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In Vitro Generation of Zebrafish PGC-Like Cells[1]

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ABSTRACT

The possibility of generating primordial germ cells (PGCs) in vitro from noncommitted embryonic cells represents an extremely useful tool in current research. Primordial germ cell in vitro differentiation has been successfully reported in mammals. However, contrary to fish, PGC specification in mammals is an inductive mechanism. This study is the first to date to describe a rapid method for PGC in vitro differentiation in teleosts. Primordial germ cell-like cells were characterized by several lines of evidence, including gene expression, cell complexity, size, and image analysis for the quantification of fluorescence under *vasa* promoter. Moreover, differentiated cells were able to colonize the genital ridge after transplantation. Differentiation treatments increased the number of PGCs in culture, causing differentiation of cells rather than inducing their proliferation. These results open up the possibility of differentiating genetically modified embryonic cells to PGC-like cells to ensure their transmission to the progeny and could be crucial for an in-depth understanding of germline differentiation in teleosts.

embryonic cell differentiation, gamete biology, primordial germ cells, zebrafish

INTRODUCTION

The use of zebrafish (*Danio rerio*) as a model species in science has exponentially increased during recent years. One important limitation in this model species, which has recently been overcome, was the inability to readily induce targeted modifications in the genome. Recent technologies such as those related to the use of zinc finger nucleases [1, 2], artificial transcription activator-like effector nucleases [3, 4], and the CRISPR/Cas9 system [5] have been successfully employed to induce targeted genetic modifications in zebrafish embryos. These approaches are essential in functional genomic studies and gene therapy.

A major goal in this field would be to increase the germline transmission frequency of genetic modifications in order to guarantee their presence in the progeny. It has been reported that in zebrafish one single primordial germ cell (PGC) can reconstitute a whole gonad after transplantation into a recipient embryo [6] and that PGCs, spermatogonial stem cells, and female germline stem cells can be successfully cultured [7, 8]. However, these cells are quite resilient to genetic manipulations. Unlike PGCs, embryonic cells can be easily modified, but their contribution to the germline is limited [9]. Embryonic stem cell-like cultures have been established [10–14], and homologous recombination has been demonstrated in them [15]. Considering all these advances, the next logical step would be to take advantage of both cell types, exploring the possibility of differentiating embryonic cells into PGC-like cells to ensure their contribution to the germline. In vitro generation of PGCs from pluripotent cells has been achieved in human [16] and mouse [17, 18] models but has not been reported in fish. Primordial germ cells in mammals and urodele amphibians are induced through cellular interactions during gastrulation instead of being specified by the inheritance of maternally provided cytoplasmic determinants [19]. It is well known that the inductive process of PGC specification in mouse embryos is mediated by the secretion of factors from the bone morphogenetic protein family [19], but the different PGC specification mechanism in teleost embryos makes in vitro generation of fish PGCs much more difficult to achieve. In mice, disruptions in the *Bmp4* gene led to defects in mouse PGC specification, and in humans the addition of recombinant BMP4 increased the number of human PGCs in a dose-dependent way [20]. Retinoic acid (RA) promotes proliferation of cultured chicken PGCs via activation of the phosphoinositide 3-kinase/protein kinase B (the latter is also known as Akt) and nuclear factor κB signaling cascade [21], and it is known to induce in vitro embryonic stem cell differentiation into PGCs in mouse [22–24]. Epidermal growth factor (EGF) has also been employed for in vitro mouse PGC generation [17], and it is required for chicken PGC proliferation [25].

In this work, we present a method for in vitro generation of zebrafish PGC-like cells from cultured embryonic cells for the first time to date. For this purpose, different combinations of growth factors were employed. The use of these factors together with a slightly modified testicular cell culture medium (TCCM) initially described by Sakai [26] produced a significant increase in the number of PGC-like cells derived from zebrafish embryonic cell cultures.

MATERIALS AND METHODS

Ethics Statement

The experiments carried out in this study using zebrafish embryos are part of a project from the Ministerio de Ciencia e Innovación AGL2009-06994. This work was approved by the University of León bioethical committee (22009).

Zebrafish Maintenance and Embryonic Cell Recovery

Adult zebrafish (*D. rerio*) *vasa* enhanced green fluorescent protein (EGFP) zf45 strain, tg {vas:egfp} transgenic line generated by the Knovel and Olsen laboratory [27], and AB line were kept in tanks (Aquatic Habitats) under standard conditions [28] and were used for embryo production. For embryo collection, adult zebrafish were kept in crossing tanks (Aquatic Habitats) at

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28°C with six fish per tank at a male:female ratio of 1:2. The embryos were allowed to develop to the appropriate developmental stage (blastula and 7 days postfertilization [dpf]) and were then divided into groups of approximately 50 individuals each. They were rinsed with 70% ethanol for 10 sec and then treated with bleach solution (0.5%) for 2 min. Then, they were immediately washed by rinsing several times with Leibovitz (L-15) medium as described by Fan and colleagues [11]. The blastoderms were manually excised from blastula-stage embryos (3.5 h postfertilization) using fine watchmaker's forceps under a microscope (Nikon). The blastomeres were washed in modified L-15 supplemented with 5% fetal bovine serum, 500 μg/ml of ampicillin, 500 μg/ml of streptomycin, and 500 IU/ml of penicillin at room temperature until further use.

Embryonic Cell Culture and In Vitro Differentiation Treatments

The embryonic zebrafish cells (50 blastoderms per well; 50 000 cells approximately) were cultured in a high cell-binding dish (Nunc; VWR) in TCCM. The TCCM included the following: human chorionic gonadotropin (final concentration, 5 IU/ml; Sigma), equine chorionic gonadotropin (2 IU/ml; Sigma), L-arginine (0.2 mg/ml from 100× stock solution; Gibco BRL), L-aspartic acid (0.02 mg/ml from 100× stock solution; Gibco BRL), L-histidine (0.015 mg/ml from 100× stock solution; Gibco BRL), L-lysine hydrochloride (0.0725 mg/ml from 100× stock solution; Gibco BRL), L-proline (0.02 mg/ml from 100× stock solution; Gibco BRL), bovine serum albumin (0.5% from 5% w/v stock solution, BSA fraction V; Sigma), and Hepes (10 mM from 1 M stock solution, pH 7.9; Sigma). Based on zebrafish growth medium [28] and described as differentiation medium in zebrafish testicular cells by Sakai [26], this medium was slightly modified for the experiment. It was supplemented with 15% fetal bovine serum (as described previously [29] to maintain the zebrafish cell line from zebrafish blastula-stage embryos), 500 μg/ml of ampicillin, 500 μg/ml of streptomycin, and 500 IU/ml of penicillin. The concentration of L-15 medium was reduced from 100% to 80% because of the addition of bovine serum albumin. All supplements were initially dissolved in 80% L-15.

One hour after plating the cells, differentiation factors were added. We studied the following four differentiation treatments (DFTs): DFT1, comprising bone morphogenetic protein 4 (BMP4) (500 ng/ml; Biovision); DFT2, comprising BMP4 (500 ng/ml) and RA (5 mM; Acros Organics); DFT3, comprising BMP4 (500 ng/ml) and EGF (50 ng/ml; Sigma); and DFT4, comprising BMP4 (500 ng/ml), RA (5 mM), and EGF (50 ng/ml). After 24 h of in vitro cell differentiation, viability and fluorescence were checked, and the characterization of the PGC-like cells was carried out (Fig. 1).

Gene Expression of PGC-Related Genes by Quantitative PCR

RNA isolation and DNase treatment. RNA was extracted from plated embryonic zebrafish cells after 24 h of being maintained in DFTs using Trizol reagent (Invitrogen) according to the manufacturer's protocol. RNA quantity and purity were determined using a spectrometer (NanoDrop 1000; Thermo Scientific). This protocol also includes a DNase I treatment step. The isolated RNA showed high purity (absorbance at 260 and 280 nm >1.8) and was stored at −80°C until further use.

Reverse transcription. Complementary DNA was obtained from RNA (1 μg) using a cDNA synthesis kit (Invitrogen) following the manufacturer's protocol. The cDNA for embryonic zebrafish cells was stored at −20°C before quantitative PCR (qPCR) analysis. The RT-PCR conditions were 95°C for 3 min; followed by 35 cycles of 95°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec; with a final extension at 72°C for 10 min.

Real-time qPCR. In order to study the effect of DFTs on zebrafish gene expression, qPCR analysis was carried out on zebrafish treated (DFT1, DFT2, DFT3, and DFT4) and TCCM nontreated embryonic cells from a *vasa*:EGFP transgenic line. Genes specifically expressed in PGCs such as *vasa* and *dead end* (*dnd*) [30, 31] were analyzed. The receptor of SDF-1a, chemokine (C-X-C motif) receptor 4b (*cxcr4b*), which is required for PGC migration [32], was also studied.

The primers for qPCR were designed using Primer Express (version 2.0; Applied Biosystems) and Primer Select (version 10.1; DNASTAR Lasergene Suite). The primer nucleotide sequences and annealing temperature from zebrafish PGC-related genes can be found in supporting information (Supplemental Table S1; all Supplemental Data are available online at www.biolreprod.org).

The qPCR conditions were optimized for the different primers to achieve similar amplification efficiencies to compare different amplicons. Product specificity was tested by melting curves, and product size was visualized by electrophoresis on agarose gel (data not shown).

Reaction mixtures had a total volume of 20 μl. They contained cDNA (2 μl), 1× SYBR Green Master Mix (10 μl; Applied Biosystems), and 500 nM of each forward and reverse primer (2 μl).

The qPCR was initiated with a preincubation phase of 10 min at 95°C, followed by 40 cycles of 95°C denaturation for 10 sec and the temperature for primer extension for 60 sec. The relationship between the threshold cycle and the dilution values of DNA was linear, with a correlation coefficient R^2 higher than 0.9 in all cases (data not shown). Each experiment was performed three times, and three technical replicates were done per sample.

Expression levels for each zebrafish gene relative to *actb2* were calculated for all zebrafish PGC-related genes using the $2^{-\Delta\Delta Ct}$ method where Ct is defined as threshold cycle, which is an algorithm to analyze relative changes in gene expression. It requires the assignment of one housekeeping gene, which is assumed to be uniformly and constantly expressed in all samples [33].

Data analysis. Data were analyzed using SPSS version 16 (IBM) and Excel (Microsoft). All results were expressed as the mean ± SE of the ΔΔCt method of three independent experiments with three replicates for each. The Student *t*-test ($\mu = 1$) was performed to identify changes in gene expression levels after DFTs, and a dendrogram with average linkage between gene expression in each treatment was made.

vasa *Detection by Fluorescent Image Analysis*

The digital image processing was performed by the development of computer vision software called bioimaging software for PGC-like cell detection by fluorescence. The software was developed by the MATLAB environment (The MathWorks, Inc., http://www.mathworks.com/products/computer-vision/), which is a mathematical software tool that offers an integrated development environment with a proprietary programming language (language M).

The aim of the program was to analyze fluorescence intensity. First, the software converts the images obtained with the microscope to gray scale, codifying in 256 levels of light intensity. The precision chosen for the artificial vision analysis was double type [34, 35].

After obtaining the intensity values, they were divided into three brightness levels of level 1 (low intensity), level 2 (medium intensity), and level 3 (high intensity), and a color scale was established (grey, green, and light green). Finally, the software drew a wireframe mesh with the color scale as set forth above, graphically showing a topographical map of intensities.

Identification of Somatic and PGC-Like Cell Populations by Flow Cytometry

Cells were maintained in vitro for 24 h in TCCM medium and TCCM medium with the different treatments (DFT1–DFT4) as described above. The cells were then prepared for flow cytometry analysis. The PGC-like cells were differentiated for higher fluorescence intensity (fluorescein isothiocyanate [FITC]), large size, and complexity (forward scatter [FSC]) compared with somatic cells.

Propidium iodide (PI) (Sigma) was added at 4 mg/ml of final concentration to detect dead cells. Immediately afterward, samples were acquired in a flow cytometer (CyAn ADP; Beckman Coulter, Inc.) adjusted for blue excitation (488 nm) and fluorescence excitation (458 nm) lines for the detection of PI (670/30 nm) and FITC (580/30 nm) fluorescence, respectively. Data analysis was performed applying WEASEL 3.1 free software (http://en.bio-soft.net/other/WEASEL.html). In total, 10 000 events were counted for each sample.

Cell Cycle Analysis in Differentiated and Nondifferentiated Embryonic Cells

For cell cycle analysis, the employed method is based on the use of DNA dyes that bind to DNA in a linear manner in order to guarantee that when DNA content of a cell increases the signal from the dye increases proportionally. The dyes used were Hoechst 33342 (H342; Sigma) and PI. For this purpose, cells were incubated at 29°C for 30 min using Hoechst 33342 at 10 mg/ml of final concentration. The supernatant was removed, and the cells were resuspended in 0.5 ml of L-15 (at room temperature). Propidium iodide was added at 4 mg/ml of final concentration to detect dead cells. Immediately afterward, samples were acquired in the CyAn ADP flow cytometer adjusted for both UV (351 nm) and blue (488 nm) excitation lines for the detection of Hoechst 33342 (450/65 nm) and PI (670/30 nm) fluorescence, respectively. All analyses were performed applying WEASEL 3.1 free software. In total, 10 000 events were counted for each sample.

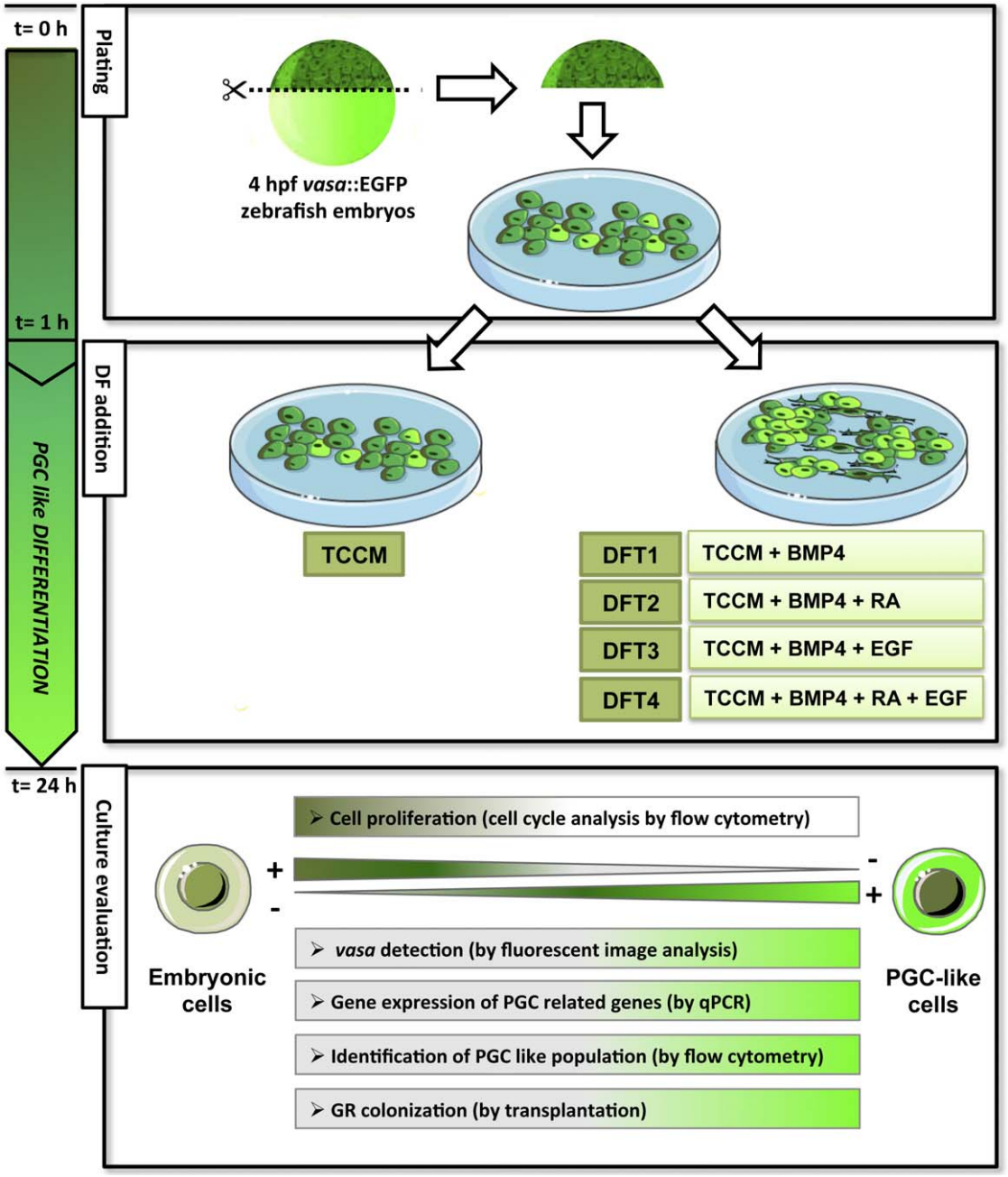


FIG. 1. Experimental design and PGC-like cell characterization. The different approaches used in this study confirm successful differentiation to PGC-like cells.

Transplantation and Analysis of Genital Ridge Colonization in Recipient Larvae

The PGC-like cells obtained from the best treatment for PGC differentiation (DFT4) were transplanted into sterilized recipient larvae (7 dpf). Thus, an endogenous germ cell population was absent, ensuring that all the PGCs present in the host were originally transplanted from the donor. For sterilization, recipient embryos were microinjected in the one-cell stage with *dnd-1* morpholino (Gene Tools) [36] at 1 μg/μl in 0.06% phenol red solution (Supplemental Figure S1). Cell transplants were performed using a glass micropipette needle (0.9-mm diameter borosilicate glass capillaries) in a fluorescence stereomicroscope (MZ16F; Leica) connected to a microinjector (Nanoliter 2010; Dismed). The PGC-like cells were transplanted into the abdominal cavity of each zebrafish larva following the procedure described by Wong and Collodi [8]. The ability of DFT4-differentiated cells to migrate and colonize the genital ridge was confirmed, and photographs were taken of chimeric larvae with a fluorescence microscope (DFC310 FX; Leica) equipped with a digital camera (LAS AF; Leica). After transplantation, germline chimeras (n = 17) were identified based on the presence and maintenance of PGC-like green fluorescent protein (GFP)-positive cells in the gonad region. All the recipients were periodically examined by fluorescence microscopy (1, 2, and 3 days after transplantation), and the percentage of transplantation success was estimated (the number of embryos with genital ridge colonization divided by the number of embryos successfully transplanted).

Methylation Analysis

DNA conversion with bisulphite. In order to test if in vitro differentiation could induce demethylation on *vasa* promoter, methylation analysis was carried out on zebrafish treated (DFT4) and noncultured embryonic cells (blastomeres). DNA was bisulphite converted using a bisulphite kit (EpiTect; Qiagen).

Bisulphite sequencing. Converted DNA was amplified by PCR using *vasa* promoter primers (forward: 5′-AATTGGGAATTTTAGTAGTATATTGA TAGT-3′ and reverse: 5′-TTTAATTTAAAACCTCATTAATCTAAATCA-

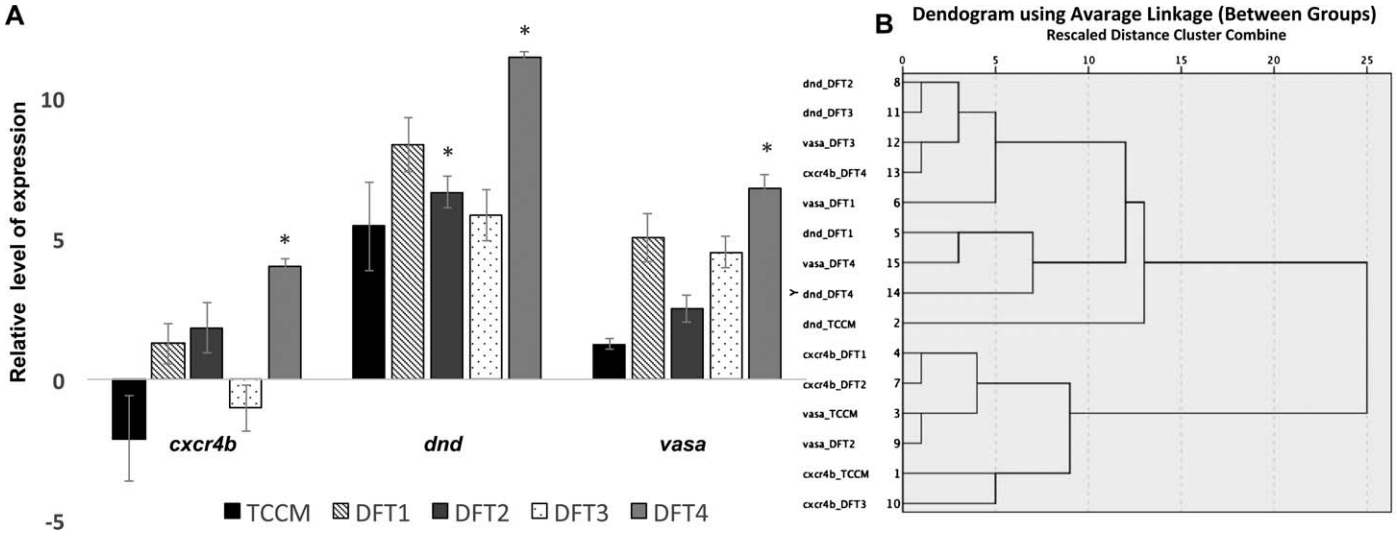


FIG. 2. Relative expression of zebrafish PGC-related genes after DFTs (DFT1, DFT2, DFT3, and DFT4). **A**) Expression levels for each gene relative to *actb2* were calculated for all samples using the $2^{-\Delta\Delta Ct}$ method. All results were expressed as the mean ± SE of the $2^{-\Delta\Delta Ct}$ method of three independent experiments with three replicates for each. The Student *t*-test ($\mu = 1$) was performed to identify changes in gene expression levels after differentiation. Asterisks show significant ($P < 0.05$) upregulation of the studied genes. **B**) The dendrogram shows the linkage between the treatment groups.

3′) described by Lindeman et al. [37]. The PCR conditions were 95°C for 7 min and 40 cycles of 95°C for 1 min, annealing temperature (56°C) for 2 min and 72°C for 2 min, followed by 10 min at 72°C. The PCR products were cloned into *Escherichia coli* (DH5α TOPO TA Cloning Kit; Invitrogen) and sequenced. The CpG Viewer (University of Leeds) software for DNA methylation analysis was used [38].

Data analysis. Data were analyzed using SPSS version 16 (IBM) and Excel (Microsoft). All results were expressed as the methylation percentage ± SE of the CpG islands of 10 different clones. The mean values were compared by a one-way ANOVA, with significance set at $P < 0.05$).

RESULTS

DFTs Increase the Expression of PGC-Related Genes

After 24 h in culture, all the studied genes (*cxcr4b*, *dnd*, and *vasa*) were significantly upregulated for DFT4 treatment. The increase in *cxcr4b* expression was significant for DFT2 and DFT4 treatments (Fig. 2A). However, *dnd* transcript significantly increased in DFT3 and DFT4 treatments. When *vasa* expression was analyzed, a significant upregulation was found in DFT1, DFT2, and DFT4 (Fig. 2A).

The qPCR results demonstrated that DFT4 produced an increase in all the studied transcripts (Fig. 2A). These included both those specifically expressed in PGCs (*vasa* and *dnd*) and those required for pro-PGC migration.

The Increase in the Expression of PGC-Related Genes Is Due to an Increase in the PGC-Like Cell Population

We performed a flow cytometry analysis to test whether the observed increase in the expression of PGC-related genes is due to an increase in cell number or simply to an alteration in the expression of the same cell population. To differentiate PGCs from somatic cells, we analyzed the size, complexity, and fluorescence intensity. Primordial germ cells are known to differ markedly from other somatic cells in their large size, complexity, and bright fluorescence when the *vasa* transgenic line is used [39].

Results demonstrated that PGC-like cells increased two to three times with TCCM medium and DFTs compared with cells dissociated from 20 to 25 somite developmental stage *vasa* transgenic line embryos (2.14 times for TCCM medium, 2.68 times for DFT1 treatment, 3.10 times for DFT2 treatment, 3.13 times for DFT3 treatment, and 3.03 times for DFT4 treatment) at the same time that the somatic population decreased, particularly when DFTs were used. The highest percentages of PGC-like cells (61.1%, 61.8%, and 59.8%) compared with the somatic population (38.7%, 37.5%, and 39.9%) were obtained after treatment with DFT2, DFT3, and DFT4, respectively. In contrast, the embryonic cells in TCCM (without BMP4, RA, and EGF) showed the lowest percentage of PGC population (42.7%), as expected (Fig. 3). Among DFTs, DFT1 showed a low population of PGC-like cells, with this result coinciding with results for gene expression (Fig. 2).

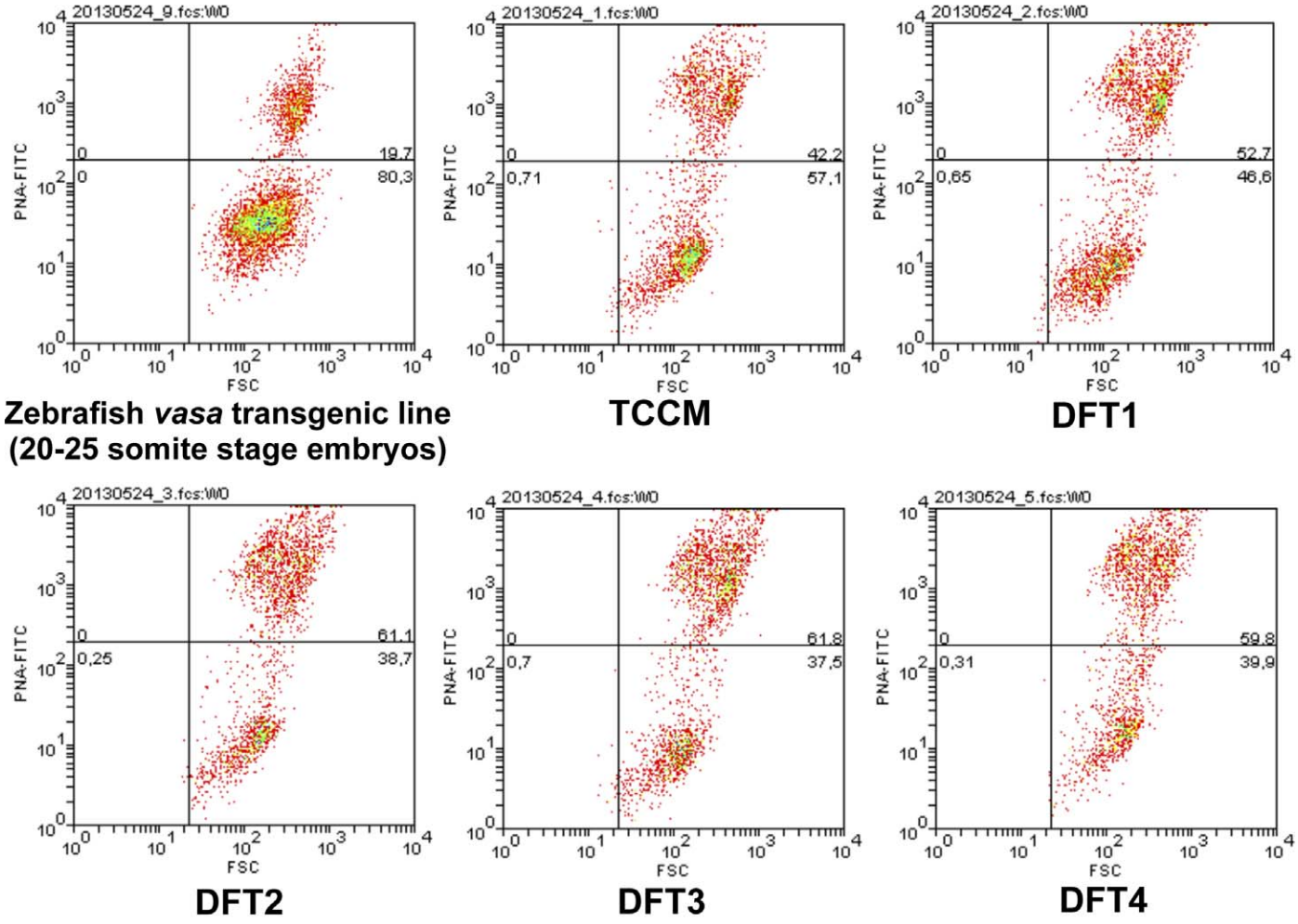
Treatments Induce Differentiation Rather Than Cell Proliferation

A cell cycle analysis was performed by flow cytometry. This was to determine whether DFTs increase the PGC-like cell population via a differentiation process or whether they induce proliferation in all cells (somatic and PGC-like cells).

The DFT2 and DFT3 treatments did not alter the number of cells in gap 2/mitosis (G2/M). Moreover, the best DFT according to previous results (DFT4) obtained the lowest event number in G2/M (Fig. 4). These results suggest that the increase in the number of PGC-like cells is due to an increase in differentiation rather than in cell proliferation.

EGFP Fluorescence Intensity, Corresponding to PGC-Like Cells, Increases after DFT4 Treatment

We developed software for the quantification of fluorescence pixels before and after DFTs. This software was developed with the aim of providing an open access tool that different laboratories could use to validate DFTs. We believe that this type of tool can help in standardizing the methodology used for the evaluation of different protocols, making results comparable among different laboratories. The bioimaging analysis results were in concordance with those for flow cytometry. Starting from field capture replicates, a total pixel amount, corresponding to the total cell population (levels 1–3), was obtained for each field capture in each treatment. The



Treatment	% PGCs	Relative fold change Zebrafish *vasa* transgenic line (20-25 somite stage)
TCCM	42.2	2.14
DFT1	52.7	2.68
DFT2	61.1	3.10
DFT3	61.8	3.13
DFT4	59.8	3.03

FIG. 3. The PGC-like cells and somatic cell populations in TCCM and DFTs (DFT1, DFT2, DFT3, and DFT4) increased the PGC population at the same time as the somatic cell population decreased. Analysis was carried out using flow cytometry. A control of 20 to 25 somite developmental stage *vasa* transgenic line embryos was used to establish the GFP-positive PGC population (greater than logarithm 10^2–10^3) according to previous studies. All analyses were performed applying WEASEL 3.1 free software. In total, 10 000 events were counted for each sample.

mean ± SE values were 20 327.33 ± 2980.55 (TCCM) and 66 264.00 ± 8900.47 (DFT4). Once selected, pixels with green fluorescence intensity (levels 2 and 3), corresponding to PGC-like cells, were subselected, with percentage mean ± SE values in respect to total pixels of 17.54% ± 7.60% (TCCM) and 38.55% ± 0.91% (DFT4). An increase of 21.01% was reported with DFT4 field captures compared with TCCM (Fig. 5).

PGC-Like Cells Can Successfully Colonize the Genital Ridge after Transplantation in a Recipient Larva

Embryonic cells that differentiated into PGC-like cells using the DFT4 treatment were transplanted in *dnd-1* morphant (sterile) 7-dpf larvae. Control (*vasa*:EGFP transgenic 7-dpf larvae) and transplanted larvae were photographically recorded 24 h after transplantation. Transplanted PGC-like cells migrated to the genital ridge in exactly the same way as the endogenous PGCs in the control larvae, showing the same

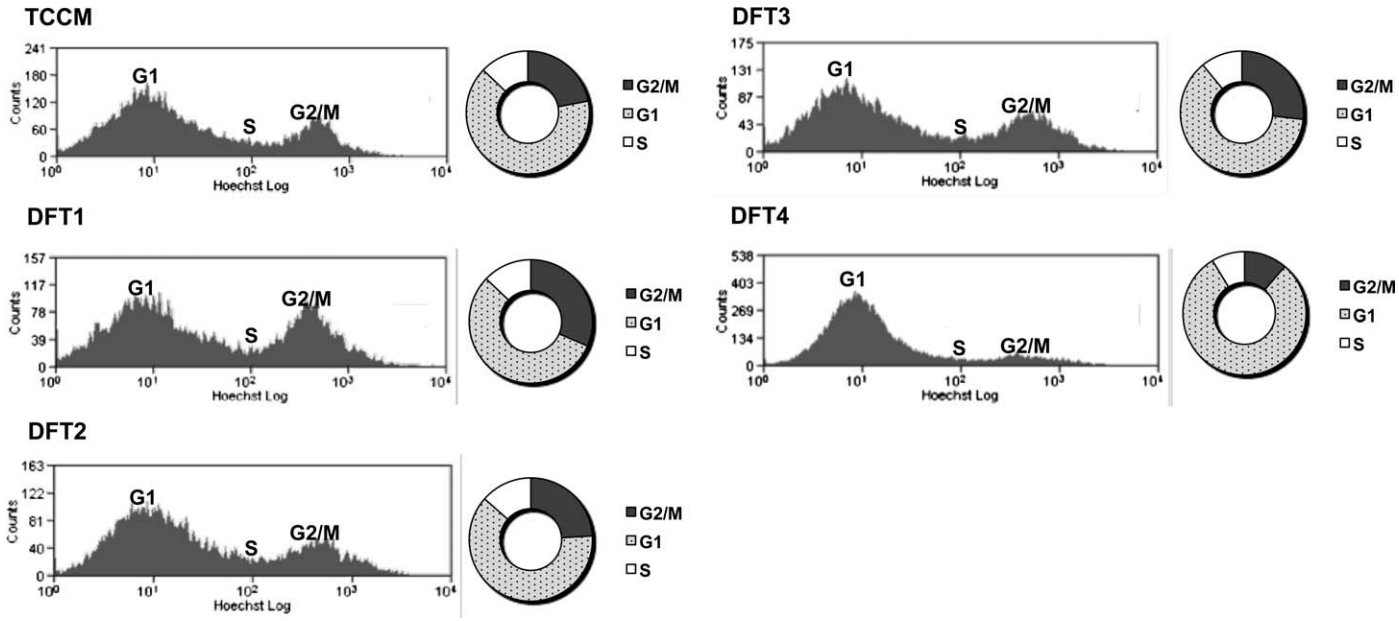


FIG. 4. Cell cycle analysis in TCCM medium and DFTs. Hoechst 33342 was used for cell cycle analysis, and cells were analyzed by flow cytometry. Except for DFT1, treatments did not increase the number of cells in G2/M; therefore, treatments induce differentiation rather than cell proliferation in cultured cells. The best DFT according to previous results (DFT4) obtained the lowest event number in G2/M. All analyses were performed applying WEASEL 3.1 free software. In total, 10 000 events were counted for each sample.

positional pattern during development (Fig. 6A). The percentage of transplantation success (1 day after transplantation) was 47.1%. The presence of PGCs in the genital ridge was confirmed 3 days after transplantation in 11.7% of the transplanted embryos, comparable to previous germline investigations [8]. Moreover, the migration capacity of PGC-like cells was tested, confirming their functionality (Fig. 6B).

DFT4 Treatment Induces vasa *Promoter Demethylation*

The DFT4 treatment induced *vasa* promoter demethylation compared with noncultured cells, decreasing from a mean ± SE of 58.00% ± 6.60% to 22.00% ± 6.29% after treatment. These results are shown in Figure 7.

DISCUSSION

The possibility of generating fish PGCs in vitro has a huge impact in biotechnology and aquaculture. In mammals, PGC in vitro differentiation has been successfully achieved [17, 18], but, contrary to fish, PGC specification in mammals is an inductive mechanism. In fish, the development of PGC markers such as *vasa* and the use of transgenic lines were the definitive tools for demonstrating the existence of inherited germplasm [19], the so-called *nuage*, containing *vasa*, *nanos 1*, *H1M*, *dnd*, *ziwi*, and other transcripts that drive PGC differentiation [40, 41]. Germplasm segregates to four cells through the 1000-cell stage, and then zygotic *vasa* expression begins [42]. The role of *nuage* in PGC differentiation was confirmed by Hashimoto et al. [40], who observed that the ablation of germplasm at the four-cell stage resulted in a decrease in the number of PGCs and abolished germline-specific gene expression. This inherited pattern of specification has dissuaded investigators from the idea of achieving PGC in vitro generation, and the factors commonly used in mammals have not been tried in fish. Until now, fish PGCs were obtained in vitro only from the already committed embryonic cells [43]. However, in this study we were able to induce zebrafish PGC-like cells in vitro from cultured embryonic cells. Different combinations of growth

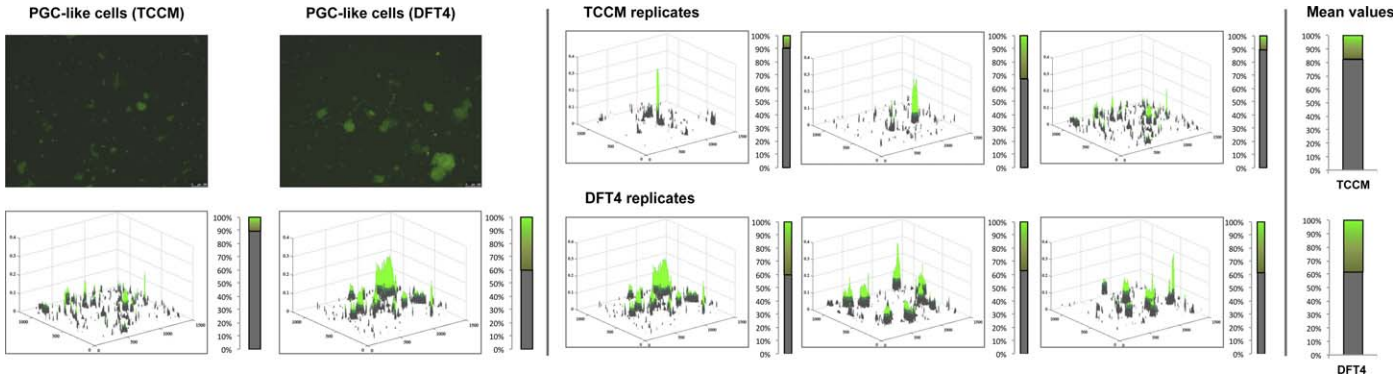


FIG. 5. Histograms obtained with bioimaging software for PGC-like cell detection by fluorescence. The digital image processing was performed with computer vision software for each field capture in TCCM and DFT4 cell cultures. Pixels with green fluorescence intensity, corresponding to PGC-like cells, were selected. An increase in percentage was reported with DFT4 field captures compared with TCCM.

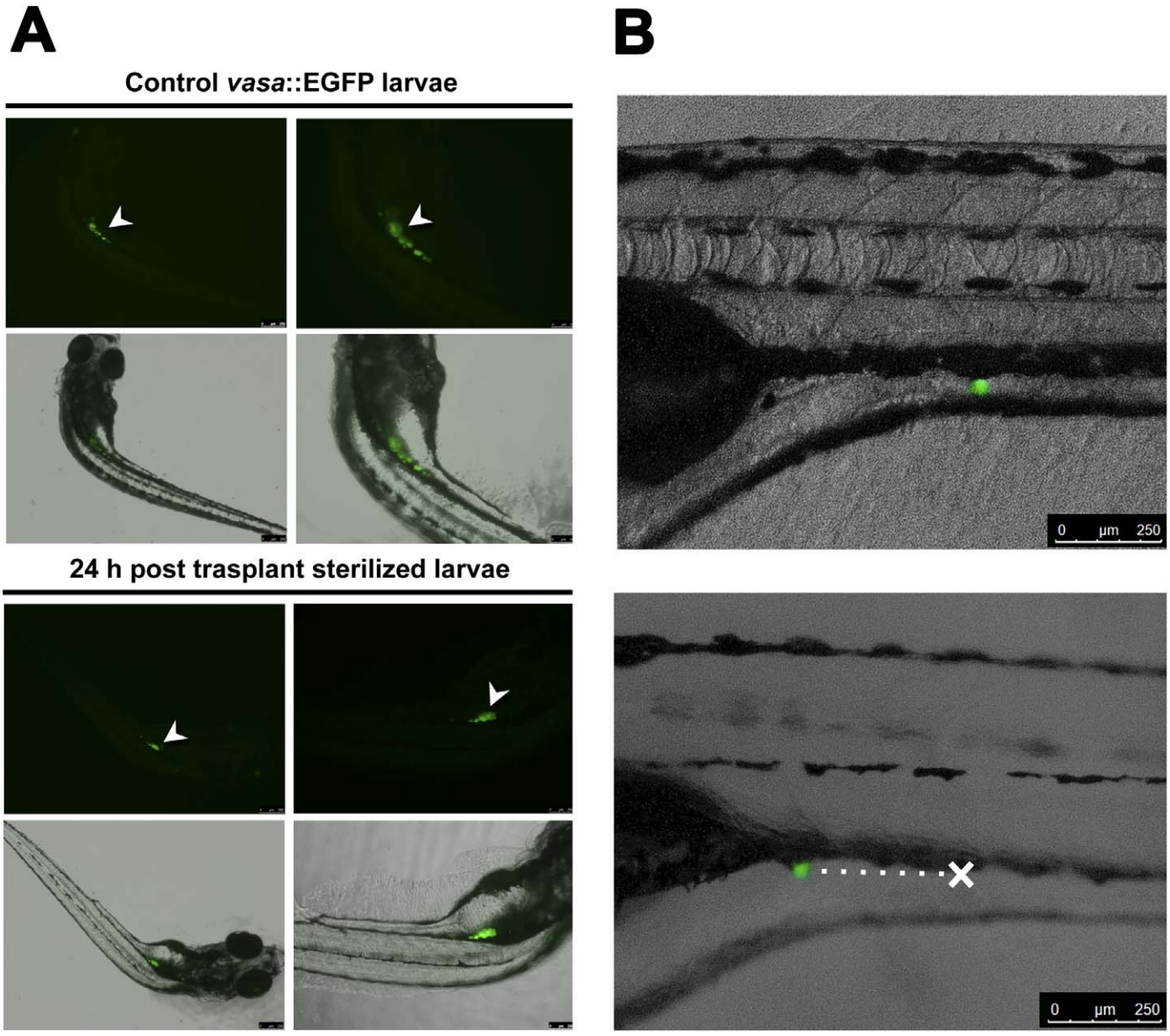


FIG. 6. **A**) The PGC-like cells can successfully colonize the genital ridge after transplantation in a recipient larva. Transplanted PGC-like cells migrated to the genital ridge as the endogenous PGCs (arrows). The PGC-like cells obtained from the best treatment for PGC differentiation (DFT4) were transplanted into infertile sterilized 7-dpf recipient larvae (injected with *dnd-1* morpholino). Control (*vasa*:EGFP transgenic 7-dpf larvae) and transplanted larvae were photographically recorded 24 h after transplantation. **B**) The PGC-like cell migration in the genital ridge after transplantation in a recipient larva. A single PGC-like (DFT4) transplanted cell migrated along the genital ridge of a sterile 7-dpf recipient larva. The migration pathway during 24 h is marked with a discontinuous line. Bar = 250 µm.

factors such as BMP4, EGF, and RA were used to achieve a significant increase in in vitro PGC generation (Fig. 1). The use of these three factors together with a slightly modified TCCM as initially described by Sakai [26] was sufficient to significantly increase the number of zebrafish PGC-like cells derived from blastomeres in vitro.

The PGC-like cells were characterized by several lines of evidence, including the expression of PGC-related genes (qPCR analysis), PGC fluorescence intensity and morphology (image analysis and flow cytometry), and PGC transplantation. Moreover, cell cycle analyses were carried out to ensure that the increase in the number of PGC-like cells after treatment was not due to increased cell proliferation.

In PGC-related gene expression studies, we selected genes expressed in PGCs, including *dnd*, which is crucial to PGC survival and migration [44], *vasa*, which is relevant in germ cell lineage and PGC development [45], and *cxcr4b*, which is involved in PGC migration [46]. The qPCR results demonstrated that DFT4 produced a significant increase in all the studied transcripts, either those specifically expressed in PGCs (*vasa* and *dnd*) or that required for pro-PGC migration (*cxcr4b*). In contrast, DFT1 produced the lowest expression levels for all studied transcripts, with these results being in concordance with the PGC-like cell number detected by flow cytometry analysis.

However, an increase in gene expression does not necessarily imply an increase in the number of PGC-like cells.

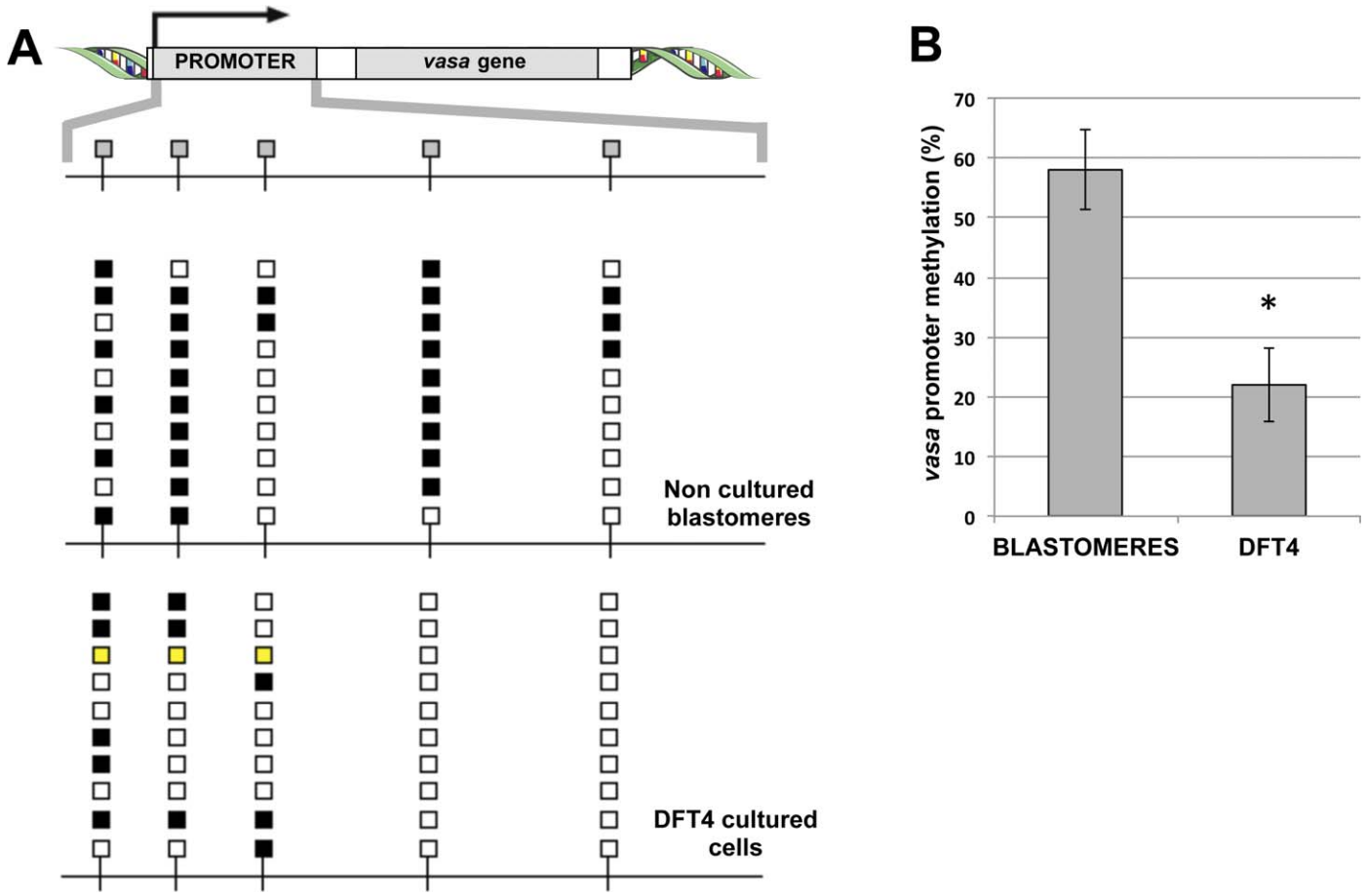


FIG. 7. Bisulphite sequencing analysis of CpG methylation in *vasa* promoter. Different methylation levels were found in DFT4-treated and nontreated embryonic cells. The asterisk shows statistical differences after a one-way ANOVA ($P < 0.05$). **A**) Black squares indicate methylated CpGs, and white squares indicate unmethylated islands. The software showed islands with an undetermined methylation pattern as yellow squares. **B**) The percentage of methylated CpGs for *vasa* promoter was shown (n = 10).

Treatments could be inducing an increase in transcription rates without increasing the number of PGC-like cells. For this reason, we performed a flow cytometry study to assess how the populations of somatic vs PGC-like cells were evolving with the different treatments. In this study, we used 20 to 25 somite developmental stage *vasa* transgenic line embryos as a control. The number of PGCs detected in the control (19.7%) was in concordance with previous investigations (20.5%) [47], as well as the GFP intensity of this population (greater than logarithm 10^2–10^3), demonstrating that the analysis of results and the cytogram for PGCs and somatic cell populations were correct. Surprisingly for us, flow cytometry results demonstrated that TCCM medium produced by itself an increase in the PGC-like cell population (42.2%). In this respect, TCCM medium has been used together with different serum and feeders by Sakai [26] to induce in vitro differentiation from spermatogonia to functional sperm, and its effect also seems to be crucial in previous stages of germline differentiation.

The addition of other factors (RA, EGF, and BMP4) to the TCCM produced a higher increase in the number of PGC-like cells. The number of PGCs increased 2.68-fold with DFT1 treatment (in respect to the control), 3.10-fold with DFT2 treatment, 3.13-fold with DMF3 treatment, and 3.03-fold with DFT4 treatment. It is interesting to point out that the increase in the PGC-like cell population occurred at the same time that the somatic population decreased. According to the expression analysis results, DFT1 showed lower differentiation capacity than other DFTs.

The simultaneous decrease in somatic cells in parallel with the increase in the PGC-like cell population clearly indicates that the in vitro treatments induce differentiation rather than proliferation. However, in order to confirm this evidence, a cell cycle analysis was also performed by flow cytometry. Our results indicated that in all samples most of the cells were in gap 0/gap 1 (G_0/G_1) according to previous investigations in embryonic stem cells [14]. Except for DFT1, treatments do not increase the number of cells in G2/M. Moreover, the best DFT according to previous results (DFT4) obtained the lowest event number in G2/M. These results suggest that the increase in the number of PGC-like cells is due to an increase in differentiation rather than in cell proliferation.

In order to provide more evidence for these observations, image analysis was performed using MATLAB for square matrix and labeling data points. These results evidenced an increase in EGFP-positive cells in treated cells in accordance with our previous results. The DFT4 treatment gave a higher number of EGFP-positive cells with high fluorescence. Although the induction of PGC differentiation in fish has been considered impossible until now due to germplasm inheritance, this study demonstrates that it could be possible in vitro. The *vasa* marker, the tool employed for the confirmation of the different PGC specification mechanisms between mammals and fish, has also been used in this study as a tool for

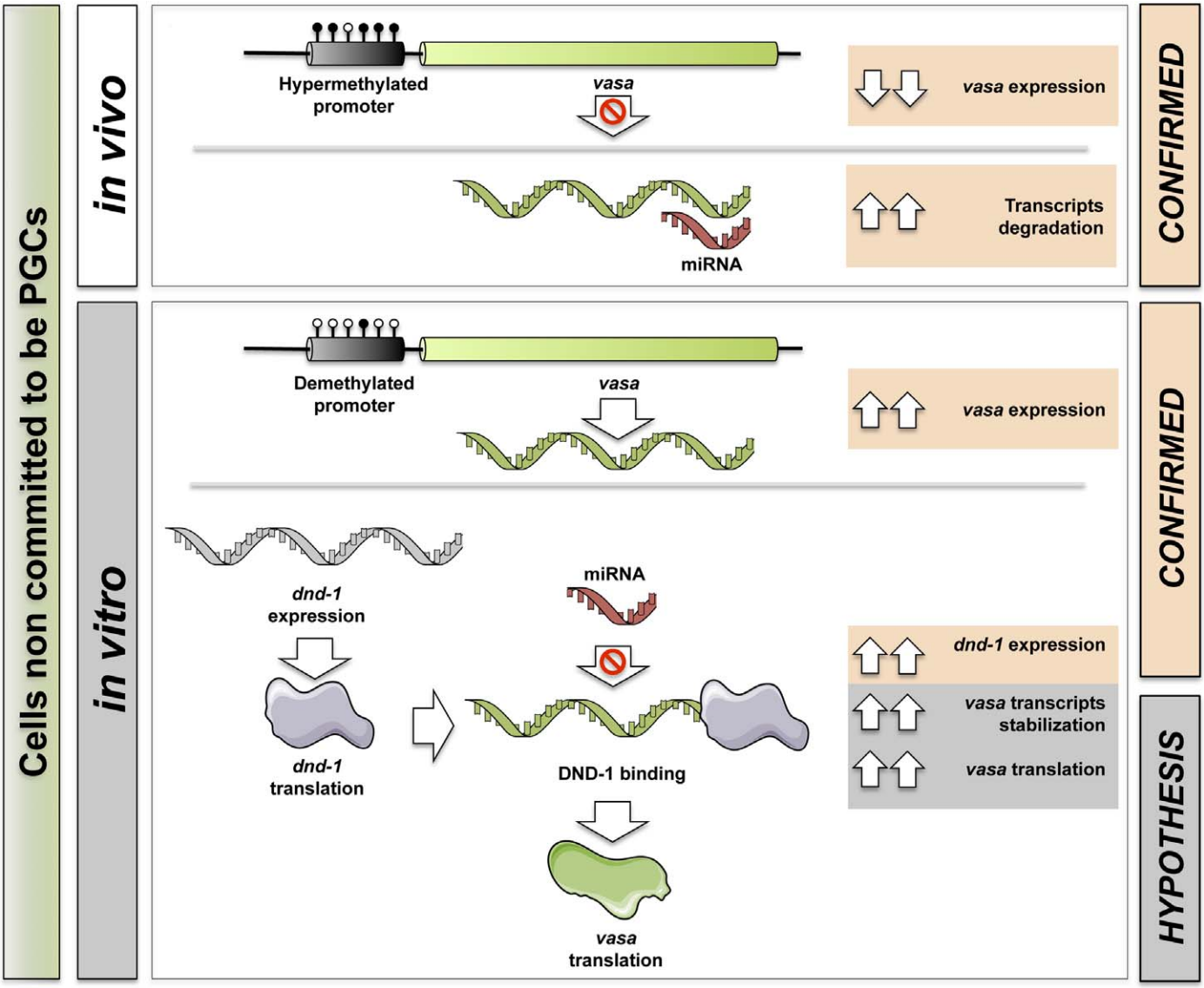


FIG. 8. Hypothesis to explain in vitro generation of zebrafish PGCs. In vitro cultured cells could be undergoing epigenetic modifications that could produce a sufficient increase in the expression of PGC markers to explain PGC in vitro generation. Moreover, in vitro conditions could artificially increase proteins involved in the stabilization of transcripts relevant for PGC viability such as DND-1. Our results demonstrate that DFTs, in particular DFT4 (considered to be the best one in this study), significantly increased the amount of *dnd-1* transcripts and induced the demethylation of *vasa* promoter.

demonstrating possible PGC generation in vitro. However, besides the innovative nature of this study, it is not contradictory to previous studies in teleost PGC specification. There is strong evidence that embryonic cells, once plated and cultured with certain media, can be significantly altered [14]. Two reasons could explain why these cells can respond in vitro rather than in vivo to the employed DFTs. In fact, both reasons are not exclusive and could even be complementary. In one way, in vitro cultured cells could be undergoing epigenetic modifications (such as *vasa* [and other PGC-related genes] promoter demethylation) that could produce a sufficient increase in the expression of PGC markers to explain PGC in vitro generation (Fig. 7). It is well known that mouse DNA methylation is a key factor in regulating the expression of crucial genes related to PGC [48].

However, more importantly, the alteration in gene expression caused by culture conditions could be affecting molecules involved in mechanisms of transcript stabilization and degradation. In vivo *vasa* transcripts are easily degraded in cells that are not committed to PGCs. We hypothesized that in vitro conditions could artificially increase proteins involved in the stabilization of transcripts relevant for PGC viability (Fig. 8). One of the most widely known mRNA-binding proteins indispensable for PGC viability is DND-1 [49]. This protein avoids micro-RNA degradation by binding to its targeted transcripts. Our results demonstrate that DFTs, in particular DFT4 (considered to be the best one in this study), significantly increased the amount of *dnd-1* transcripts. Moreover, a demethylation phenomenon was also demonstrated after DFT4 treatment (Fig. 7), suggesting the synergic effect between both processes previously mentioned.

The different approaches used in this study confirm successful differentiation to PGC-like cells. The successful transplantation of PGC-like cells provided the final evidence for the success of these treatments for in vitro PGC generation. Considering the relevance of the obtained results, we envision that future work could address the improvement of differentiation efficiencies using different combinations of factors. In

order to have a standardized procedure for PGC differentiation assessment to compare work performed in different laboratories, we have provided the program developed in this study for image evaluation as supporting information (Supplemental Figure S2).

The derivation of PGC-like cells has critical implications in stem cell biology and biotechnology. This work has broken away from the idea that teleost PGC induction cannot be achieved in vitro. Once this possibility is open, genetic manipulations can be performed in embryonic stem-like cell cultures (more easily transfected), and after modification and selection, cells can be forced to differentiate into PGCs using DFT2 and DFT4 rapidly, improving their germline contribution after transplantation in a recipient embryo. This is even more promising considering that recent work [6] reported methods to ensure complete germ cell replacement using a single PGC transplantation approach. These technologies are crucial to consolidate zebrafish as a potent model species in science, and we anticipant that this work will be a step forward in this direction. Studying these differentiation media in other teleosts with commercial or scientific interest should be carried out in order to evaluate if these results have a species-specific effect or not.

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