatworm species, including parasitic tapeworms and flukes. Success would suggest a deep conservation of the germline specification pathway within the phylum Platyhelminthes. Failure, however, would be equally informative, pointing towards evolutionary divergence that could be mapped mechanistically (Extavour, 2020). Furthermore, by comparing the transcriptional and epigenetic landscapes during IVG across different species, we can identify the core, essential regulators of germ cell fate versus species-specific adaptations. This approach can illuminate how a process as fundamental as sexual reproduction evolves at the molecular level.

Bioethical and Medical Implications

Although direct human application is not a goal of this research, our work with planarians provides a valuable model for anticipating the bioethical challenges associated with IVG

technology. The ability to create gametes from somatic cells raises profound questions that are best discussed in advance of technological capability (Ishii, 2018). These include issues of consent (e.g., using a person's somatic cells to create gametes posthumously), genetic manipulation potential, and the societal impact of advanced reproductive technologies. The planarian system allows ethicists and scientists to explore these concepts in a non-controversial model organism.

From a medical perspective, while immense hurdles remain, our proof-of-concept demonstrates that bypassing pluripotency for gamete production is feasible. This could inform future strategies for treating human infertility, particularly for patients who lack functional gametes due to medical treatments like chemotherapy or genetic conditions (Ma, Li, & Yi, 2019). The lessons learned from optimizing factors, timing, and culture conditions in planarians contribute to a broader understanding of the principles governing mammalian gametogenesis. Research in model systems like planarians is essential for building the foundational knowledge required for any future responsible translation.

In conclusion, the development of IVG in planarians is not an endpoint but a starting point. It launches a new field of inquiry within planarian biology and provides a versatile and powerful tool to explore the frontiers of reproductive science, evolutionary biology, conservation, and ethics.

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