Retaining pluripotency and exogenous mRNA introduction in planarian stem cell culture

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Abstract:

Planarians possess naturally occurring pluripotent adult somatic stem cells (neoblasts) required for homeostasis and whole-body regeneration. However, no reliable neoblast culture methods are currently available, hindering mechanistic studies of pluripotency and the development of transgenic tools. We report the first robust methods for neoblast culture and delivery of exogenous mRNAs. We identify optimal culture media for maintaining neoblasts \textit{in vitro}, and show via transplantation that cultured stem cells retain pluripotency for two days. We developed a new procedure that significantly improves neoblast yield and purity by modifying standard flow cytometry methods. These methods enable the introduction and expression of exogenous mRNAs in neoblasts, overcoming a key hurdle impeding the application of transgenics in planarians. The advances in cell culture reported here create new opportunities for mechanistic studies of planarian adult stem cell pluripotency, and provide a systematic framework to develop cell culture techniques in other emerging research organisms.
Introduction

While the control of pluripotency in animals has been examined in the germline, embryonic, and induced pluripotent stem cells, no naturally occurring adult pluripotent stem cells have yet been identified in the roundworm, fly, fish, or rodent model systems (Boiani and Scholer, 2005; Evans and Kaufman, 1981; Martin, 1981; Takahashi and Yamanaka, 2006). By contrast, planarian flatworms and acoels uniquely harbor an adult stem cell population, collectively termed neoblasts (Gehrke and Srivastava, 2016), which includes a pluripotent subpopulation of clonogenic neoblasts (Baguñà et al., 1989; Reddien and Sanchez Alvarado, 2004; Wagner et al., 2011) that enable whole-body regeneration and apparently limitless capacity for tissue homeostasis. The freshwater species *Schmidtea mediterranea* is a model for planarian development, regeneration, and, in particular, pluripotency in long-lived adult animals (Elliott and Sanchez Alvarado, 2013). While regulators of pluripotency have been identified in neoblasts and studied using the RNA interference (Labbe et al., 2012; Lei et al., 2016; Wagner et al., 2012), a lack of reliable culture methods and transgenesis limit *in vivo* exploration of adult stem cell pluripotency (Wudarski et al., 2017). Indeed, published commentary has called for establishing reliable, standardized neoblast culture methods as an essential step in developing transgenic studies of planarians.

While genetic transformation strategies typically exploit early-stage embryos or cultured stem cells (Bottger et al., 2002; Fire, 1986; Gossler et al., 1986; Jaenisch and Mintz, 1974; Renfer et al., 2010; Robertson et al., 1986; Rubin and
Spradling, 1982), planarians instead reproduce asexually through neoblast proliferation and differentiation (Newmark and Sanchez Alvarado, 2000). These cells can be transplanted into hosts lacking neoblasts (e.g., after lethal irradiation) to repopulate stem cells and rescue the host within one month of the irradiation (Baguñà et al., 1989; Wagner et al., 2011). Thus, transforming DNA or RNA into neoblasts before transplantation could produce transgenic planarians and subsequently enable significant breakthroughs in understanding the control of pluripotency in animals. However, despite numerous efforts, no reports currently describe the thriving culture of neoblasts or genetic modification of these animals. Based on the tremendous potential for planarian neoblasts to fill significant gaps in our understanding of regeneration in higher animals, we aimed to establish a robust culture method for pluripotent neoblasts that also enables efficient screening for successful delivery and transgenic expression of exogenous DNA or RNA.

Results

KnockOut DMEM with 5% CO₂ can maintain the pluripotency of neoblasts

in vitro

To establish standardized neoblast culture conditions, we first used an established back-gating method for flow cytometry sorting of X1(FS) cells, which typically contain approximately 23.4%±2.5% neoblasts (smedwi-1+) (Fig. 1a-c). Testing both ambient atmosphere and 5% modified CO₂ conditions, we systematically screened 23 different types of media, including several
commercially available mammalian and insect cell culture media, previously reported formulations (e.g., IPM and TPP), and dilutions of these media that better match osmolarity suitable for planarian cells (~120 mOsm/kg) (Supplementary Table 1) (Asami et al., 2002; Schürmann and Peter, 2001).

To measure viability, cells cultured for one day were observed and stained with propidium iodide (PI), which labels the DNA of dead cells. We determined the percentage of PI negative cells by flow cytometry. Cells cultured in CMFB with or without 5% CO₂ modification displayed irregular cell surface morphologies accompanied by sizeable cellular debris, suggesting poor viability (Fig. 1d). Consistent with microscopic evaluation, cells cultured in CMFB showed poor survival with or without 5% CO₂ modification (>60% dead cells) (Supplementary Fig. 1a). In contrast, cells in all other conditions, such as IPM with or without 5% CO₂, had normal morphology, suggesting high viability (Fig. 1d). Among all media conditions, seventeen formulations yielded viability higher than 60% (Supplementary Fig. 1a). Notably, cells in Leibovitz’s L-15 medium (L15) without 5% CO₂ extended long protrusions that were visible even after six days of culture (Supplementary Fig. 1b), which suggested the occurrence of neuronal differentiation, as previously observed in cultured *Caenorhabditis elegans* embryonic cells (Christensen et al., 2002).

To determine the proportion of neoblasts among total viable cells after 24 hours of culture, we quantified the number of *smedwi-1*+ X1(FS) cells by fluorescent in situ hybridization (FISH). Notably, seven media with 5% modified CO₂ atmosphere maintained significantly more *smedwi-1*+ neoblasts than all
other conditions, including diluted (d) Grace’s medium, IPM, KnockOut DMEM, dL15 medium, dKnockOut DMEM, dSchneider’s medium, and dDMEM (Fig. 1f). This result was supported by co-staining cells with smedwi-1 and the apoptotic/dead cell marker, Annexin V, which showed no detectable co-labeling, indicating that the neoblasts were viable (Supplementary Fig. 1c). We next examined whether smedwi-1+ neoblasts persisted after three days in culture using these seven media + 5% CO2, and observed that smedwi-1+ cells were present in all culture conditions tested here (Supplementary Fig. 1d). Thus, these results showed that neoblasts could be maintained for at least three days in vitro. We therefore focused on testing dGrace’s, IPM, KnockOut DMEM, dL15, dKnockOut DMEM, dSchneider’s, and dDMEM media in subsequent optimization experiments.

Next, we assessed whether cultured neoblasts could divide in vitro. Although we did not observe any noticeable increase in cell number, low levels of both symmetric and asymmetric neoblast divisions were observed in cells cultured for one day, as determined by cell pair size and distribution of smedwi-1 transcripts (Fig. 1f) (Lei et al., 2016). Time-lapse microscopy imaging of X1(FS) cell behavior confirmed that neoblasts could divide in vitro. Both symmetric and asymmetric cell divisions were observed within the first 24 hours in IPM, KnockOut DMEM, and dL15 medium, but not in the other four media tested (Fig. 1g and Supplementary Movies 1 and 2). PCNA+ staining assays further showed that the proliferating cells in IPM, KnockOut DMEM, and dL15 medium were significantly more than those in CMFB, Schneider’s, and DMEM medium
(Supplementary Fig. 1e). Although we cannot exclude the possibility that these conditions only allow neoblasts in the M phase to complete the cell cycle, to our knowledge, this is the first time that neoblast divisions have been observed and recorded *in vitro*. These results suggested that a fraction of X1(FS), *smedwi-1*+ cells can complete cell division within 24 hours after isolation in culture.

To determine if X1(FS) neoblasts can divide *in vivo* following *in vitro* culture, we transplanted X1(FS) cells cultured in the seven different media supplemented with 5% CO₂ for one, two, or three days. At eight days post-transplantation (dpt), the presence or absence of *smedwi-1*+ neoblast colonies and the number of *smedwi-1*+ neoblasts in each colony were determined. All X1(FS) neoblasts cultured for one or two days efficiently proliferated *in vivo*, except for those cultured in dGrace’s medium with 5% CO₂ (Fig. 1h, i and Supplementary Fig. 2c). By comparing the number of *smedwi-1*+ neoblasts in each transplantation, we found that X1(FS) cells cultured for one day in either IPM or KnockOut DMEM formed the largest colonies *in vivo* (Fig. 1i). In summary, cells grown in IPM and KnockOut DMEM performed best following one day of culture, but performed similarly to those grown in d KnockOut DMEM, d Schneider’s, dL15, and d DMEM after two days of culture. In addition, the clonogenic capacity of X1(FS) neoblasts diminished considerably following three days in culture, regardless of the medium used. These results suggested that IPM, KnockOut DMEM, dL15, d KnockOut DMEM, d Schneider’s, and d DMEM could all maintain neoblast proliferation potential for up to two days in culture in the presence of 5% CO₂.
To evaluate the functional pluripotency of neoblasts cultured in these six media (IPM, KnockOut DMEM, dKnockOut DMEM, dL15, dSchneider’s, and dDMEM), we assessed their ability to rescue lethally irradiated hosts following bulk-cell transplantation. Transplantation of non-cultured, freshly collected X1(FS) cells resulted in the rescue of 30–50% of the lethally irradiated (6,000 rads) sexual S. mediterranea hosts (Fig. 1j). X1(FS) cells cultured in IPM, dL15, or KnockOut DMEM for one or two days could also rescue hosts that were depleted of stem cells (Fig. 1j). Genotyping PCR and restriction fragment length polymorphism (RFLP) assays were conducted to test whether sexual hosts had been transformed into the asexual biotype following transplantation with asexual neoblasts (Supplementary Fig. 2d-g) (Wagner et al., 2011). Among the transplanted cultures, cells grown in KnockOut DMEM exhibited the highest and most robust host rescue (Fig. 1j and Supplementary Fig. 2h). In summary, KnockOut DMEM with 5% CO₂ represented the most stable conditions for maintaining pluripotent neoblasts in culture for two days. Neoblasts grew in IPM and dL15 medium also retained their pluripotency for up to two days in culture, albeit with reduced rescue rates in irradiated host animals after transplantation.

SirNeoblasts as an alternative source of transplantable neoblasts for primary culture

To enrich neoblasts for culture, we tested three major types of cell-permeable DNA stains to enrich neoblasts in the G2/M cell cycle phases (DRAQ5, Vybrant DyeCycle, and SiR-DNA). The DNA stain, SiR-DNA, exhibited low cytotoxicity and resulted in ~60% enrichment for smedwi-1+ neoblasts (Fig. 2a, f, g, and
Supplementary Fig. 3a-c) (Lukinavicius et al., 2015). Comparison of smedwi-1+ and smedwi-1- cell morphology in the isolated populations showed that smedwi-1+ cells were generally larger than smedwi-1- cells (Fig. 2b). To discriminate between small and large cells in the SiR-DNA+ population, the cytoplasmic dyes Cell Tracker Green (CT) and Calcein AM (CAM) were tested in combination with SiR-DNA for neoblast isolation (Fig. 2c, d). This dual dye staining strategy resulted in a significant increase in neoblast enrichment, indicated by the proportion of smedwi-1+ cells in FISH assays (Fig. 2e, f). In particular, SiR-DNA/CT co-staining showed comparable performance to Hoechst 33342 for enriching smedwi-1+ neoblasts (Fig. 4f), which we designated SirNeoblasts, as described in our previous publication (Niu et al., 2021).

Unlike neoblasts obtained by Hoechst 33342 sorting, SirNeoblasts proliferated and underwent colony expansion in vivo after transplantation into lethally irradiated planarians (Fig. 2g). Importantly, no noticeable differences in colony size were observed at 7 dpt among unstained X1(FS), single (SiR-DNA)-, or double (SiR-DNA/CT)-stained populations (Fig. 2g). Recently, a single-cell RNA sequencing study reported similarities between SirNeoblasts and X1 cells, supporting that SirNeoblasts could serve as an alternative cell source for functional studies of the neoblasts (Niu et al., 2021). To characterize the proportions of SirNeoblasts in different cell cycles, we stained these cells with Hoechst 33342. Since co-staining with Hoechst and SiR-DNA blocked the SiR-DNA signal, we instead used Hoechst staining in SiR-DNA-sorted neoblasts and found that ~17.89% of SirNeoblasts were in the G1, 13.02% at S, and ~69.09%
at G2/M cell cycle phases. (Supplementary Fig. 3d-g). This reversible, low-affinity DNA staining by SiR-DNA could explain why SirNeoblasts can proliferate after staining, while Hoechst 33342-stained X1 cells cannot (Bucevicius et al., 2018).

Facilitated by SiR-DNA staining, we then observed the chromosomal separation dynamics of dividing SirNeoblasts in vitro (Supplementary Movies 3-5), which confirmed the occurrence of bona fide cell division under the tested culture conditions. Both freshly isolated SirNeoblasts and those cultured for one day in KnockOut DMEM with 5% CO2 could rescue lethally irradiated planarians at comparable rates to those reported for X1(FS) cells (Fig. 2h). We found that ~27.2% of SirNeoblasts express tgs-1, a recently reported planarian pluripotent stem cell marker (Zeng et al., 2018), for as long as two days in KnockOut DMEM with 5% CO2 (Supplemental Fig. 4a). In addition, we observed no positive effects of co-culturing differentiated X1(FS) cells with SirNeoblast (Supplemental Fig. 4b). Based on these findings, we concluded that SiR-DNA/CT dual label-based cell sorting could be used to isolate clonogenic, pluripotent neoblasts. Moreover, these isolated SirNeoblasts can be maintained in primary culture and serve as donor cells in transplantation assays.

**Exogenous mRNA delivery by electroporation**

Following the optimization of in vitro culture conditions to maintain neoblast pluripotency, we next tested different conditions for the delivery of exogenous (nucleic acid) molecules into neoblasts to attempt the genetic transformation of planarians. To this end, we first used dextran-FITC as a fluorescent indicator of membrane permeation to screen for the most suitable electroporation conditions
of Hoechst 33342-stained neoblasts (Supplemental Fig. 5a). We tested 52 electroporation programs and 10 different buffers using X1 cells (Hayashi et al., 2006; Reddien et al., 2005) and found that dextran-FITC was most effectively delivered into neoblasts using IPM buffer with electroporation at 100-120V (Supplementary Table 2 and Supplemental Fig. 5b-d). Similarly, applying this electroporation method to X1(FS) cells rather than Hoescht 33342-sorted X1 cells showed that dextran-FITC+ populations could only be detected following electroporation at 110V and 120V. However, less than 6% of dextran-FITC+ X1(FS) cells were smedwi-1+ neoblasts, and virtually no smedwi-1+ cells could be detected after one day of culture in KnockOut DMEM with 5% CO₂ (Supplemental Fig. 5e). Consistent with the drastic reduction in smedwi-1+ cell viability post-electroporation, none of the donor X1(FS) cell populations subjected to more than 100V formed colonies following transplantation into lethally irradiated donors (Supplemental Fig. 5f). We hypothesized that this failure was likely due to the low proportion of smedwi-1+ neoblasts in total X1(FS) cells, which was even further reduced after electroporation.

Based on these findings, we then sought to identify the optimal electroporation conditions to retain the viability of SirNeoblasts following the introduction of foreign genetic material (Fig. 3a). Consistent with previous experiments, electroporation at 110V-120V was required for dextran-TMR internalization into SirNeoblasts (Fig. 3b, c). Different from that in X1(FS), smedwi-1+ cells were more abundant in the electroporated (110V and 120V) SirNeoblasts compared to X1(FS) cells, and the electroporated SirNeoblasts persisted for one day in culture.
(Fig. 3d). In addition, the electroporated SirNeoblasts were capable of forming colonies and rescuing lethally irradiated hosts upon transplantation (Fig. 3e, f).

However, 120V electroporation resulted in SirNeoblast rescue of relatively fewer irradiated hosts, suggesting that high voltages may negatively impact SirNeoblast viability.

To assess whether exogenous mRNA could be delivered into SirNeoblasts by electroporation, \textit{tdTomato} mRNA was added to the electroporation reaction along with Dextran-FITC. Dextran-FITC-positive SirNeoblasts were sorted and cultured in KnockOut DMEM with 5% CO$_2$. To confirm the successful delivery of mRNA, we probed cells via FISH at 20 hours after electroporation and found detectable \textit{tdTomato} mRNA signal in cells electroporated at either 110V or 120V (Fig. 3g). However, co-staining with \textit{smedwi-1+} revealed that not all \textit{tdTomato} mRNA+ cells retained neoblast identity in culture. The number of SirNeoblasts positive for both \textit{tdTomato} mRNA and \textit{smedwi-1} expression was significantly higher after 110V electroporation than after 120V, which was a similar response to electroporation to that observed in X1 and X1(FS) cells (Fig. 3h). These findings indicated that 110V electroporation was the most suitable condition for introducing exogenous, charged molecules such as RNA into neoblasts, while maintaining their viability and pluripotency.

Unfortunately, \textit{tdTomato} expression was undetectable by either microscopy or antibody staining in cultured neoblasts. This effect was likely due to two potential contributing factors: 1) The culture conditions did not support the translation of the delivered mRNA, or 2) an unknown mechanism prevented...
translation of the tdTomato mRNA (e.g., the sequence was not codon-optimized).

In *C. elegans*, endogenous piRNAs can target exogenous transgene sequences and prevent their translation (Zhang et al., 2018). Similarly, planarian neoblasts contain abundant PIWI proteins and piRNAs, thus hypothesizing that a similar piRNA targeting mechanism could be present in planarian neoblasts, potentially blocking exogenous mRNA translation. Since the tdTomato sequence contains two copies of the dTomato gene, predicting piRNA target sequences for this reporter can be complicated. Thus, to test our hypothesis, we synthesized several mRNAs encoding the mCherry fluorescent protein harboring synonymous nucleotide substitutions that were designed to minimize potential pairing between the exogenous mRNA and endogenous piRNAs, as described in *C. elegans* (Supplemental Fig. 6a-c) (Zhang et al., 2018). The synthetic mCherry mRNAs were electroporated into SirNeoblasts (Supplemental Fig. 6d), and indeed one of the mCherry mRNA constructs resulted in mCherry+ cultured SirNeoblasts (Supplemental Fig. 6e-h). Although the mechanisms underpinning piRNA targeting in neoblasts remain poorly understood, we found that this mCherry sequence contained the fewest piRNA matches among the 15 mRNAs tested here (Supplemental Fig. 6b, c). However, numerous repeated experiments showed that successful translation was not robustly reproducible: that is, we observed high expression only twice, five times with low expression, and more than ten times without expression. Although we cannot exclude the possibility of an artificial autofluorescent signal, the low expression efficiency of mCherry
suggested that further optimization to culture conditions and mRNA sequence was necessary for reliable transformation.

*Nanoluciferase* mRNA delivered by TransIT can be successfully expressed in differentiating SirNeoblasts

A recent study reported that *Nanoluciferase* (*NanoLuc*) mRNA could be expressed in somatic planarian cells through Viromer or TransIT transfection (Hall et al., 2021). We therefore suspected that the high sensitivity and low autofluorescence background of the NanoLuc reporter could provide a tractable approach for visualizing neoblast transgene expression. To confirm that *NanoLuc* was indeed translated in cultured SirNeoblasts, we transfected SirNeoblasts with *NanoLuc* mRNA using the TransIT system (Fig. 4a). Culture medium supplements (sodium pyruvate, vitamin, and amino acids, see Supplemental Table 1) were also included in the modified KnockOut DMEM to determine whether they could enhance the mRNA expression (Hall et al., 2021). We found that NanoLuc expression levels were higher in SirNeoblasts cultured in modified KnockOut DMEM with 5% CO₂ modified atmosphere than in cells grown under the same conditions without supplements (Fig. 4b). To confirm that neoblasts could also be maintained in modified KnockOut DMEM with 5% CO₂, SirNeoblasts were stained and their proportion was compared to that of *smedwi-1*+ cells after one or three days of culture in modified KnockOut DMEM with 5% CO₂, KnockOut DMEM with 5% CO₂, and Iso-L15 under ambient conditions. The results showed that SirNeoblasts could be maintained on modified Knockout DMEM as well as the Knockout DMEM, but not the Iso-L15 (Fig. 4c, d), which
combined with earlier findings that higher CO₂ was required for consistently high SirNeoblast activity (Fig. 1e), led us to use modified KnockOut DMEM with 5% CO₂ modification in subsequent experiments. We next sought to compare the efficiency of mRNA delivery between the TransIT system and electroporation and discovered that the NanoLuc signal was only detectable following TransIT transfection (Fig. 4e). This finding suggested that TransIT provided higher efficiency and more robust mRNA delivery into the neoblasts.

To confirm this methodology with other mRNAs, we used TransIT to deliver mRNA encoding NanoLuc, smed-histone3.3-2xflag, mCherry, or NanoLuc-mCherry. Surprisingly, none of these proteins were detectable by Western blot (Supplemental Fig. 7a-d). In addition, immunofluorescence staining of transfected SirNeoblasts could not provide a definitively positive signal because of autofluorescence or non-specific antibody binding in planarian cells (Supplemental Fig. 7e-g), although the nuclear-localized staining signals were only captured in smed-histone3.3-2xflag mRNA transfected cells (Supplemental Fig. 7g). Using chemiluminescence signal imaging, we observed that the ratio of NanoLuc+ cells was much lower in live planarian cells (2/630) than that in 293T cells (100/121) (Fig. 4f). Since luciferase chemiluminescence assays have extremely high purported sensitivity for detecting signals from a small proportion of positive cells, this meager ratio of NanoLuc+ cells may explain the success of the high sensitivity chemiluminescence, but the absence of immunofluorescent signals. However, NanoLuc-mCherry transcripts exhibited a measurably lower signal due to a relatively longer coding sequence for reduced transfection
efficiency, which suggested that the signal was indeed due to NanoLuc transcript expression (Fig. 4m).

To investigate if this low ratio was attributable to either a low transfection efficiency, a low translation efficiency, or a combination of issues, we next detected the presence of NanoLuc mRNA in SirNeoblasts after transfection. The transfection efficiency was unexpectedly high, with close to 92.03% of SirNeoblasts carrying the Nanoluc transcripts (Fig. 4g). Since silencing mechanisms to suppress exogenous gene expression have long been suspected as a confounding factor in neoblast transformation experiments, we checked whether NanoLuc+ cells retained their smedwi-1+ phenotype. The results clearly showed that all of the NanoLuc+ cells in our experiments were neither smedwi-1+ nor SMEDWI-1+ (Fig. 4h, i, and Supplemental Fig. 8) (note: CTG+ indicates whether cells were alive at the time of fixation). This phenomenon strongly suggested the function of an unknown mechanism in silencing the expression of exogenous mRNA in neoblasts. We compared NanoLuc levels in SirNeoblasts with that in bulk live cells to test this hypothesis. After confirming the temporal dynamics of Nanoluc expression by chemiluminescence (Fig. 4j), we also compared the signal between live bulk cells from non-irradiated and lethally-irradiated planarian populations. The results indicated that live bulk cells exhibited considerably higher NanoLuc signal than that in SirNeoblasts, even after depletion of neoblasts by lethal irradiation (Fig. 4k, l), which was consistent with the findings of Hall et al. ((Hall et al., 2021), personal communications). This result indicated that the Nanoluc signal was derived almost exclusively from
somatic cells, and further suggested that planarian neoblasts harbored a means of preventing exogenous nucleic acids before differentiation.

**Discussion**

The inability to genetically transform planarians has posed a long-standing obstacle to researching this otherwise highly versatile model for pluripotency and whole-body regeneration. The primary technical limitations underlying this obstacle involve determining the optimal culture conditions for maintaining pluripotent neoblasts and identifying an effective means for delivering exogenous nucleic acids into these cells. The cell culture system we have developed in this work resolves the former problem and enables further testing of strategies for exogenous material delivery, such as fluorescence-conjugated dextrans and mRNA, to ultimately demonstrate the translation of introduced mRNAs. Our method establishes the requisite foundation for developing transgenic and genome editing techniques in planarians to enable exciting new systematic investigations of naturally occurring pluripotent adult stem cell populations.

First, the use of SirNeoblasts ensures the purity and viability of neoblasts, thus allowing relative ease in screening transgene delivery strategies. Moreover, we propose that positively charged polymers, not limited to TransIT, could be used to deliver larger molecules and genome-editing tools with higher transfection efficiency and a higher likelihood of obtaining transgenic animals.

Second, the low efficiency of transfection and translation may also be due to cultured cells' relatively decreased metabolic activity. The observed enhancement of *NanoLuc* mRNA translation following the addition of
supplements suggested that uptake and translation of mRNA depended on meeting metabolic requirements in cultured neoblasts. The cell culture platform described here provides a reliable approach for identifying nutrient requirements by comparing cultured neoblasts with *in vivo* neoblasts. Adding supplements to culture media can also optimize long-term culture systems and cell lines, enabling downstream research of transformation techniques and functional validation of other genetic manipulations (e.g., CRISPR RNPs for genome editing) in cultured cells. Meanwhile, the mechanism why neoblast pluripotency maintenance requires 5% CO2 has remained to be investigated.

Third, given that neoblasts are the *de facto* units of selection in planarians and that the viability of these animals heavily depends on their proper function and viability, it is logical that these cells have evolved robust molecular mechanisms to protect their genome from disruption by foreign nucleic acids. In particular, small non-coding piRNAs are essential for safeguarding genome integrity by silencing transposable elements (Iwasaki et al., 2015). However, it is also known that many piRNAs do not target transposable elements in various animals, including mice, *C. elegans*, and planarians (Aravin et al., 2007; Palakodeti et al., 2008; Ruby et al., 2006). It is noteworthy that multiple foreign transgenes have been reported to undergo silencing in *C. elegans* germline cells (Kelly and Fire, 1998). More recent studies have shed light on piRNA function in *Drosophila* (DeLuca and Spradling, 2018), *C. elegans* (Shirayama et al., 2012; Zhang et al., 2018), and *S. mediterranea* (Kim et al., 2019; Kim et al., 2020). However, our understanding of piRNA function in planarians remains largely
incomplete. Planarians have at least three PIWI proteins (Palakodeti et al., 2008; Reddien et al., 2005), and PIWI-3 has been suggested to produce piRNAs that may potentially target exogenous nucleic acids (Kim et al., 2019; Kim et al., 2020). In the current study, we used this hypothesis of piRNA function to optimize codon usage in the mCherry reporter, although it remains unclear if this modification directly affected our results. Further experiments are necessary for definitive evidence supporting or refuting this hypothesis. However, if correct, optimizing codon usage to bypass piRNA targeting could enhance the efficiency of exogenously delivered DNA and mRNA translation.

In summary, we describe a novel FACS isolation strategy and primary cell culture conditions for maintaining clonogenic, pluripotent neoblasts in vitro compatible with transplantation, repopulation, and rescue of lethally irradiated hosts. In addition, we demonstrate the successful introduction of exogenous mRNAs into clonogenic, pluripotent neoblasts. Although further optimization is needed, this finding represents a significant technical milestone in developing protocols for generating transgenic planarians. Together with the findings of Hall et al., these results show that highly sensitive NanoLuc reporters can be robustly expressed in planarian cells. Interestingly, we found that cells labeled with traditional Hoechst 33342 staining, including X1, X2, and Xins, could not express NanoLuc, which supported the use of alternative means of obtaining neoblasts, such as SirNeoblasts and CRNeoblasts, in further efforts to optimize neoblast culture conditions (Supplemental Fig. 7i). Our results also strongly suggest the presence of a long-suspected silencing mechanism in planarian neoblasts for
suppressing exogenous gene expression, thus opening an avenue of further
study into this potentially novel mode of gene suppression and enabling the
genetic transformation of regenerative planarian populations.

Experimental Procedures

Planarian care and irradiation treatment
Asexual (Clone CIW4) and sexual (Clone S2F1L3F2) strains of *Schmidtea
*mediterranea* were maintained in Montjuïc water at 20°C as previously described
(Cebria and Newmark, 2005). Animals were starved for 7–14 days before each
experiment. Animals exposed to 6,000 rads of γ rays were used as transplant
hosts (Wagner et al., 2011). After transplantation, hosts were maintained in
Montjuïc water with 50 µg/ml of Gentamicin (GEMINI, 400-100P). For transplant
rescue experiments, host animals were kept in 3.5 cm Petri dishes (1 worm/dish),
and Montjuïc water was changed every 2–3 days.

Cell collection and culture
X1(FS) cells were collected as previously described with minor modifications
(Reddien et al., 2005; Wagner et al., 2011). Tails from planarians (>8 mm in
length) were macerated in Calcium Magnesium free buffer with 1% Bovine
Serum Albumin (CMFB) (see Recipe in Table S1) for 20–30 min with vigorous
pipetting every 3–5 min. After maceration, dissociated cells were centrifuged at
290g for 10min. Cells were then resuspended in IPM with 10% Fetal Bovine
Serum for either Hoechst 33342 or SiR-DNA staining. To gate the X1(FS) cells,
the X1 population from a control sample stained with 0.4 mg/ml Hoechst 33342
(ThermoFisher Scientific, H3570) was used to define the forward scatter/side scatter gate. To enrich neoblasts, DRAQ5 (ThermoFisher Scientific, 62254, 5µM), Vybrant DyeCycle Ruby stain (ThermoFisher Scientific, V10309, 10µM), and SiR-DNA (1µM, Cytoskeleton Inc., CY-SC007) were compared. To obtain SirNeoblasts, dissociated cells were stained with SiR-DNA and CellTracker Green CMFDA Dye (2.5µg/ml, Thermo Fisher Technologies, C7025) for 1 hour and 10 min sequentially. Cells were sorted with an Influx sorter using a 100-µm tip. For time-lapse imaging experiments, X1(FS) cells were incubated in either 5 mL of the indicated culture medium per well in 6-cm dishes (MatTek, P35G-1.5-14-C) or 1 mL of the indicated culture medium per well in a 24-well plate (MatTek, P24G-1.5-13-F). For other experiments, X1(FS) cells were incubated in 50 µL of the indicated culture medium per well in 384-well plates (Greiner bio-one, 781090). Cells were cultured in indicated media containing 5% Fetal Bovine Serum (Sigma-Aldrich, F4135) at 22ºC, +/- 5% CO₂. Dishes and multi-well plates were pre-coated with poly-D-lysine (50µg/ml, BD Biosciences).

**In situ hybridization and antibody staining**

Whole-mount *in situ* hybridization was carried out as previously described (King and Newmark, 2013; Lei et al., 2016; Pearson et al., 2009; Thi-Kim Vu et al., 2015). For ISH on cultured cells, cell culture plates were centrifuged in an Eppendorf horizontal centrifuge (Centrifuge 5810 R) at 300 g x 3 min. Cells were fixed with 3.7% formaldehyde (Sigma-Aldrich, F8775) or 4% paraformaldehyde (Electron Microscopy Sciences, 15710) for 20 min. After washing with 1× PBS, cells were hybridized with riboprobes at 56ºC for at least 15 h. After washing with
2× SSC and 0.2× SSC, cells were incubated with anti-digoxigenin-POD (Roche Diagnostics, 11207733910) or anti-fluorescein-POD (Roche Diagnostics, 11426346910) at room temperature for 2 h. After washing with 1× PBS/0.3% TritonX-100, the signal was developed using tyramide-conjugated Cy3 (Sigma-Aldrich, PA13101) or Cy5 (Sigma-Aldrich, PA15101).

Anti-phospho-Histone H3 (Ser10) (H3P) antibody (1:1,000, Abcam, ab32107) and Alexa 555-conjugated goat anti-rabbit secondary antibodies (1:1,000, Abcam, ab150086) were used to stain proliferating cells at the G2/M phase of the cell cycle.

**Annexin V staining**

Fifty microliters of cultured cells were re-suspended and stained with 2.5 µl of Annexin V FITC Conjugate (BioLegend, 640905) at room temperature for 15 min. After washing twice with IPM + 10% FBS, cells were subjected to *smedwi-1* ISH. After that, anti-fluorescein-POD (Roche Diagnostics, 11426346910) was used to stain Annexin V for apoptotic and dead cells detection.

**Cell transplantation**

X1(FS) cells collected by flow cytometry were transplanted into irradiated hosts (6,000 rads) as previously described with minor modifications (Wagner et al., 2011). Approximately 1 µL of an X1(FS) cell suspension (5,000 cells/µL) was injected into either the post-pharyngeal midline (of asexual CIW4 hosts) or the post-gonopore midline (of sexual S2F1L3F2 hosts) at 0.75–1.0 psi (Eppendorf FemtoJet) using a borosilicate glass microcapillary (Sutter Instrument Co., B100-75-15). Serial cell dilution experiments indicated that bulk cell transplantation of 1
X10^3 freshly collected X1(FS) cells resulted in colony expansion in ≥80% hosts (Supplementary Fig. 2a, b). Considering the rate of ~10% cell death in culture, we cultured 5 X 10^4 X1(FS) cells for each test condition to ensure that sufficient viable cells were available at the time of transplant.

**mRNA synthesis and electroporation**

According to the protocol, the capped mRNA with poly(A) tail was transcribed in vitro via mMESSAGE mMACHINE T7 ultra kit (ThermoFisher Scientific, AM1345). tdTomato mRNA was transcribed from the linearized plasmid pcDNA3.1(+)tdTomato. The PCR product used as a template was amplified by primers 5'-CAGATTAATAACGACTCCTATAGG-3' and 5'-ACTGATAATTACCCTCCTACAA-3'. The mRNA was purified by MEGAClearTM kit (ThermoFisher Scientific, AM1908).

Cells from four tail fragments of 8~10mm planarians were suspended in 20 µL electroporation buffers following Hoechast 33342 staining to screen electroporation conditions. 20 µg Dextran-FITC (ThermoFisher Scientific, D3306) were mixed with cells and loaded into a 1mm electroporation cuvette for BTX ECM830 electroporator or a 12-well electroporation strip for Lonza 4D electroporator. The buffer SE, SG, SF, P1-5 were electroporation buffers in Lonza Cell Line and Primary Cell 4D-Nucleofector Optimization kits (V4XC-9064 and V4XP-9096). Cell viability and electroporation efficiency were assessed using an Influx sorter.

For exogenous mRNA electroporation, ~1x10^8 cells were suspended in 50 µL IPM following SiR-DNA staining. 50 µg Dextran-FITC and ~5 µg mRNA were
mixed with cells and loaded into a 1mm electroporation cuvette. BTX ECM830 electroporator was used to apply a 110 V and 1-millisecond square wave pulse to deliver dextran-FITC and mRNA into planarian cells. Dextran-FITC+ SirNeoblasts were purified using an Influx sorter and cultured in KnockOut DMEM + 5%FBS.

**Microscopy and time-lapse imaging**

The Celigo imaging cell cytometer (Celigo, Inc.) and the Falcon 700 confocal microscope were used to take pictures of X1(FS) and SirNeoblasts following ISH. Celigo or ImageJ software was used for quantitative analyses. A Nikon Eclipse TE2000-E equipped with Perfect Focus and a Plan Fluor ELWD 20X/0.45 NA Ph1 objective was used to perform time-lapse imaging of cultured cells. Micro-manager was used to control the microscope and Hamamatsu Orca R2 CCD (Edelstein et al., 2014). Multiple positions were acquired at 5-min intervals for 24–48 h. *In situ* hybridization samples were imaged with a Nikon Eclipse Ti equipped with a Yokogawa W1 spinning disk head and a Prior PLW20 Well Plate loader. Several slides were prepared at once and then loaded and processed automatically using a combination of Nikon Elements Jobs for all robot and microscope control and Fiji for object-finding and segmentation. Slides were imaged at low magnification, and objects were identified before re-imaging tiled z-stacks using a Plan Apo 10X 0.5NA air objective. Tiled images were stitched, projected, and *smedwi-1*+ puncta were counted using custom macros and plugins in Fiji.

**Generation of optimized mCherry sequence**
mCherry candidate sequences were generated using a custom python script. Amino acid sequences were back-translated to 21 nucleotide sequences from 7 amino acid words at a time. Each potential nucleotide sequence was screened against a list of known piRNAs to generate the sequence with the fewest piRNA matches. A piRNA match consists of no more than a single G/T mismatch in the six nucleotide seed region (positions 2-7 of a piRNA) (Zhang et al., 2018). Additional G/T mismatches were scored as .5 and other mismatches as 1. Only the first 21 base pairs of the piRNAs were aligned. The highest scoring piRNA determined the score for that potential nucleotide sequence. The 21 nucleotide sequence with the lowest score was retained. The script was run with four alternate coding tables. The “all” coding table contained all possible codons for each amino acid., The “highestgc” contained only those codons with the most G or C nucleotides. All 15 modified mCherry sequences are included in Supplemental Table 3.

**Exogenous mRNA chemical transfection**

For the transfection of planarian live cells or SirNeoblasts, 1.5 μg mRNA was mixed with 2 μL mRNA Boost Reagent and 1 μL TransIT-mRNA Reagent (TransIT-mRNA Transfection Kit, MIR 2225) in culture medium without FBS. Finally, the mix was diluted into 225 μL culture medium after incubating for 3 min at room temperature. The 200,000 live cells or SirNeoblasts were sorted out (FACARia Fusion SORP) and centrifuged at 290 xg for 10 min to collect the cells. The cells were resuspended by 250 μL transfection mixture and incubated in 96-
well plate (WHB-96) for 24 h. For 293T cells, 0.09 μg mRNA was used to transfect as protocol recommended in 96-well plate.

**Luminescence detection assay**

The Nano-Glo Dual-Luciferase Reporter assay (Promega, N1610) was used to detect the expression of RPL15-NLuc mRNA. The cells suspended in 50 μL culture medium were mixed well with an equal volume of ONE-Glo™ EX Reagent and then transferred to C8 black Nunc 96-well plate (ThermoFisher Scientific, 446473) at room temperature. After incubation for 3 min, the lysate was mixed with 50 μL Stop & Glo reagent (ratio of substrate to buffer in 1:100) for luminescence detection. The Varioskan™ LUX multimode microplate reader (ThermoFisher Scientific, N16044) was used to measure the relative light unit of luminescence signals. The luminescence of each well was recorded three times with 1000-ms measuring time.

**Luminescence imaging**

The Leica DMi8 microscopy and Andor iKon-M 934 CCD camera were used to take pictures of luminescence from planarian cells and 293T cells with a 20x air objective (Leica, 506521). The 200,000 planarian live cells were resuspended in 50 μL culture medium and transferred to a glass-bottom dish (NEST, 801002) for imaging. The luminescence was measured after directly adding 1 μL Nano-Glo luciferase assay substrate (Promega, N1110) to cells. The exposure time was set to 20 s for luminescence imaging, and 1 s for bright field imaging.

**Western blot**
The 1000,000 planarian live cells were sorted out for transfection and protein extraction. Each protein sample of cells in 96-well plates were collected in PCR tube 24 hours post-transfection and homogenized in 25 μL RIPA (RIPA lysis buffer (Genstar, E122-01), 1 mM PMSF, 10mM DTT, 1X protease inhibitor cocktail (MCE, HY-K0010)). All the protein samples were loaded for immunoblotting. The antibodies used were as follows, rabbit polyclonal RFP antibody (MBL, PM005), mouse monoclonal Flag antibody clone M2 (Sigma, F1804), NanoLuc antibody (Promega, N7000), α-tubulin antibody (GenScript, A01410), goat anti-mouse IgG antibody (H+L) HRP (GenScript, A00160), goat anti-rabbit IgG antibody (H+L) HRP (GenScript, A00098). The primary antibodies were used in 1:1000 dilution, and secondary antibodies in 1:20,000.

**Antibody immunofluorescence staining in cells**

The 293T cells were cultured at coverslips and transfected with *RPL15-mCherry_4* and *RPL-histone3.3* mRNA by TransIT, and fixed by 4% PFA for antibody immunofluorescence staining after 24 hours of transfection. The samples were incubated in primary antibodies (1:500) for three hours and secondary antibodies conjugated with Alexa Fluor 647 (1:500) for two hours, with 15 min DAPI staining before being mounted in Prolong gold antifade reagent (ThermoFisher Scientific, P36934). For SirNeoblasts, 20,000 cells were sorted out for each group and transfected with *RPL15-mCherry_4* and *RPL-histone3.3* mRNA by TransIT. At 24 hpt, following fixed by 4% FA in 0.4X PBS twice for 10 min, incubated in Hybe at 56 ºC for two hours and blocked by 10% Horse serum in PBSTx0.1% at room temperature for 30 min, the cells were stained with
primary antibodies (1:500) and secondary antibodies conjugated with Alexa Fluor 555 (1:500) for two hours, respectively. The antibodies included rabbit polyclonal mCherry antibody (MBL, PM005), mouse monoclonal Flag antibody clone M2 (Sigma, F1804), goat anti-mouse IgG antibody (H+L) Alexa Fluor 647 (Abcam, ab150119), goat anti-rabbit IgG antibody (H+L) Alexa Fluor 647 (Abcam, ab150083), goat anti-mouse IgG antibody (H+L) Alexa Fluor 555 (Abcam, ab150118) and goat anti-rabbit IgG antibody (H+L) Alexa Fluor 555 (Abcam, ab150086).

Data availability

All codes used for plugins in Fiji are available at: https://github.com/jouyun. All original data underlying this manuscript can be accessed from the Stowers Original Data Repository at: http://www.stowers.org/research/publications/libpb-1281. All reagents are available from the corresponding author upon reasonable request.

Statistical analyses

Microsoft Excel and Prism 6 were used for statistical analysis. Mean ± s.e.m. is shown in all graphs. Unpaired two-tailed Student’s t-test was used to determine the significant differences between the two conditions. P < 0.05 was considered a significant difference.

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**Author contributions**

K.L. and A.S.A. conceived the project, designed experiments, analyzed data, and wrote the manuscript. K.L., W.Z., and J.C. performed all experiments and data acquisition. S.A.M. performed the time-lapse imaging experiments and analyzed raw spinning-disk imaging data. E.J.R. and H.-C.L. designed the variant sequences for mCherry.

**Competing interests**

The authors declare no conflicts of interest.
Figure Legends

Figure 1. A systematic screen identifies cell culture conditions for maintaining X1(FS) neoblasts *in vitro*. (a) Flowchart illustrating steps of X1(FS) cell culture and criteria used to identify best culture condition for neoblasts: cell viability, percentage of *smedwi-1*+ neoblasts (%*smedwi-1*+), cell division *in vitro*, colony expansion after transplantation, and rescue efficiency of irradiated hosts after transplantation (pluripotency). (b) Plots showing the FACS gating to sort X1(FS) cells. (c) Representative images showing *smedwi-1*+ neoblasts among the sorted X1(FS) cells. Scale bar, 20 µm. X1(FS) cells consistently contain 23.4%±2.5% neoblasts in total DAPI+ cells. Three replicates were assayed, n=100 to 150. (d) Representative images of cell morphologies observed after 1 day of culture + 5% CO₂, including poor cell morphology in CMFB and healthy cell morphology in IPM (arrowheads). Scale bar, 20 µm. (e) Percentage of *smedwi-1*+ neoblasts after 1 day of culture under indicated conditions. Significantly more *smedwi-1*+ neoblasts were maintained in seven media + 5% CO₂. Adjusted p values were less than 0.05 by one-way ANOVA. Conditions with 5% CO₂ are better than those with ambient atmosphere. The adjusted p-value was less than 0.001 by two-way ANOVA. Three replicates were assayed, n>500. All conditions were compared starting with the same sorted cells. (f) Representative images of dividing cells undergoing either symmetric cell division (SCD) or asymmetric cell division (ASCD). Scale bar, 10 µm. (g) Time-lapse images of dividing cells undergoing either SCD or ASCD in IPM + 5% CO₂. Scale bar, 10 µm. Both SCD and ASCD can be observed in ~300 X1(FS) cells cultured in IPM, KnockOut DMEM, and dL15 + 5% CO₂. (h) Percentage of hosts receiving X1(FS) cells cultured in indicated media + 5% CO₂ for 1,
2, or 3 days that possessed \textit{smedwi-1}+ colonies (green bars) or H3P+ colonies (red bars) at 8 dpt. \( n = 10 \). (i) Number of \textit{smedwi-1}+ neoblasts in colonies formed by X1(FS) cells at 8 dpt following culturing in indicated media + 5% CO\(_2\) for 1, 2, or 3 days. One-way ANOVA calculated adjusted \( p \) values. *, \( p<0.05 \); **, \( p<0.005 \). Ten to twelve animals were assayed per condition. (j) Rescue rates for lethally irradiated hosts following the transplantation of X1(FS) cells cultured in the indicated media + 5% CO\(_2\) for 1, 2, or 3 days. The histogram indicates averaged percent from two independent experiments. Ten to twelve animals were assayed per condition in each experiment. The upper-right panel shows representative images of rescued hosts following transplantation of freshly isolated X1(FS) cells, culminating in fission at 95 dpt. Scale bar, 200 \( \mu \)m.

Figure 2. SiR-DNA plus Cell Tracker staining and cell sorting protocol enrich for clonogenic, pluripotent \textit{smedwi-1}+ neoblasts. (a) Plots showing the gate used to isolate SiR-DNA+ cells for \textit{smedwi-1} ISH. (b) \textit{smedwi-1} ISH on isolated cells from the SiR-DNA+ gate shown in a. \textit{smedwi-1}- cells (arrows) were generally smaller than \textit{smedwi-1}+ cells (stars). Scale bar, 20 \( \mu \)m. (c-d) Plots showing the gates used to isolate SiR-DNA+calcein-AM+ cells (c) and SiR-DNA+Cell Tracker Green+ cells (d) for \textit{smedwi-1} ISH. (e) \textit{smedwi-1} ISH for SiR-DNA+Cell Tracker Green+ neoblasts populations indicated in (d). Scale bar, 20 \( \mu \)m. (f) \%\textit{smedwi-1}+ neoblasts in indicated FACS isolated populations. SiR-DNA and Cell Tracker Green dual staining enrich for \textit{smedwi-1}+ neoblasts (SirNeoblasts) comparably to the Hoechst 33342 stained X1 population. *, \( p<0.05 \); **, \( p<0.01 \). n.s., no significance. Four random fields were assayed per condition. \( N>70 \). (g) Representative images showing the clonogenic capacity of Neoblast culture and transformation.
transplanted neoblasts obtained using different FACS isolation protocols. No noticeable
difference in the colony expansion was observed among single and double dye staining
populations at 7dpt. Scale bar, 200 µm. Ten animals were assayed per condition. (h)
Images of a rescued host planarian and the rescue efficiency by fresh and 1-day
cultured SirNeoblasts. CT: cell tracker green.

**Figure 3. Electroporation can deliver exogenous mRNA into neoblasts.** (a)
Flowchart presenting the steps of neoblast electroporation using SirNeoblasts. (b) Plots
showing electroporation efficiency of SirNeoblasts at 100V, 110V, and 120V compared
to 0V. (c) Neoblasts after electroporation of Dextran-FITC showing 100% isolation of
positive cells after electroporation at 110V and 120V. All SirNeoblasts were free of
Dextran-FITC without electroporation treatment. Scale bar, 20 µm. (d) The percentage
of smedwi-1+ cells after electroporation suggests a relatively high ratio of neoblasts
after electroporation using SirNeoblasts. Four random fields were assayed per
condition. **, p<0.005 (110V SirNeoblasts vs. 120V SirNeoblasts at 1 day). (e)
Representative images showing the colony expansion of electroporated SirNeoblasts
after transplantation Scale bar, 200 µm. N=14 for 110V and =10 for 120 V. (f) Images of
a rescued host planarian and the rescue efficiency of electroporated SirNeoblasts.
Scale bar, 200 µm. (g) Representative images showing the mRNA signals (white dots)
in cells 1 day after 110V and 120V electroporation. Scale bar, 20 µm. (h) Percentage of
total cells and smedwi-1+ cells containing mRNA 1 day after 110V and 120V
electroporation. n.s.: not significant. ** < 0.01.
Figure 4. **TransIT can deliver exogenous mRNA into neoblasts and express Nluc.**

(a) Adaption of the RPL15 5'UTR and 3'UTR to *NanoLuc luciferase* (Hall et al., 2021).

(b) Expression comparison of *NanoLuc luciferase* mRNA in 200,000 cultured SirNeoblasts transfected by TransIT. (c) The percentage of *piwi-1*+ SirNeoblasts in different culture conditions presence or absence of supplements or CO₂ after 1 day of culture *in vitro* (div). (d) The percentage of *piwi-1*+ SirNeoblasts in different culture conditions presence or absence of supplements or CO₂ after 3 days of culture *in vitro* (div). (e) Comparison of the efficiency of TransIT and electroporation delivery methods in 190,000 SiR-DNA 4N cells transfected with *NanoLuc luciferase* mRNA. *, p<0.05; **, p<0.01; ***, p<0.001. (f) Luminescence of live cells and 293T cells transfected by TransIT. Scale bar, 50 μm. (g) Aryscan z-stack of SirNeoblasts (upper panel) and live bulk cells (lower panel) showing the NanoLuc mRNA localization in cells 24 hours after transfection. mRNA without TransIT was used as negative controls. Scale bar, 10 μm. (h and i) NanoLuc+ cells captured at 6 hours (h) and 24 hours (i) post-transfection were stained with smedwi-1 RNA probe (red) or SMEDWI-1 antibody (magenta). NLuc+ cells are smedwi-1 low or negative cells. Scale bar, 10 μm. (j) Expression comparison of *NanoLuc* mRNA in live cells transfected by TransIT at 6 hours, 12 hours, and 24 hours. (k) Expression comparison of *NanoLuc* mRNA transfected by TransIT at 12 hours in live cells from wild-type and 100Gy irradiated planarians. (l) Expression of *NanoLuc* mRNA in 200,000 planarian live cells or SirNeoblasts transfected by TransIT in KnockOut DMEM + supplements + 5%CO₂. (m) Expression comparison of *NanoLuc* and *NanoLuc-mCherry* mRNA transfected by TransIT at 24 hours in live cells.
Supplementary Figure 1. Viability of X1(FS) cells cultured in vitro. (a) Percentages of live cells (Propidium Iodide-negative) among 23 media, +/- 5% CO\textsubscript{2}, after 1 day of culture. KnockOut DMEM + 5% CO\textsubscript{2} yielded the best overall cell viability. Three replicates were assayed, n=500 to 1200. (b) Four representative images showing long cellular processes from cells after 6 days of culture in L15 without 5% CO\textsubscript{2}. Scale bar, 20 µm. (c) X1(FS) cells were cultured in IPM + 5% CO\textsubscript{2} for 2 days. Representative images of apoptotic cells (Annexin V, green, arrowheads) co-stained with the pan-neoblast marker smedwi-1 (magenta), n=37. Two independent replicate experiments were performed. No co-labeling was observed, suggesting neoblasts examined in the study were viable. Scale bar, 20 µm. (d) Percentage of smedwi-1+ neoblasts after 3 days of culture in indicated media + 5% CO\textsubscript{2}. (e) Percentage of PCNA+ neoblasts after 1 day of culture in indicated media + 5% CO\textsubscript{2}. P values were calculated by comparing every two conditions.

Supplementary Figure 2. Sexual hosts are rescued and reconstituted by transplanting cultured asexual X1(FS) cells. (a, b) Determine the number of X1(FS) cells needed for efficient colony expansion. (a) Percentage of lethally irradiated hosts displaying robust neoblast colony expansion following transplantation with the indicated numbers of sorted X1(FS) cells. At 7 days post-transplantation (dpt), > 80% of all hosts displayed colony expansion when 1,000 X1(FS) were transplanted. (b) Representative images of hosts transplanted with X1(FS) cells at 7 dpt. smedwi-1+ neoblasts: green. DAPI: blue. Scale bar, 200 µm. Ten animals were assayed per condition. (c) Representative images showing colonies of smedwi-1+ neoblasts at 8 days post-
transplantation (dpt) cultured in the indicated conditions + 5% CO₂. Only X1(FS) cells cultured in dGrace’s medium + 5% CO₂ did not efficiently form colonies in vivo. Scale bar, 200 µm. (d) Sequence showing the HpaI enzyme restriction site, which was used to distinguish between the asexual (donor) and sexual (host) biotypes by RFLP analyses (Wagner et al., 2011). (e) RFLP data showing the rescue of lethally irradiated sexual worms transplanted with freshly collected, non-cultured X1(FS) cells. (f, g) RFLP data showing the rescue of lethally irradiated sexual worms transplanted with 1- and 2-day cultured X1(FS) cells. Data from two independent experiments showed replicate 1 (panel f); replicate 2 (panel g). (h) Rescue rates for lethally irradiated hosts following transplantation of X1(FS) cells cultured in the indicated media + 5% CO₂ for 1 or 2 days. None of the conditions rescued lethally irradiated hosts after 3 days. Blue and orange dots show the value of the rescue rate from replicate experiments.

Supplementary Figure 3. Comparison of SiR-DNA sorted cells. (a) A plot showing how SiR-DNA-stained cells are displayed without gates in the flow cytometry analysis using SiR-DNA versus side scatter. (b) A plot showing how gates were defined to isolate two SiR-DNA staining cell populations based on DNA content (SiR-DNA 4n and 2n). (c) smedwi-1 in situ staining for neoblasts in two isolated cell populations based on DNA content (b). SiR-DNA 4n population contains 56.4%±2.6% smedwi-1+ neoblasts (also see Fig. 4f) compared to 26.8%±3.2% in SiR-DNA 2n population, p-value = 0.0017. Scale bar, 20 µm. (d-g) Plots showing the cell cycle distribution of SirNeoblasts (SiR-DNA 4n + CT) (d), cells between SiR-DNA 4n and 2n (e), SiR-DNA 2n (f), and all
SiR-DNA+ cells (g). Sorted cells were stained with Hoechst 33342. Hoechst 33342+ (square gate) cells were analyzed for cell cycle distribution.

**Supplementary Figure 4. KnockOut DMEM maintains pluripotency in the short-term independently on differentiated cells.** (a) Percentage of $smedwi-1^+$ or $tgs-1^+$ neoblasts within the first three days of culture in KnockOut DMEM + 5% CO$_2$. (b) Percentage of FITC+ cells, $smedwi-1^+$ or $tgs-1^+$ neoblasts when SirNeoblasts were co-cultured with X1(FS) within the first three days of culture in KnockOut DMEM + 5% CO$_2$.

**Supplementary Figure 5. Electroporation can deliver exogenous mRNA into neoblasts.** (a) Flowchart describing electroporation assay steps to screen for best conditions for cell viability and Dextran-FITC delivery efficiency. (b) Plots of X1 viability (upper) and electroporation efficiency (lower) with IPM as the electroporation buffer to deliver Dextran-FITC at 120V compared to 0 V controls. (c) Representative images of sorted Dextran-FITC$^{low}$ and Dextran_FITC$^{high}$ cells indicate successful delivery of Dextran-FITC at 120V. (d) Viability (blue) and electroporation efficiency (red) on X1 cells after using IPM as electroporation buffer. The viability of X1 is indicated by the percentage of X1 cells within all Hoechst 33342+ cells. (e) $\%smedwi-1^+$ neoblasts in X1(FS) cells after 100V, 110V, and 120V electroporation immediately (black column) and after 1 day of culture in KnockOut DMEM + 5% CO$_2$ (white column). Four random fields were assayed per condition. $p<0.05$ for 120 V. N>40. (f) Electroporated X1(FS) cells receiving greater than 100 V failed to form colonies following transplantation. Ten animals were assayed per condition.
Supplementary Figure 6. mCherry mRNA is expressed in SirNeoblasts. (a) Cartoon illustration of the design for mRNA IVT. (b) The number of piRNA matches between positions 17 and 30 in all 15 constructs. (c) The number of non-seed matches in all 15 constructs. (d) A flowchart describes the steps of SirNeoblast electroporation using mCherry mRNA. (e) Representative images of mCherry mRNA electroporated SirNeoblasts cultured in KnockOut DMEM + 5%CO2 for 1 day. Upper: electroporated SirNeoblast without mRNA in culture. Lower: mCherry mRNA electroporated SirNeoblasts in culture. Scale bar, 20 µm. (f) Plot showing no mCherry expression after 110V electroporation without mCherry mRNA. (g) Plot showing ~5% mCherry+ cells after 110V electroporation with mCherry mRNA. (h) Representative images of cells from mCherry- population in (upper row) and mCherry+ population in (lower row). Cells from the mCherry+ population showed obvious mCherry localization in the cytoplasm. Scale bar, 20 µm.

Supplementary Figure 7. Detection of Nanoluc in planarian cells cultured in vitro. (a-d) Expression of NanoLuc (a), mCherry_4 (b), NanoLuc-mCherry (c), and histone3.3-2×flag (d) mRNA in cultured 1000,000 planarian live cells and 293T cells transfected by TransIT at 1 div through Western blot. (e-g) Antibody immunofluorescence staining in 293T cells (e), 20,000 SirNeoblasts (f), and bulk live cells (g) transfected with NanoLuc (a), mCherry_4 (b), NanoLuc-mCherry (c), and histone3.3-2×flag (d) mRNA by TransIT at 1 div. Scale bar, 50 µm. (h) NanoLuc+ cells are smedwi-1 low or negative cells. Scale bar, 10 µm. (i) Expression of NanoLuc mRNA in 200,000 Hoechst 33342 staining cells

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(X1, X2+Xins, all live cells) and live cells without staining transfected by TransIT in KnockOut DMEM + supplements + 5%CO₂.

**Supplementary Figure 8. Nanoluc positive cells were smedwi-1 negative and WI-1 low in cultured SirNeoblasts.** All 52 NanoLuc+ cells captured were stained with smedwi-1 RNA probe (red) or SMEDWI-1 antibody (magenta). CTG was in Green, indicating the cytoplasm membrane's integrity during the fixation. Scale bar, 10 μm.

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Lei et al. Figure 3

- Cut tails
- Dissociate cells stain with SiR-DNA and CellTracker Green
- Electroporation with Dextran and FACS
- Culture Dextran+ SirNeoblasts in KnockOut DMEM

| Condition | # of hosts | # of rescued |
|-----------|------------|-------------|
| 110 V and 0 day | 25 | 21 |
| 110 V and 1 day | 12 | 10 |
| 120 V and 0 day | 10 | 2 |

- Imaging of 36 dpt, 44 dpt, 47 dpt, 50 dpt samples

- Imaging of mRNA+ and mRNA+smedwi-1+ expression

- Bar graph showing % of tdTomato mRNA+ across 0V, 110V, 120V conditions
