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Ex vivo transfection of trout pronephros leukocytes, a model for cell culture screening of fish DNA vaccine candidates

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ABSTRACT

DNA vaccination opened a new era in controlling and preventing viral diseases since DNA vaccines have shown to be very efficacious where some conventional vaccines have failed, as it occurs in the case of the vaccines against fish novirhabdoviruses. However, there is a big lack of *in vitro* model assays with immune-related cells for preliminary screening of *in vivo* DNA vaccine candidates. In an attempt to solve this problem, rainbow trout pronephros cells in early primary culture were transfected with two plasmid DNA constructions, one encoding the green fluorescent protein (GFP) and another encoding the viral haemorrhagic septicaemia virus (VHSV) glycoprotein G (G_{VHSV}) – the only viral antigen which has conferred *in vivo* protection. After assessing the presence of GFP- and G_{VHSV}-expressing cells, at transcription and protein levels, the immune response in transfected pronephros cells was evaluated. At 24 h post-transfection, G_{VHSV} up-regulated *migm* and *tcr* transcripts expression, suggesting activation of B and T cells, as well, a high up-regulation of *tnfα* gene was observed. Seventy-two hours post-transfection, we detected the up-regulation of *mx* and *tnfα* genes transcripts and Mx protein which correlated with the induction of an anti-VHSV state. All together we have gathered evidence for successful transfection of pronephros cells with pAE6G, which correlates with *in vivo* protection results, and is less time-consuming and more rapid than *in vivo* assays. Therefore, this outcome opens the possibility to use pronephros cells in early primary culture for preliminary screening fish DNA vaccines as well as to further investigate the function that these cells perform in fish immune response orchestration after DNA immunisation.

1. Introduction

One of the best methods to fight against viral infections is the prevention by means of vaccination. However, although many efforts have been paid on vaccination, results are less encouraging. DNA vaccination opened a new era in the control and prevention of viral diseases and demonstrated to be very efficacious where conventional vaccines had failed. In fact, three DNA vaccines have been licensed since 2005 in the area of veterinary medicine [1], one for horses West Nile virus [2], another for dogs melanoma [3] and another for salmonid fish infectious hematopoietic necrosis rhabdovirus (IHNV) [4]. However, DNA vaccines still face several challenges that constitute an impairment for their approval in many countries, for example in Europe [5].

Regarding the DNA vaccine against IHNV, which is based on a plasmid encoding the surface antigen of the virus (the glycoprotein G gene) [6], it is remarkable that this vaccine has turned into an invaluable tool to study and understand how DNA vaccines function and confer protection in fish [7]. However, so far it has not been possible to develop any other fish DNA vaccines as efficacious as that for IHNV and other novirhabdoviruses. This is mainly due to the incomplete understanding of how fish immune system works as well as to the lack of appropriate *in vitro* cell systems to analyse and screen the immune response induced by potential DNA vaccine candidates.

In vivo rainbow trout DNA vaccination investigations with the glycoprotein G of IHNV have demonstrated the transfection of migrating antigen-presenting cells or direct transfection of distal tissue cells, since its presence has been detected in trout head kidney tissue after intramuscular injection, being mainly located in areas of melanomacrophage aggregates [8]. However, to date evidence for plasmid DNA transfection of *ex vivo* pronephros cells in primary cultures has not been given, a potential useful tool for screening candidates for the best immune response induced by fish DNA vaccines. Nevertheless, the expression of G_{VHSV}, another fish novirhabdovirus that causes severe problems in European

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salmonid aquaculture, has been reported in trout primary gill cells [9].

The pronephros or head kidney is the major haematopoietic tissue in fish [10] and many studies have confirmed the usefulness of pronephros cell cultures for immunological and pathological studies. For instance, it has been demonstrated that fish pronephros responds differently to viral [11–13], bacterial [12,14] and fungal [12] pathogen associated molecular patterns (PAMPs), as well as to immunogenic viral antigens [15] and immunoestimulants like unmethylated oligodeoxynucleotides (ODNs) [16] and imiquimod [17]. This suggests that fish pronephros cell cultures exert different immune responses upon activation by different factors.

Here we report, for the first time, a successful DNA transfection of pronephros cells in early primary culture using a plasmid encoding a reporter gene (*GFP*) and another plasmid encoding the G_{VHSV} gene, which it is known to protect *in vivo* against VHSV lethal challenge [18]. Overall, the results have shown that pronephros cells, mainly monocyte/macrophage-like cells, can successfully express both genes, at transcriptional and protein levels, after transfection using conventional methods. In addition, the immune response triggered by G_{VHSV} could be evaluated. All together, this outcome evidenced that *ex vivo* pronephros cell transfection could be used for the screening of vaccine candidates in a less time-consuming manner than *in vivo* protection/challenge assays. Furthermore, these results open the possibility to use pronephros cells cultures to further investigate the function that these cells perform in the fish immune response after DNA immunisation.

2. Materials and methods

2.1. Fish

Adult rainbow trout (*Oncorhynchus mykiss*) of approximately 150 g. obtained from a VHSV-free commercial farm (Piszolla Group, Zaragoza, Spain), were maintained at the University Miguel Hernandez (UMH) facilities at 12–14 °C. Prior to experiments, fish were acclimatised to laboratory conditions during 2 weeks.

2.2. Pronephros cells primary culture

Fish were sacrificed by overexposure to tricaine (tricaine methanesulfonate) (0.2 g/l) and then ex-sanguinated. Head kidney (pronephros) was aseptically removed, placed in a 15 ml tube with RPMI-1640 medium (Dutch modification) (Gibco, Invitrogen Coorp., Grand Island, NY) supplemented with 0.1% FBS gamma irradiated (Cultek, Madrid, Spain), 100 U ml^{-1} penicillin (PAA Laboratories, Pasching, Austria), 100 μg ml^{-1} streptomycin (PAA laboratories) and 10 U ml^{-1} heparin (Sigma, Madrid, Spain), disaggregated with a Pasteur pipette and passed through a Falcon 100 μm nylon cell strainer (BD Biosciencies, Madrid, Spain) using a plunger of a 5 ml syringe. Cell viability was determined with trypan blue and cell concentration was adjusted to 5×10^5 cells/well using a haemocytometer. Cells were seeded in RPMI medium 1640 supplemented with 10% FBS and 2% trout serum in 24-well plates at a concentration of 5×10^5 cells/well in a final volume of 500 μl and then acclimatised for 1 day at 20 °C prior transfection in a 5% CO_2 atmosphere.

2.3. Plasmids

The plasmid pAE6 [19] containing the 5′ regulatory sequences of carp β-actin gene (Gene bank accession number M24113) was used in this study. The pAE6 plasmid constructions encoding the sequences of the *GFP* (pAE6GFP) or G_{VHSV} gene (pAE6G) were obtained as previously described [19,20]

2.4. Transfection assays

Cell transfections were carried out as described previously for other fish cells [21,22]. Briefly, pronephros cells were transfected with 1.25 μg of pAE6, pAE6G or pAE6GFP plasmids complexed with 1.5 μl of TurboFect™ *in vitro* transfection reagent (Fermentas, York, UK), and the plates were further incubated at 20 °C for 24 or 72 h.

Table 1
Primer sequences used for gene expression analysis.

Primer name	5′–3′ sequence	Reference/accession no.
ef1α-fw	ACCCTCCTCTTGGTCGTTTC	[24]
ef1α-rv	TGATGACACCAACAGCAACA	
ef1α-probe	GCTGTGCGTGACATGAGGCA	
mx1-3 fw	TGAAGCCCAGGATGAAATGG	[22]
mx1-3 rv	TGGCAGGTCGATGAGTGTGA	
mx1-3 probe	ACCTCATCAGCCTAGAGATTGGCTCCCC	
tnfα fw	AGCATGGAAGACCGTCAACGAT	[50]
tnfα rv	ACCCTCTAAATGGATGGCTGCTT	
tnfα probe	AAAAGATACCCACCATACATTGAAGCAGATTGCC	
cd83 fw	TTGGCTGATGATTCTTTCGATATC	AY263797
cd83 rv	TGCTGCCAGGAGACACTTGT	
cd83 probe	TCCTGCCCAATGTAACGGCTGTTGA	
migm fw	AAAGCCTACAAGAGGGAGACCGAT	[51]
migm rv	AGAGTTATGAGGAAGAGTATGATGAAGGTG	
migm probe	CTCGTGTTGACTGACTGTCCATGCAGCAAC	U04616.1
tcr fw	AGCACCCAGACTGCCAAGCT	EU072699.1
tcr rv	GAGGAGCCCTGGAACTTCCA	
tcr probe	TCTTCATCGCTAAGAGTACCTTCTATGGCCTGGT	
GFP fw	GGGCACAAGCTGGAGTACAAC	U76561
GFP rv	CACCTTGATGCCGTTCTTCTG	
G_{VHSV} fw	GGGCCTTCCTTCTACTGGTACTC	[52]
G_{VHSV} rv	CGGAATCCCGTAATTTGGAAT	
G_{VHSV} probe	CTGTTGCTGCAAGGCGTCCCCT	
N_{VHSV} fw	GACTCAACGGGACAGGAATGA	[52]
N_{VHSV} rv	GGGCAATGCCCAAGTTGTT	
N_{VHSV} probe	TGGGTTGTTCACCCAGGCCGC	

ef1α: elongation factor 1 alpha; *mx*: isoforms 1 and 3 of the trout antiviral Mx protein; *tnfα*: tumour necrosis factor alpha; *cd83*: *cluster of differentiation 83*; *tcr*: T-cell receptor; *migm*: membrane immunoglobulin M; *GFP*: green fluorescent protein; G_{VHSV}: G protein of VHSV; N_{VHSV}: N protein of VHSV; fw: forward; rv: reverse.

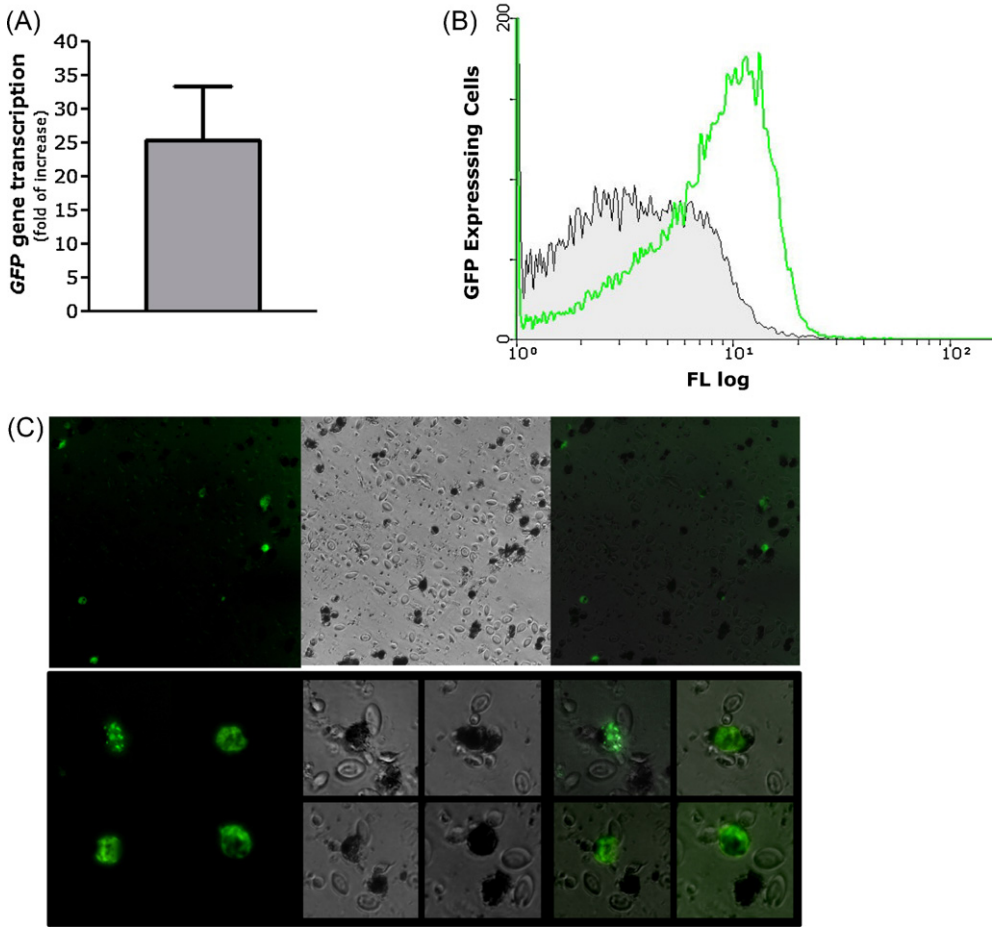

Fig. 1. Transfection of pronephros cells monitored by green fluorescent protein (GFP) mRNA transcript levels by qPCR (A), GFP-expressing cells by flow cytometry (B) and GFP-expressing cells by fluorescence microscopy (C). Cells were transfected with pAE6GFP, at 20 °C. GFP gene transcripts levels 24 h post-transfection (A). Gene expression was normalised against *ef1α* gene and relative to control (non-transfected) cells. Gene expression data represents the mean fold changes ± SD ($n = 5$). Representative histogram of the number of GFP-expressing cells (B), 72 h post-transfection, estimated by flow cytometry. Green line, transfected cells. Grey, control cells. Representative micrographs of GFP-expressing cells (C), 24 h post-transfection. Visible, light and merged images from left to right, respectively. Three different fish were analysed for flow cytometry and fluorescence microscopy.

Cells were treated with 30 μg ml^{-1} of Polyribocytidylic acid (pIC) (Sigma), as a positive control for immune response evaluation. After the incubation period, cells for quantitative PCR (qPCR) assays were centrifuged at 371 g for 10 min, the medium removed and total RNA extracted as described in Section 2.5. On the other hand, cells for flow cytometry assays were centrifuged at 157 g for 10 min, the medium removed and flow cytometry analysis proceeded as described in Section 2.7.

2.5. RNA isolation and cDNA synthesis

Total RNA was extracted from the treated, transfected and/or infected pronephros cells using the "RNeasy® Plus Mini" kit (Qiagen, Valencia, CA), following manufacturer's instructions. Isolated RNAs were resuspended in DNase and RNase free water (Sigma) and stored at −80 °C until used. Two hundred nanograms of total RNA were used for cDNA synthesis using the Moloney murine leukaemia virus reverse transcriptase (M-MLV) (Invitrogen, San Diego, CA, USA) and Random Hexamers (Applied Biosystems, NY, USA), following manufacturer's instructions.

2.6. Gene expression analysis

Quantitative real time PCR (qPCR) assays were performed using the ABI PRISM® 7300 Sequence Detector System (Applied Biosystems). Reactions were carried out in a final volume of 20 μl, containing 900 nM of each primer, 300 nM of the TaqMan® probe conjugated with the fluorescein FAM at the 5′ end and with the quencher TAMRA at the 3′, 20 ng of RNA and 1× TaqMan® Universal Master Mix (Applied Biosystems). Primers and probes sequences used are indicated in Table 1. For *GFP* gene primers SYBR Green (Applied Biosystems) was used. Primers for *cd83*, *tcr* and, *GFP* and probe for *migm* genes amplification were designed using the Primer Express™ software (Applied Biosystems). Thermal cycling conditions followed the standard default protocol of the instrument. Gene expression results were analysed using the $2^{-\Delta\Delta Ct}$ method [23]. Endogenous control for quantification was the *ef1α* gene [24]. Gene expression was normalised against *ef1α* gene and relative to control cells. Gene expression data represent the mean fold changes ± SD and correspond to 5 different fish for 24 h assays and 3 different fish for 72 h assays, each performed in duplicate.

2.7. GFP, G_{VHSV} and Mx protein expression by flow cytometry and fluorescent microscopy

The expression of GFP, G_{VHSV} and Mx proteins in *ex vivo* transfected pronephros cells in primary culture was evaluated 72 h post-tranfection. Then, cells were centrifuged at 157 g for 10 min and the medium removed. For G_{VHSV} protein detection a cocktail of anti-G_{VHSV} monoclonal antibodies (MAbs) (C10, 3F1A2 and

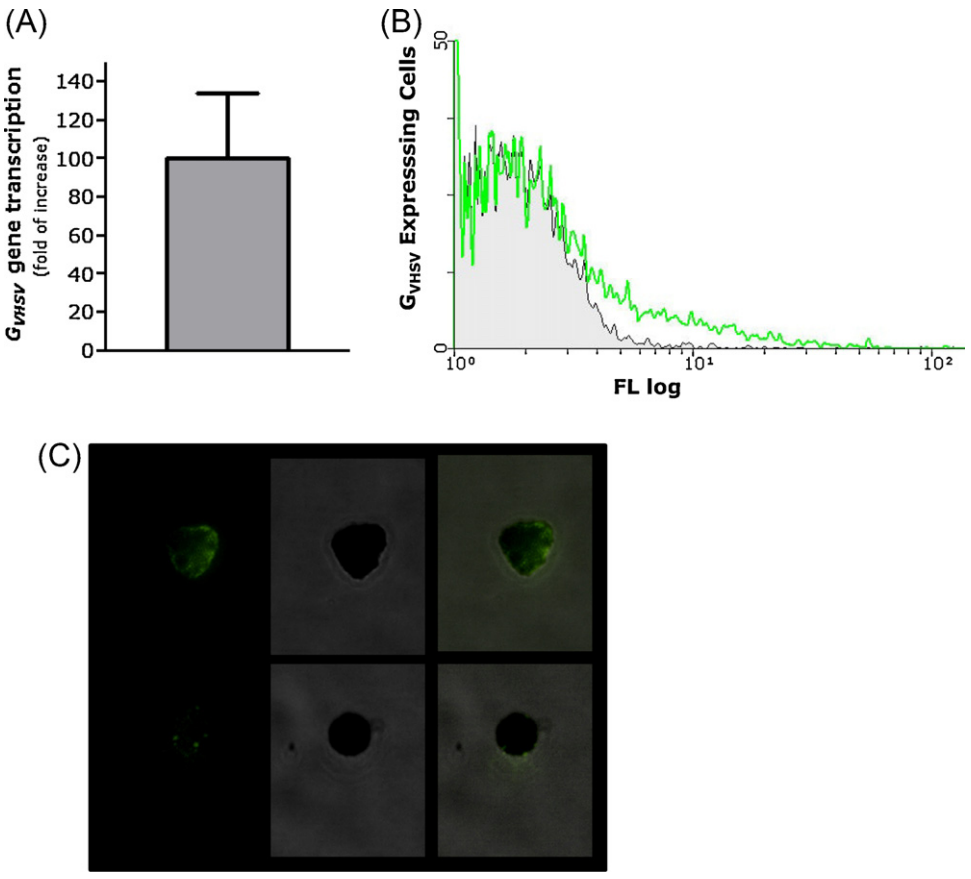

Fig. 2. G_{VHSV} transfection of pronephros cells. G_{VHSV} mRNA transcript levels (A), number of G_{VHSV}-expressing cells by flow cytometry (B) and G_{VHSV}-expressing cells visualisation by indirect immunofluorescence. Cells were transfected with pAE6G, and transfection evaluated after 72 h at 20 °C. G_{VHSV} gene transcripts levels (A). Gene expression was normalised against *ef1α* gene and relative to control (non-transfected) cells. Gene expression data represent the mean fold changes ± SD ($n = 5$) Representative G_{VHSV}-expressing cells estimated by flow cytometry (B). Green line, transfected cells. Grey, control cells. Representative G_{VHSV}-expressing cells micrographs (C) at visible, light and merged images from left to right, respectively. Three different fish were analysed for flow cytometry and fluorescence microscopy.

I16) [25] diluted 500-fold in FACS buffer (PBS–1%BSA–0.1%sodium azide (Sigma) was used. For Mx protein detection, cell pellets were incubated with the antiserum to rainbow trout Mx protein [26] diluted 500-fold in FACS buffer with 0.1% Triton X100 (Merck, Darmstadt, Gemany) for 1 h at RT. Afterwards, the cells were washed with FACS buffer and incubated for 30 min with fluorescein-labeled rabbit anti-mouse immunoglobulinG (IgG) Ab (Sigma) diluted 300-fold in FACS buffer, with 0.1% Triton X100. Finally, the cells were washed three times (157 g for 10 min) and resuspended in FACS buffer. Cell associated fluorescence was analysed with a BD FACS Canto™ II flow cytometer (BD Biosciences, NJ, USA). For each sample, 10,000 cells were analysed. Background fluorescence profiles were obtained using non-transfected pronephros cells. Results were visualised using WinMDI 2.9 (http://en.bio-soft.net/other/WinMDI.html). Three different fish, each in duplicate, were analysed.

GFP and G_{VHSV} expression in GFP and G_{VHSV} transfected pronephros cells were viewed and photographed 24 or 72 h post-transfection, respectively with an inverted fluorescence microscope (Nikon Eclipse TE2000-U, Nikon instruments Inc., NY) provided with a digital camera (Nikon DS-1QM).

2.8. Infection of rainbow trout pronephros cells with VHSV

Rainbow trout pronephros cells in primary culture, grown in 24-well plates, were transfected with pAE6 or pAE6G, or treated with pIC during 72 h at 20 °C. After the incubation period, cells were infected with VHSV (VHSV-07.71 isolate [27] grown as previously reported [28]) at a multiplicity of infection (m.o.i.) of 10^{-2} and incubated at 14 °C for 24 h. After incubation, total RNA was extracted and VHSV replication evaluated by qPCR as described in Sections 2.5 and 2.6, respectively, using the specific primers and probe for the gene encoding the protein N of VHSV (N_{VHSV}) (Table 1).

2.9. Statistical analysis

Data were analysed using a paired *t* test (Graph Pad Prism 5 software) to determine the differences between treated and control groups. Statistical differences were considered significant when $p < 0.05$.

3. Results

3.1. Evaluation of transfection of pronephros cells

As a first approach, pronephros cells were transfected with the plasmid encoding for the GFP reporter gene (pAE6GFP). At 24 h post-tranfection, GFP transcripts were detectable in all of the cultures indicating a successful *ex vivo* pronephros cell transfection. The average levels of GFP transcript expression are shown in Fig. 1A. Next, the expression of GFP was estimated at the protein level by flow cytometry and resulted to be ≈36% (Fig. 1B). Finally, melanomacrophage-like cells were identified as the main source of GFP-expressing cells by fluorescence microscopy (Fig. 1D).

Having established that the GFP gene was efficiently expressed at transcript and protein levels in transfected pronephros cells

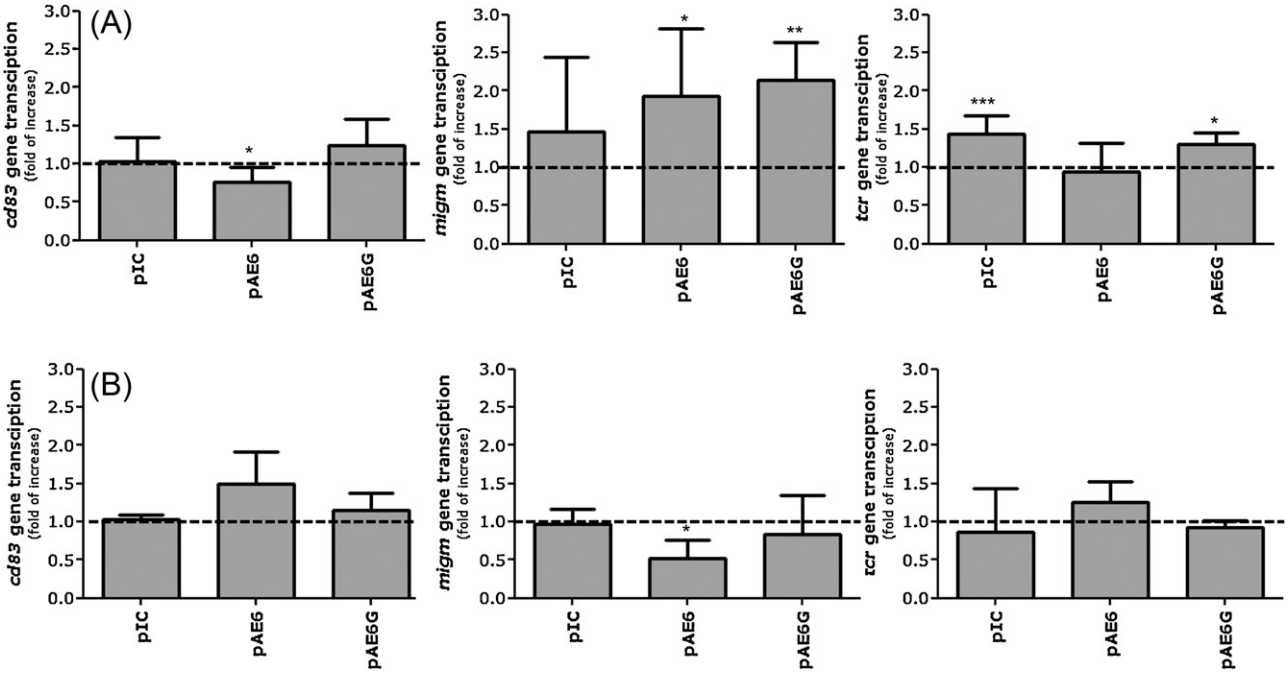

Fig. 3. *cd83*, *migm* and *tcr* genes transcript levels in pronephros cells treated with pIC or transfected with pAE6 or pAE6G, 24 (A) or 72 (B) h, respectively. Gene expression was normalised against *ef1α* gene and relative to control cells. Data represent the mean fold changes ± SD, $n = 5$ (A) or $n = 3$ (B). Asterisk denotes statistically significant differences between the different treatments and the control cells (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

in primary culture, their capability to express transfected G_{VHSV} was then evaluated. Transcripts (Fig. 2A) and G_{VHSV}-expressing pronephros cells (Fig. 2B) could be detected at 24 and 72 h post-transfection, respectively. The percentage of G_{VHSV}-expressing cells was ≈10% as determined by flow cytometry. Transfected macrophage-like cells were observed (Fig. 2C).

On the other hand, no direct correlation between transcript and protein expression levels was observed in G_{VHSV} or GFP genes (Figs. 1 and 2).

3.2. Pronephros cell types in transfected cell cultures

The presence of different cell types in pronephros cell cultures, before and after transfection with the plasmid encoding G_{VHSV}, was evaluated by quantifying the transcript levels of the *cd83* (cluster of differentiation 83, an antigen presenting cell marker), *migm* (membrane immunoglobulin M, a B cell marker) and *tcr* (T cell receptor, a T cell marker) genes 24 and 72 h post-transfection. Twenty-four hours post-transfection, the expression of transfected G_{VHSV} significantly up-regulated *migm* and *tcr* genes expression, but not that of *cd83*, suggesting activation of B and T cells (Fig. 3A). In addition, cell transfection with the empty plasmid (pAE6) also up-regulated the *migm* gene expression although not as much as pAE6G. Regarding cells treated with pIC, only up-regulation of the *tcr* transcripts could be observed, suggesting T cell proliferation. Seventy-two hours post-transfection, none of the treatments significantly changed any of the genes studied (Fig. 3B).

3.3. Immune response of pronephros cells induced after transfection with pAE6G

Twenty-four hours post-transfection, the pIC treated cells, but not the pAE6 or pAE6G transfected cells, significantly up-regulated the transcript expression of *mx* gene compared to untreated cells (Fig. 4A). However, 72 h post-transfection, only the pAE6G transfected cells significantly up-regulated both the *mx* gene and protein expression (Fig. 4B and C, respectively). On the other hand, at 24 h post-transfection pIC-treated and pAE6G-transfected cells up-regulated the *tnfα* gene transcript expression (Fig. 4A), while at 72 h post transfection only pIC up-regulated *tnfα* gene expression (Fig. 4B). pIC results are in accordance with previous studies, where pIC elicits an immediate, although low, inflammatory response [12,29], Separately, pAE6GFP transfected cells did not show a significant change in the expression of the immune-related genes (data not shown).

3.4. VHSV infectivity in transfected pronephros cells

Low N_{VHSV} transcript levels in cells transfected with pAE6G showed reduced VHSV infectivity (≤40%), when compared with untreated pronephros cells. Therefore, transfection of pronephros cells with pAE6G induced protection against VHSV infection. In contrast, pronephros cells treated with pIC or transfected with pAE6 propagated the virus almost as efficiently as untreated cells (VHSV infectivity ≥60%) (Fig. 5).

4. Discussion

The use of DNA vaccines has gained increasing interest since some of them are being routinely used with successful results [30]. However, because of the complexity of *in vivo* assays for vaccination studies, it would be helpful to have alternative *in vitro* cellular models for DNA vaccine screening.

In this context, we have evaluated the possibility of using rainbow trout pronephros cells in early primary culture as an approach to test fish DNA vaccine candidates. Because of the existence of possible culture-related phenotypic changes [31], we have used rainbow trout pronephros cells shortly after being extracted, when their cellular phenotype should be very similar to that found *in vivo*. Overall, the results have shown that pronephros cells in those primary cultures can successfully express both GFP and G_{VHSV} genes with respective moderate (36%) and low (10%) efficiencies, by using conventional transfection methods. Similarly, 11–30% transfection efficiencies have been obtained for EPC cells [32]. By contrast,

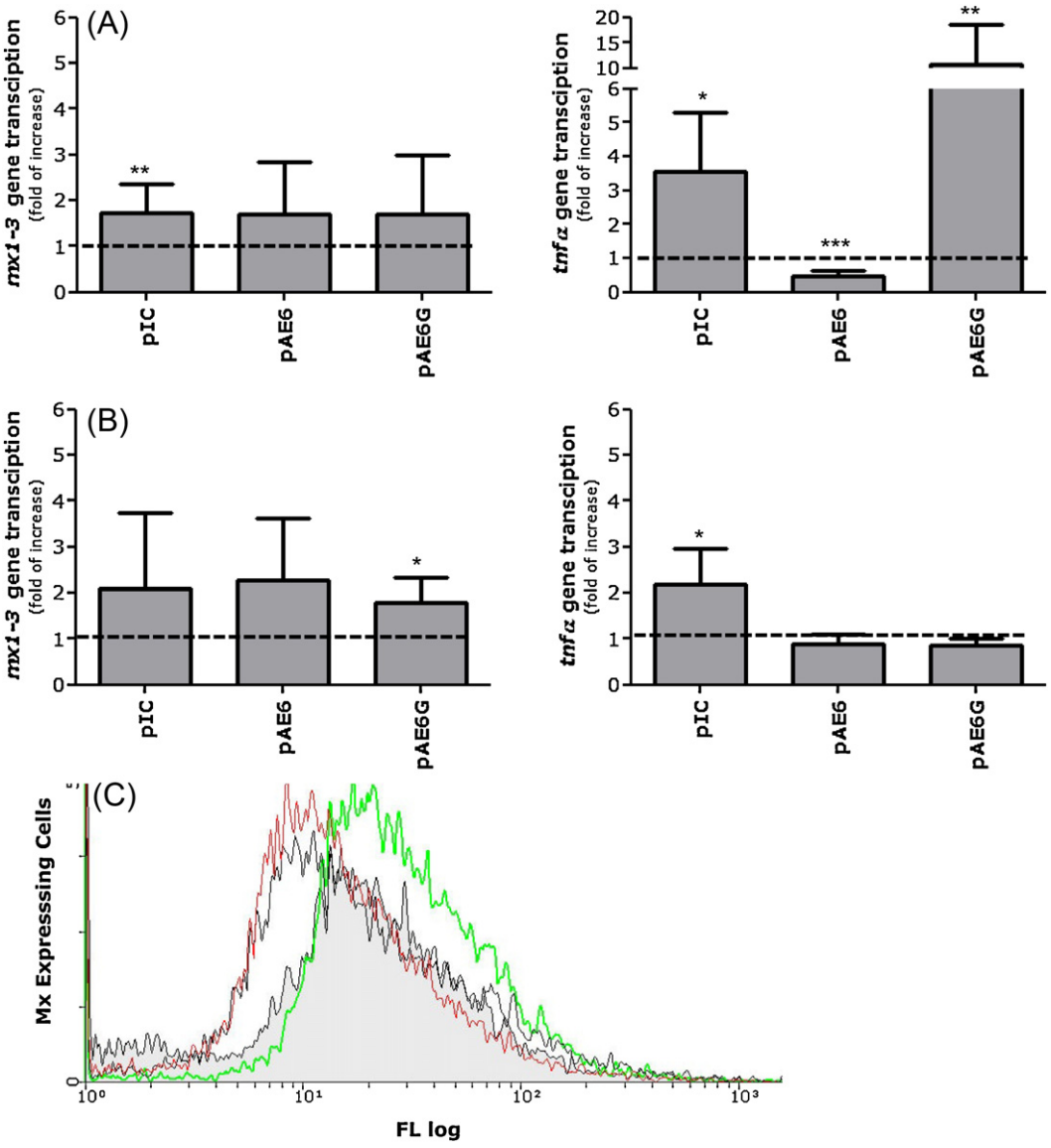

Fig. 4. Immune response induced after transfection of pronephros cells with pAE6G. Transcript levels of *mx1-3* and *tnfa* were measured in cells treated with pIC or transfected with pAE6 or pAE6G, 24 (A) or 72 (B) h, respectively, at 20 °C. Transcripts expression was normalised against *ef1α* gene and relative to control cells. Data represent the mean fold changes ± SD and correspond to $n = 5$ (A) or $n = 3$ (B) different fish. Asterisk denotes statistically significant differences between the different treatments and the control cells (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Mx protein expression was quantified by flow cytometry in cells treated with pIC or transfected with pAE6 or pAE6G (72 h, at 20 °C). Representative Mx-expressing cells estimated by flow cytometry (C), pAE6G transfected cells (green line), pAE6 transfected cells (black line), pIC treated cells (red line) and control cells (light grey filled).

higher transfection efficiencies (11–90%) have been found in the TO cell line (originated from Atlantic salmon head kidney) [33] using nucleofection [34]. On the other hand, the highest transfection efficiency reported for a fish cell primary culture (gill cells) using a plasmid encoding G_{VHSV} was 0.5% [9].

A first transfection evaluation by means of GFP- and G_{VHSV}-expressing cells visualisation showed that melano-macrophage-like cells were the main target for plasmid DNA transfection. This result agrees with those obtained *in vivo* where after immunising fish by intramuscular injection with a plasmid encoding the G gene of IHNV, the viral protein was mainly located in melanomacrophage aggregates [8]. The presence of transfected macrophage-like cells, probably the main source of antigen presenting cells (APC) in fish [17,35], suggests that APC could be processing endogenously produced transfected antigens prior to peptide presentation by the major histocompatibility complex (MHC) class I, as it occurs in mammals [36]. This phenomenon might play an important role in the orchestration of the fish protective immune response to genetic immunisation by intramuscular injection. Therefore, transfection of *ex vivo* pronephros cell cultures could be a new tool to *in vitro* study the four possible antigen-processing 'pathways' identified in mammalian dendritic cells (DCs) [37] including the cross-presentation.

On the other hand, the expression of transfected G_{VHSV} in pronephros cells induced a modest up-regulation of *migm* and *tcr* genes suggesting an activation of B and T cells, and therefore, an effective antigen pre-processing and presentation by APC-like cells. However, further protein analysis should be carried out to corroborate this statement. In contrast, a decrease in *migm* and *tcr* genes expression in G_{VHSV} transfected pronephros cells was observed three days post-transfection. It has been described in mammals that, once activated by the presence of mitogens or antigens, B cells begin to proliferate to become plasmablasts and this activated stage is characterised by a decrease in MHC class II and mIgM expression

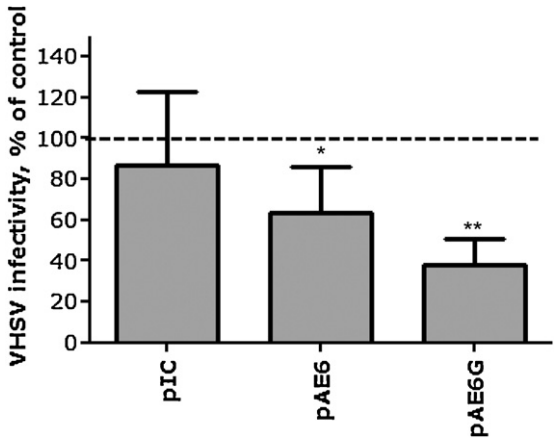

Fig. 5. VHSV infectivity estimated by N_{VHSV} gene expression analysis. Pronephros cells were treated with pIC or transfected with pAE6 or pAE6G (72 h at 20 °C), and then infected with VHSV with a multiplicity of infection (m.o.i.) of 10^{-2} pfu/cell. VHSV infectivity was quantified 1 day post-infection. Gene expression was normalised against *ef1α* gene and relative to control cells. Data represent the mean fold changes ± SD ($n = 3$) Asterisk denote statistically significant differences between the different treatments and the control cells ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

[38]. If similar B cell activation occurred in fish, this model could explain the later decrease in *migm* gene expression observed in G_{VHSV} transfected pronephros cells.

In vivo immunisation of trout with fish novirhabdoviruses glycoprotein G have demonstrated the induction of the type I IFN system at the injection site [18,35,39–41]. To evaluate the IFN related immune response in our *ex vivo* transfected pronephros cells we chose the IFN-inducible Mx because it has been proved to be a very specific and sensitive marker for type I IFN induction [42–45]. Furthermore, a direct correlation between *in vitro* G_{VHSV} expression and the induction of Mx and type I IFN [22,46] has been observed. Similarly, an up-regulation of the *mx* gene (both at transcript and protein levels) was detected in our study. Furthermore, we also report that transfected G_{VHSV} pronephros cells presented resistance to VHSV infection compared to non-transfected cells.

On the other hand, pro-inflammatory cytokines such as TNFα have been reported to be up-regulated at the injection site in *in vivo* DNA immunisation studies [18] as well as in *in vitro* G_{VHSV} cell transfection assays [22]. Besides, TNFα is a cytokine mainly produced by macrophages that plays a primary role in the regulation of immune cells [47]. Here, we report high levels of *tnfα* expression after G_{VHSV} transfection, comparable to those found in RTG-2 cells transfected with G_{VHSV} [22] and in trout head kidney leukocytes stimulated by lipopolysaccharide (LPS) [12,48]. Since regulation of trout TNFα might be closely linked to the differentiation status of monocyte/macrophage [49], our levels of *tnfα* expression in G_{VHSV} transfected pronephros cells could indicate macrophage maturation after G_{VHSV} transfection.

In conclusion, our results reported evidence for transfection of pronephros cells *in vitro* and open the possibility to investigate the function that these cells perform in fish immune response after DNA immunisation.

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