*Highlights

Four Nk-lysin genes were characterized for the first time in zebrafish (*Danio rerio*)

A different expression pattern was observed under different experiments/conditions

A certain cell specialization in the production of Nk-lysins could be proposed

Two Nk-lysins were significantly up-regulated after SVCV challenge

Zebrafish Nk-lysins: first insights about their cellular and functional diversification

**Pereiro P.¹, Varela M.¹, Diaz-Rosales P., Romero A., Dios S., Figueras A., Novoa
B.***

Instituto de Investigaciones Marinas (IIM), CSIC, Vigo, Spain

¹These authors contributed equally to the work presented in this paper.

*Corresponding author: Dr. Beatriz Novoa

Tlf: +34 986 214 463

Fax: +34 986 292 762

E-mail: beatriznovoa@iim.csic.es

Abstract

Nk-lysins are antimicrobial proteins produced by cytotoxic T lymphocytes and natural killer cells with a broad antimicrobial spectrum (including bacteria, fungi and parasites). Nevertheless, the implication of these proteins in the protection against viral infections is still poorly understood. In this work, four different Nk-lysin genes (*nkla*, *nkla*, *nkla* and *nkld*) were identified in the zebrafish genome. That means that zebrafish is the species with the higher repertoire of Nk-lysin genes described so far. The differential expression pattern of the Nk-lysins in several tissues, during ontogeny, among the different kidney cell populations, as well as between $Rag1^{-/-}$ and $Rag1^{+/+}$ individuals, could suggest a certain specialization of different cell types in the production of different Nk-lysin. Moreover, only two of these genes (*nkla* and *nkld*) were significantly up-regulated after viral infection, and this observation could be also a consequence of a functional diversification of the zebrafish Nk-lysins.

Keywords: Nk-lysin; Granulysin; Zebrafish; Rag1; Ontogeny; SVCV

Introduction

Nk-lysins (or granulysin in humans) are antimicrobial cationic proteins produced by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells and stored in cytolytic granules. These cells are important effectors of the immune system and play a relevant role in defense against a variety of targets (virus or bacteria-infected cells as well as tumor cells). An array of molecules, such as perforin, granulysin/Nk-lysin and numerous serine proteases (granzymes), are stored in cytoplasmic granules and released upon target cell recognition via Major histocompatibility complex (MHC) class I in order to induce apoptosis in infected cells or tumors (Lowin et al., 1994; Nagata and Golstein, 1995; Trapani and Smyth, 2002). MHC class I proteins present antigens to CTLs in the form of peptides on the surface of tumor or virus and bacteria-infected cells (Townsend et al., 1986) that are needed for the immune regulation of the NK-cells activity (Ljunggren and Kärre, 1990). The interaction of T-cell receptor (TCR) on CTLs with tumor or viral peptide–MHC class I complexes activates a signaling cascade that leads the granule polarization and release of granule toxins by exocytosis (Trapani and Smyth, 2002). In addition to their cytotoxic function, a direct and MHC class I-independent antimicrobial activity of these cells has been observed (Levitz et al., 1995; Oykhman and Mody, 2010).

Perforin is a pore-forming member of the membrane-attack-complex/perforin (MACPF) protein family with the ability to form transmembrane channels and cause osmotic lysis (necrosis) of the target cells (Trapani and Smyth, 2002). Moreover, perforin allows the entry of other cytotoxic components (mainly pro-apoptotic proteases known as granzymes) into the target cell for inducing apoptosis (Trapani and Smyth, 2002; Cullen et al., 2010). Although Nk-lysin was relegated to a secondary role during several years, its broad and direct antimicrobial spectrum (including bacteria, fungi and parasites) (Andersson et al., 1995; Wang et al., 2000; Jacobs et al., 2003; Endsley et al., 2004; Hirono et al., 2007) and the ability to lyse intracellular *Mycobacterium tuberculosis* following permeation of the cellular membrane by perforin (Stenger et al., 1998) has contributed to increase the interest in this molecule. This peptide could be required in the elimination of some intracellular pathogens, since perforin is able to kill the infected cells but not the intracellular *M. tuberculosis*. Nk-lysin is a member of the saposin-like protein (SAPLIP) family and therefore, possesses membrane-binding activity and capability to altering the membrane integrity (Ruysschaert et al., 1998). The presence of

this molecule in NK cells and of related peptides from the SAPLIP family in cytoplasmic granules of *Entamoeba histolytica* (amoebapores) suggests that these proteins are ancient but highly conserved during the evolution as an important antimicrobial host defense mechanism (Stenger et al., 1999).

Perforin-granzymes synergy is the main mechanism used by the cytotoxic cells to combat viral infections. The role of Nk-lysin in viral clearance is still poorly understood and even contradictory results were observed in some cases. The main objective of this work was to identify and characterize the zebrafish Nk-lysins, as well as to conduct some analysis in order to investigate the potential implication of these molecules in viral clearance. Moreover, the expression studies of the four zebrafish Nk-lysins in different tissues, during ontogeny, among different cell populations isolated from kidney and Recombination activation gene 1 deficient (Rag1^{-/-}) mutant zebrafish, shed some light about the specialization of different cell types in the production of certain Nk-lysins.

2. Material and methods

2.1. Sequence retrieval and characterization of zebrafish Nk-lysin genes

An exhaustive BLAST search (Altschul et al., 1997) was performed against the *Danio rerio* full genome (version Zv9) using Nk-lysin nucleotide sequences from fish (including an Nk-lysin gene from *D. rerio*) that were retrieved from the public NCBI nucleotide database (<http://www.ncbi.nlm.nih.gov/nucleotide>). Synteny conservation between zebrafish and several teleost species (*Astyanax mexicanus*, *Tetraodon nigroviridis*, *Takifugu rubripes*, *Gasterosteus aculeatus* and *Oryzias latipes*) as well as with the human granulysin gene was investigated using Genomicus v75.01. Four Nk-lysins genes located in tandem were identified. The full-length coding sequence of these genes was confirmed by PCR using specific primers (Supplementary data Table 1) and subsequent linking of the PCR product into pCR2.1-TOPO vector (Invitrogen) for their cloning using One Shot TOP10F' competent cells (Invitrogen) following the protocol instructions. cDNA sequencing of 4 selected clones from each Nk-lysin was conducted using an automated ABI 3730 DNA Analyzer (Applied Biosystems, Inc. Foster City, CA, USA).

The presence of signal peptide was analyzed with the SignalP 3.0 Server (<http://www.cbs.dtu.dk/services/signalp-3.0/>) (Emanuelsson et al., 2007) and the presence of specific domains with SMART 4.0 (<http://smart.embl.de/>) (Letunic et al., 2004). Molecular weight and isoelectric point were determined using the Compute pI/Mw tool from ExPASy (Gasteiger et al., 2003). The potential disulphide bonds between cysteines were analyzed using the server DiANNA 1.1 (Ferrè and Clote, 2005) and the 3D-structures of zebrafish Nk-lysins were predicted using I-TASSER server (Roy et al., 2010) selecting the model with the best C-score and viewed by PyMOL (<http://www.pymol.org>). The Template Modelling Score (TM-score), a measure of structural similarity between two proteins, was also considered in order to identify those structural analogs with known crystal architecture in the Protein Data Bank (PDB; <http://www.rcsb.org/pdb/>).

2.2. Sequence conservation and phylogenetic analysis

An alignment between *D. rerio* Nk-lysins and several Nk-lysins/Granulysins sequences from other fish and vertebrates was conducted using the ClustalW server (Thompson et al, 1994). Sequence similarity and identity scores were calculated with the software MatGAT (Campanella et al., 2003) using the BLOSUM62 matrix. Moreover, a phylogenetic analysis was conducted using several proteins belonging to the saposin-like proteins (SAPLIPs) family (Nk-lysins/Granulysin, Prosaposins, Acyloxyacyl hydrolases – AOA –, Pulmonary surfactant protein B – SP-B – and Amoebopores). The alignment was constructed also with ClustalW and the tree was drawn using Mega 6.0 software (Tamura et al., 2013). Neighbor-Joining algorithm (Saitou and Nei, 1987) was used as clustering method, the distances matrix was computed using Poisson correction method and complete deletion of the positions containing alignment gaps and missing data was conducted. Statistical confidence of the inferred phylogenetic relationships was assessed by performing 10,000 bootstrap replicates. The GenBank accession numbers of the sequences used in this section are listed in Supplementary data Table 2.

2.3. Animals

Adults, embryos and larvae from wild-type zebrafish were obtained from our experimental facility, where zebrafish are cultured following established protocols

(Westerfield, 2000; Nüsslein-Volhard and Dahm, 2002) (also see http://zfin.org/zf_info/zfbook/zfbk.html).

The sacrifices were performed via MS-222 overdose (500 mg/L⁻¹). Fish care and challenge experiments were reviewed and approved by the CSIC National Committee on Bioethics under approval number (07_09032012).

2.4. Experimental treatments

In order to analyze the constitutive expression of Nk-lysin genes in different tissues of adult zebrafish, spleen, liver, kidney, gill, caudal fin, whole head, muscle and intestine were sampled and pooled, yielding a total of 4 pools of 5 fish per organ.

With the aim of identifying the immune cells involved in the production of the different Nk-lysins, a total leukocyte suspension from kidney (using 10 adult zebrafish) was prepared by passing the tissues through a 100-µm nylon and the cells were diluted in Leibovitz's L-15 medium supplemented with Primocin (Invivogen) and 2% Fetal Calf Serum (all components were from Life Technologies). Cells were centrifuged at 400g (4 ° C) for 10 min and diluted in Leibovitz's L-15 medium supplemented with Primocin (Invivogen) and 10% Fetal Calf Serum for being analyzed by flow cytometry based on forward and side scatter on a FACSCalibur flow cytometer (Beckton Dickinson) equipped for cell-sorting (Traver et al., 2003). A total of 300,000 events were sorted in 1x PBS from the regions corresponding to myeloid (R2), lymphoid (R3) and precursor (R4) population, and they were pelleted by centrifugation at 400g for 5 min at 4 °C and processed for gene expression. The cells from the total population (non-sorted) were also processed. The correct distribution of the cell populations was corroborated by analyzing the expression level of different cell markers: *marco* (macrophages), *mpx* (neutrophils), *cd4* and *cd8a* (T lymphocytes). Primer sequences are listed in Supplementary data Table 1. In addition, the expression level of these four genes was studied in kidney from Rag1^{-/-} mutants and compared with Rag1^{+/+} wild-type adult zebrafish using 4 biological replicates (4 fish/replicate).

To determine the expression levels of the different genes during zebrafish ontogeny, wild-type zebrafish larvae were sampled at the following dpfs: 1 dpf, 2 dpf, 3 dpf and at 3-day intervals from 5 to 29 dpf. Due to differences in animal size, 10–15 animals were necessary to yield biological replicates from 3 dpf to 14 dpf, whereas only 6–8

individuals from 17 to 29 dpf were used for biological replicates. A total of 3 biological replicates per sampling point were obtained.

Fish challenge experiments were also conducted in order to investigate the induction of the Nk-lysin genes upon viral infection. The rhabdovirus, spring viraemia of carp virus (SVCV isolate 56/70) was used in these experiments. The virus was propagated on EPC cells (ATCC CRL-2872) and titrated in 96-well plates. The TCID₅₀/mL was calculated according to Reed and Muench (1938) and the experimental challenges were performed at 23°C. Zebrafish larvae were infected through microinjection (using a glass microneedle using Narishige MN-151 micromanipulator and Narishige IM-30 microinjector) into the duct of Cuvier as described in Varela et al. (2014a) at 3 days post-fertilization (dpf). 2 nL of 3×10⁶ TCID₅₀/mL of SVCV were microinjected per larvae and PBS microinjection was used as a control in larvae experiments. At 24 hours post-stimulation (hps) 3 biological replicates composed by 10 larvae each were collected. Moreover, adult zebrafish were intraperitoneally injected with 10 µL of a SVCV suspension (3×10⁶ TCID₅₀/ml) and the corresponding controls were injected with the same volume of viral medium (Eagle's minimum essential medium supplemented with 2% fetal bovine serum, penicillin and streptomycin). Kidneys were sampled from anesthetized fish at 3, 6 and 24 hps and 4 biological replicates (4 fish/replicate) per sampling point were obtained.

2.5. RNA extraction, cDNA synthesis and real-time quantitative PCR analysis

Total RNA from the different samples was extracted using the Maxwell[®] 16 LEV simplyRNA Tissue kit (Promega) with the automated Maxwell 16 Instrument in accordance with instructions provided by the manufacturer. This kit also includes a DNase treatment step for removing potential genomic DNA contamination. cDNA synthesis was performed with the SuperScript II Reverse Transcriptase (Invitrogen) using 0.5 µg of RNA and following the manufacturer indications. Nk-lysins expression profiles, the transcription of the cells markers in the cell population samples, and the expression of the nucleoprotein (N) gene from SVCV in the challenge experiments, were determined using real-time quantitative PCR (qPCR). Specific qPCR primers were designed using the Primer3 program (Rozen and Skaletsky, 2000) and their amplification efficiency was calculated using seven serial two-fold dilutions of cDNA from unstimulated zebrafish with the Threshold Cycle (CT) slope method (Pfaffl, 2001).

The identity of the amplicon was confirmed by sequencing. Primer sequences are listed in Supplementary data Table 1. Individual qPCR reactions were carried out in 25 µl reaction volume using 12.5 µl of SYBR GREEN PCR Master Mix (Applied Biosystems), 10.5 µl of ultrapure water (Sigma-Aldrich), 0.5 µl of each specific primer (10 µM) and 1 µl of five-fold diluted cDNA template in MicroAmp optical 96-well reaction plates (Applied Biosystems). All reactions were performed using technical triplicates in a 7300 Real-Time PCR System thermocycler (Applied Biosystems) with an initial denaturation (95°C, 10 min) followed by 40 cycles of a denaturation step (95°C, 15 s) and one hybridization-elongation step (60°C, 1 min). No-template controls were also included on each plate to detect possible contamination or primer dimers formed during the reaction. An analysis of melting curves was performed for each reaction. Relative expression of each gene was normalized using the 18S ribosomal RNA as reference gene, which was constitutively expressed and not affected by the experimental treatments, and calculated using the Pfaffl method (Pfaffl, 2001). Fold change units were calculated by dividing the normalized expression values in SVCV-infected or Rag1^{-/-} individuals by the normalized expression values of the controls. In the case of Nk-lysins tissue-distribution, cell population analysis, ontogeny and SVCV N gene transcription analysis, expression units were represented as normalized values.

2.6. Statistical analysis

Expression results were represented graphically as the mean ± standard error of the biological replicates. In order to determine statistical differences, data were analyzed with the computer software package SPSS v.19.0 using the Student's t-test. Significant differences were displayed as *** (0.0001<p<0.001), ** (0.001<p<0.01) or * (0.01<p<0.05).

3. Results

3.1. Gene organization and sequence analysis

Four Nk-lysin genes (*nkla*, *nkla*, *nkla* and *nkld*) were identified in tandem on zebrafish chromosome 17. A high degree of synteny conservation was observed between *D. rerio* and cave fish (*A. mexicanus*), which possesses two Nk-lysins on chromosome KB882179.1 (Fig. 1). The genes *edrf1*, *tonsl*, *psmc1a*, *zranb1b* and *akap6* were found

surrounding the Nk-lysin genes in both cases, but in an inverted sense. Interestingly, synteny conservation between zebrafish and the other teleost species was not observed, as well as with human granulysin. On the other hand, *T. nigroviridis*, *T. rubripes* and *G. aculeatus* showed only one Nk-lysin gene on their genomes and a high degree of synteny conservation was found, even in the case of *O. latipes*, in which Nk-lysin genes were not identified.

The full-length coding sequences of the *D. rerio* Nk-lysin genes were confirmed by PCR and sequencing and consisted of 435 (*nkla*), 426 (*nkla*), 360 (*nkla*) and 366 (*nkla*) nucleotides, encoding proteins composed by 144, 141, 119 and 121 amino acids, respectively (Fig. 2A). The sequences were submitted to GenBank under accession numbers KP100115–KP100118. A signal peptide and the saposin B (SapB) domain were identified in all cases. The corresponding molecular weights and isoelectric points (pI) of the mature proteins were 14.7 kDa/6.23, 14.4 kDa/7.13, 11.7 kDa/6.21 and 11.7 kDa/6.87. The four Nk-lysins contain 6 cysteine residues in their amino acid sequence and, as consequence, three disulfide bonds were predicted to be formed in each case. The hypothetical 3D-structures of these peptides revealed the characteristic four/five-helical-bundle structure observed in the family of saposin-like proteins (Fig. 2B).

3.2. Homology and phylogenetic analysis

Vertebrate Nk-lysins showed 6 well conserved cysteines in their sequences, with the exception of the human granulysin, in which the first one is missing (Fig. 3A). Nevertheless, mammals possess one or two additional cysteines in previous positions. With regard to the identity/similarity matrix (Supplementary data Table 3), *Nkla* and *Nkln* showed the highest scores among the zebrafish Nk-lysins (70.1/81.3%), whereas *Nkln* and *Nkln* reflected also high values (66.9/79.3%). When the zebrafish proteins are compared with the three Nk-lysins described in catfish (*I. punctatus*), *Nkla* and *Nkln* were found to be more homologues to catfish *Nk13* (32.7/50.3% and 36.1/50.0%, respectively), whilst *Nkln* and *Nkln* were more related to *Nk12* (39.5/57.4% and 39.7/57.3%, respectively). Interestingly, the zebrafish Nk-lysin showing the highest scores with regard to the Nk-lysins from teleost species possessing only one Nk-lysin copy was in all cases *Nkln*. *Nkln* and *Nkln* were also more similar to mammalian Nk-lysins/granulysin than *Nkla* and *Nkln*.

A phylogenetic analysis containing several proteins belonging to the SAPLIP family revealed that Nk-lysins from mammals and birds are more closely related with Prosaposins and SP-B than with teleost Nk-lysins (Fig. 3B). AOAHs showed the lowest phylogenetic relationships with regard to the other Saposin-like proteins. Concerning zebrafish Nk-lysins, *Nkla* and *Nklb* grouped together, as well as *Nklc* with *Nkld*. Interestingly, *Nklc* and *Nkld* were phylogenetically positioned closer to Nk-lysins from other teleost, whereas *Nkla* and *Nklb* formed a separated cluster.

3.3. Nk-lysins tissue-distribution pattern

A very different expression pattern of the four Nk-lysins was observed in adult zebrafish tissues (Fig. 4A). Whereas *nkla* was mainly expressed in muscle, *nkla* and *nkla* showed their higher expression level in intestine and spleen, respectively. The last Nk-lysins gene, *nkld*, showed less tissue-specificity, being gill, kidney and spleen the organs with a higher constitutive transcription of this gene. Gene expression of the four Nk-lysins was detected in all the tested tissues. The analysis of proportion of Nk-lysins transcripts in each tissue revealed that *nkla* is the most expressed gene in liver, kidney, gill (a similar proportion of *nkla* was observed in this organ), tail, head and muscle, but *nkla* was the predominant in intestine and *nkla* in spleen (Fig. 4B). *nkld* showed a very low expression in all cases.

3.4. Nk-lysins expression analysis in different cell populations from kidney

To analyze the expression of the four zebrafish Nk-lysins in the cell populations from kidney, the cells were sorted by size and granularity, and their individual expression levels were determined by qPCR. Additionally, the mRNA transcription of each gene was also analyzed in cells from the total population (non-sorted). Three different regions, corresponding to the myeloid (R2), lymphoid (R3) and precursor (R4) populations, were considered in the study (Fig. 5A). The sorted fractions were analyzed using gene markers (*marco*, *mpx*, *cd4* and *cd8a*) in order to corroborate their correct distribution (Supplementary Figure 1). Our results showed that the region R4, corresponding to the precursor population, was, in absolute terms, the main producer of Nk-lysins, followed by the lymphoid and myeloid populations (Fig. 5B). Taking into consideration the transcription of Nk-lysins in the total population, the expression level was lower with regard to the precursor and lymphoid populations, but higher compared with the myeloid region. Also, in absolute terms, the precursor population was the main

producer of *nkla* and *nkld* mRNA, whereas the lymphoid population was the region producing the higher quantity of *nkla* and *nkld* mRNA. Regarding to the relative proportions of each zebrafish Nk-lysin expression in the different populations, *nkla* was the most transcribed Nk-lysin in the myeloid (69%) and precursor (93%) regions, followed by *nkla* (26 and 6%, respectively) and *nkld* (5 and 1%, respectively), whereas *nkla* was the most expressed in the lymphoid population (49%), followed by *nkla* (38%) and *nkld* (13%) (Fig. 5C). *nkld* showed the lowest expression level in all the populations, although its expression was detected in all cases.

3.5. Expression of Nk-lysins in *Rag1*^{-/-} compared with *Rag1*^{+/+}

The mRNA level under basal conditions was analyzed in kidney from wild-type and *Rag1*^{-/-} adult zebrafish. *Rag1*^{-/-} mutant zebrafish lack VDJ recombination and, therefore, these individuals lack of T cell receptor (TCR) and immunoglobulin (Ig) expression (Wienholds et al., 2002; Petrie-Hanson et al., 2009). As a consequence, these fish do not have mature T and B lymphocytes but present NK-like cells (the existence of mammalian NK cell homologues in fish remains still unclear) and non-specific cytotoxic cells of the innate immune system. The results showed that whereas *nkla* was significantly less expressed in *Rag1*^{-/-} individuals (FC= 0.42), *nkla*, *nkld* and *nkld* mRNA levels were higher in this mutant line (Fig. 6A). This difference was especially remarkable in the case of *nkld*, reaching a FC about 7 with regard to wild-type fish. Interestingly, although *nkla* was underrepresented in *Rag1*^{-/-}, the relative values indicated that this gene is the second Nk-lysin more expressed in these mutants after *nkld* (71%), representing a 18% of the total Nk-lysins mRNA levels (Figure 6b). As was mentioned in the section 3.3, *nkla* was the most expressed Nk-lysin in kidney from wild-type fish. In absolute terms, the total level of Nk-lysins transcripts was more than double in *Rag1*^{-/-} compared with wild-type (Fig. 6C).

3.6. Nk-lysins mRNA levels during ontogeny

We also analyzed the expression level of *nkla*, *nkla*, *nkld* and *nkld* during zebrafish early development (Fig. 7). Transcripts of these genes were detected at all sampling points between 1 and 29 dpf. Nevertheless, the expression pattern observed was very different between Nk-lysins. *nkla* showed a time-increasing profile, reaching its higher mRNA

level at 29 dpf. A totally opposed tendency was observed in the case of *nkld*, where the highest values were obtained at 1dpf. *nklb* and *nklc* gradually increased their expression from 5dpf, reaching a maximum peak of transcription at 26 dpf in both cases, but with some sharp declines along the ontogeny, especially in the case of *nklb* at 14 dpf. A strong drop in the expression levels of these two genes was observed at 29 dpf, just after the peak at 26 dpf.

3.7. Nk-lysins induction after SVCV challenge

SVCV infection in 3 dpf larvae did not significantly affect the expression level of any Nk-lysin at 24h post-challenge (Fig. 8). On the other hand, when adult zebrafish were intraperitoneally infected with SVCV and the induction of Nk-lysins was analyzed in kidney samples at 3, 6 and 24 h post-challenge, the result revealed that only *nkla* and *nkld* were significantly affected by this treatment at the tested sampling points (Fig. 9). Whereas *nkla* was significantly modulated at 24h, reaching a fold-change (FC) with regard to the control individuals of 4.85, *nkld* expression was up-regulated at 6h (FC= 4.05), and returning to basal levels after 24h. The efficiency of the viral infection was confirmed in both experiments through the transcription analysis of the SVCV N gene (Supplementary Figure 2).

4. Discussion

The present work reports for the first time, the characterization and expression studies of the *D. rerio* Nk-lysin repertoire. Four Nk-lysin genes (*nkla*, *nklb*, *nklc* and *nkld*) were identified in the zebrafish genome positioned in tandem on chromosome 17. The synteny analysis revealed a high degree of synteny conservation between the individuals belonging to the superorder *Ostariophysi* (zebrafish and cave fish), but not with those teleost included in the superorder *Acanthopterygii*, as well as with the human granulysin. It is also interesting to highlight that zebrafish and cave fish presented more than one copy of Nk-lysin in their genome (four and two, respectively), in comparison with the other teleost and higher vertebrates, which only seem to possess one Nk-lysin/granulysin. These observations could suggest a specific differentiation of the superorder *Ostariophysi*, since three Nk-lysin genes were also characterized in channel catfish (*Ictalurus punctatus*) located in the same genomic region (Wang et al., 2006). Differences in the synteny and retention/loss of duplicated sequences between both superorders have been previously reported (Taylor et al., 2003; Garcia de la Serrana et

al., 2014). Therefore, zebrafish is the species with the higher repertoire of Nk-lysin genes described so far. This diversity could indicate a specialization of the different proteins into different functions and, as consequence, the preferential expression of these genes by different cell types.

Nk-lysins have been characterized only in a small set of fish, including the three genes from channel catfish (Wang et al., 2006) and only one isoform from two flatfish species, Japanese flounder - *Paralichthys olivaceus* - (Hirono et al., 2007) and half-smooth tongue sole - *Cynoglossus semilaevis* - (Zhang et al., 2013). This is, therefore, the first characterization of Nk-lysin genes in a fish model species, being a very interesting issue due to the absence of an Nk-lysin/granulysin homolog in murine models (Clayberger et al., 2012). As consequence, this work will serve as a start point in order to fully understand the role of these molecules in the immune response using a laboratory animal model.

Zebrafish Nk-lysins (Nkla, Nklb, Nklc and Nkld) were composed by 144, 141, 119 and 121 amino acids, respectively, and a signal peptide was identified in all of them, indicating, as was expected, that they are proteins with the ability to be secreted. The conserved and characteristic Sap domain of the SAPLIPs was also detected in the four Nk-lysins, as well as the six conserved cysteine residues involved in the 3-D conformational structure typically observed in the saposin-like proteins, a four- or five-helix bundle (Munford et al., 1995; Willis et al., 2011). This structure allows the interaction with biological membranes and the ability to altering the membrane integrity (Ruysschaert et al., 1998). Saposins activate lipid-degrading enzymes, such as glucosylceramidase and sphingomyelinases, and this lead an increase in the level of intracellular ceramide, a molecule involved in the induction of apoptosis (Stenger et al., 1999).

When an identity/similarity matrix and a phylogenetic analysis were conducted, Nkla and Nklb were found to be more similar between them, as occurs with Nklc and Nkld. Nklc and Nkld showed a higher relationship with the remaining fish Nk-lysins and moreover, they were also more similar to mammalian Nk-lysins/granulysin, indicating a higher evolutionary conservation of *nklc* and *nkld* genes. Interestingly, these more conserved genes were in general constitutively lower expressed in the majority of the tested tissues, with some exception. *nkla* showed the highest mRNA level in all the

tissues with the exception of spleen and intestine, in which *nk1c* and *nk1b* were the predominant forms, respectively. What could be the explanation for this tissue distribution? Gene duplication and subfunctionalization provide specialized paralogs of an ancient gene (Prince and Pickett, 2002; Emes et al., 2003) and as consequence, variations in the expression of the different genes could be observed among different tissues and cell types, and even between distinct subpopulations belonging to the same cell type. As consequence, their responses to different treatments or conditions and their functions can also differ among paralogs, as was previously observed in multitude of immune-related molecules (Kofoed and Vance, 2012; Sastalla et al., 2013; Forn-Cuni et al., 2014; Varela et al., 2014b). Interestingly, *nk1a* and *nk1b* were mainly expressed in muscle and intestine, respectively. Therefore, these results suggest that zebrafish Nk-lysins are also expressed in other cells than CTLs and NK cells, as was also observed by analyzing their transcription in the cell populations identified in kidney.

Different cell populations from zebrafish kidney showed a differential expression pattern of Nk-lysins, and these differences could suggest a certain specialization of the cell types in the production of these proteins. Nevertheless, B and T lymphocytes and NK cells belong to the lymphoid lineage and therefore to withdraw conclusions regarding the involvement of different cells in the production of a specific zebrafish Nk-lysin using only classical flow cytometry separation, should be considered cautiously. Moreover, it is not clear yet the existence of NK cells in fish. Although, during the last decade, some investigations, based on the presence of Novel immune-type receptors (NITRs), the “functional orthologs” of mammalian natural killer receptors (NKR), have suggested the existence of these cells in teleost fish (Yoder, 2009). As NK cells and CTLs could be specialized in the production of different zebrafish Nk-lysins, the expression analysis in kidney from *Rag1*^{-/-} individuals compared with *Rag1*^{+/+} was conducted in this work. As was mentioned above, *Rag1*^{-/-} mutant zebrafish do not have mature T and B lymphocytes. As consequence, NK cells would be the main producers of Nk-lysin in this mutant line. In a previous work the expression of three forms of Nk-lysin (named as 1, 2 and 3) was analyzed in kidney from *Rag1*^{-/-} and *Rag1*^{+/+} zebrafish, and the results reflected that these transcripts were expressed in both cases (Petrie-Hanson et al., 2009). Our results showed that *nk1a* was more expressed in wild-type fish, whereas *nk1b*, *nk1c* and *nk1d* showed a higher transcription level in *Rag1*^{-/-} individuals. Therefore, it could be possible that CTLs are mainly specialized in the

production of *Nkla* and NK cells in the synthesis of the other Nk-lysins, although all zebrafish Nk-lysins were detected both in wild-type and *Rag1*^{-/-}. Moreover, the higher absolute expression level of Nk-lysins in *Rag1*^{-/-} fish could be related with an increase in the number of NK cells in this mutant line, although more investigation is needed to determine if these observations are correct.

It is also interesting to highlight the difference in the expression pattern of the four Nk-lysins during wild-type zebrafish ontogeny. *nkla*, on the basis of our results more linked with CTLs, showed a time-increasing transcription especially from 20 dpf to 29 dpf. The specific immune system, both cell-mediated and humoral immunity, is non-functional during the early larval stages and becomes fully competent several weeks after hatching (Lam et al., 2004). The opposite response was observed in the case of *nkld*, which higher transcription level was reached at 1 dpf. Another interesting issue for further investigations could be if this stronger expression level is due to mRNA maternal transference. *nk1b* and *nk1c* followed a similar tendency, with a general time-increasing expression, but the mRNA level dropped abruptly at 29 dpf, coinciding with the sharp increase in the expression of *nkla*. It is possible that the appearance of mature CTLs affects the number of NK cells and/or the transcription of certain genes and therefore, these changes in the cell populations could be altering the expression level of Nk-lysin genes.

The implication of Nk-lysins/granulysin in the immune response against a broad spectrum of microorganisms is clear, as was mentioned in the introduction section. Interestingly, although CTLs and NK cells are the main producers of Nk-lysin and these cells are directly implicated in the destruction of virus-infected cells, the role of this molecule in viral infections is still poorly understood. When the SVCV infection was conducted in zebrafish larvae (3 dpf) not significant modulations were observed in any Nk-lysin at 24 post-challenge. It is possible that at 3 dpf the cells secreting these molecules (NK cells and CTLs) are not yet ready to combat the virus, being still in an immature state as referred above. Up-regulations in the expression of *nkla* and *nkld* were observed in kidney from adults after SVCV challenge, whereas *nk1b* and *nk1c* expression was not significantly affected at the tested sampling point. Previous works have tried to shed light about the implication of Nk-lysin/granulysin in viral diseases, but the results were contradictory in many cases. Whereas Marek's disease virus-infected chickens (oncogenic herpesvirus) showed an up-regulation of Nk-lysin

expression (Sarson et al., 2008), inhibitions in the expression of this gene were observed in infectious bursal disease virus-infected chickens (immunosuppressive viral disease) (Rauf et al., 2011). On the other hand, it has been shown that synthetic human granulysin accelerated the death of MRC-5 cells (human lung fibroblast) infected with Varicella-zoster virus (Hata et al., 2001) but, in contrast, acutely human immunodeficiency virus-infected CD4⁺ T cells were not affected by granulysin and the recombinant peptide did not alter the viral infectivity and replication kinetic (Mackewicz et al., 2000). Moreover, the induction of granulysin in CD8⁺ T cells by IL-21 and IL-15 was suppressed by HIV-1 (Hogg et al., 2009). Recently, an Nk-lysin from the flatfish *C. semilaevis* was characterized and some studies concerning to bacterial and viral diseases were conducted (Zhang et al., 2013). Overexpressions in the level of this gene were observed in head kidney and spleen after megalocytivirus intraperitoneal injection in *C. semilaevis*, especially at 24 h post-challenge. Moreover, the intramuscular administration of an expression plasmid encoding Nk-lysin was able to significantly reduce the viral copy number in head kidney and spleen, although mortality data were not provided (Zhang et al., 2013). Up-regulations in the transcription of the antimicrobial peptide Nk-lysin were also observed using microarray analysis at 24 h after VHSV-challenge in turbot (Pereiro et al., 2014). It appears that, depending on the type of virus, the response and protection capabilities are different.

5. Conclusions

This work represents the first formal characterization of the complete set of Nk-lysin genes in *D. rerio*. Due to the absence of an Nk-lysin gene in murine models, this work opens the door to future investigations aimed at assessing the role of these molecules in the immune system of a model species. Our results show a different expression pattern of the four Nk-lysins in different tissues, during the ontogeny, in different cell populations from zebrafish kidney, as well as between Rag1^{-/-} and Rag1^{+/+} individuals. This could suggest a certain specialization of different cell types in the production of different Nk-lysins. On the other hand, only two of these genes (*nkla* and *nkld*) were up-regulated after SVCV challenge, indicating a possible role of these genes in the protection against viral diseases.

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Figure legends

Fig. 1

Comparative gene location of human Granulysin and Nk-lysin genes in several teleost species. Human Granulysin, located on chromosome 2, do not share synteny conservation with regard to the Nk-lysin genes in teleost. On the other hand, the genes flanking the four zebrafish Nk-lysins, located on chromosome 17, showed a high degree of synteny conservation with cave fish (*A. mexicanus*), but not with the other fish. This revealed a clear synteny differentiation between the superorder Ostariophysi (*D. rerio* and *A. mexicanus*) and the superorder Acanthopterygii (*T. nigroviridis*, *T. rubripes*, *G. aculeatus* and *O. latipes*).

Fig. 2

(A) Nucleotide and amino acid sequence of zebrafish Nk-lysins open reading frames. The predicted signal peptides are underlined, whereas the SapB domains are shaded in the amino acid sequences. (B) Predicted tertiary structure of zebrafish Nk-lysins using I-TASSER server.

Fig. 3

(A) Multiple amino acid alignment comparing the zebrafish Nk-lysins with other vertebrates. Asterisks mark the identical amino acids in all the sequences. Conserved cysteines, essential for structural integrity forming disulfide bridges are presented in light shaded boxes. (B) Phylogenetic relationship between the Nk-lysins/Granulysin proteins from bony fish and other vertebrates with other proteins belonging to the SAPLIP family (prosaposins, AOA, SP-B and amoebopores). (NCBI GenBank accession numbers of sequences used are listed in Supplementary data Table 2).

Fig. 4

(A) Constitutive expression of Nk-lysins genes in tissues from healthy adult zebrafish. For basal expression of each Nk-lysins form, tissues were sampled and pooled, yielding a total of 4 pools of 5 fish per organ. The relative expression level of each gene was normalized to the expression level of the 18 S ribosomal RNA gene in the same tissue. The graphs represent the mean $\pm$ standard error of 4 independent samples. (B) Relative proportion (%) of the Nk-lysins transcripts in different zebrafish tissues.

Fig. 5

(A) Flow cytometry analysis of the cell populations from zebrafish kidney by size (forward scatter; FSC-H: voltage E00/mode Lin) and granularity (side scatter; SSC-H: voltage 350/mode Log). R2, R3 and R4 represent the myeloid, lymphoid and precursor population, respectively. (B) Cumulative normalized expression of the zebrafish Nk-lysins in the different cell populations and in total cells (non-sorted). The expression of each Nk-lysins was normalized to the expression of the housekeeping gene (18 S ribosomal RNA gene). (C) Relative proportion (%) of the Nk-lysins transcripts in each population and in total cells.

Fig. 6

(A) Expression of the different zebrafish Nk-lysins in kidney from adult Rag1^{-/-} and Rag1^{+/+} individuals. The experiment was composed by 4 biological replicates (4 fish/replicate). The expression of each Nk-lysin was normalized to the expression of the housekeeping gene (18 S ribosomal RNA gene). Fold change was calculated by dividing the normalized expression values in Rag1^{-/-} zebrafish by the normalized expression values obtained in Rag1^{+/+} individuals. Significant differences were displayed as *** (0.0001<p<0.001), ** (0.001<p<0.01) or * (0.01<p<0.05). (B) Cumulative normalized expression of the zebrafish Nk-lysins in kidney from Rag1^{+/+} and Rag1^{-/-}. (C) Relative proportion (%) of the Nk-lysin transcripts in Rag1^{+/+} and Rag1^{-/-} zebrafish.

Fig. 7

Nk-lysins expression during the ontogeny of zebrafish. Samples (3 biological replicates composed by a pool of larvae) were taken at different days post-fertilization (dpf) and the relative expression level of each gene was normalized to the expression level of the 18 S ribosomal RNA gene at the same sampling point.

Fig. 8

(A) Comparative expression analysis of Nk-lysin genes in SVCV-infected and healthy (control) zebrafish larvae. The larvae (3 dpf) were infected through microinjection into the duct of Cuvier and the controls were injected with PBS. At 24 hps 3 biological replicates composed by 10 larvae each were collected. The expression of each Nk-lysin was normalized to the expression of the housekeeping gene (18 S ribosomal RNA gene). Fold change was calculated by dividing the normalized expression values in SVCV-infected larvae by the normalized expression values obtained in control larvae. Not significant differences were observed regarding to the control fish. (B) Cumulative normalized expression of the zebrafish Nk-lysins in SVCV-infected and control larvae (C) Relative proportion (%) of the Nk-lysin transcripts in SVCV-infected and control larvae.

Fig. 9

Expression analysis of Nk-lysins in kidney after SVCV-challenge in adult zebrafish. Samples were taken at 3, 6 and 24 hps and 4 biological replicates (4 fish/replicate) per sampling point were obtained for the intraperitoneally infected and control fish. The

expression of each Nk-lysin was normalized to the expression of the housekeeping gene (18 S ribosomal RNA gene). Fold change was calculated by dividing the normalized expression values in SVCV-infected fish by the normalized expression values obtained in the controls. Significant differences were displayed as *** ($0.0001 < p < 0.001$), ** ($0.001 < p < 0.01$) or * ($0.01 < p < 0.05$).

Supplementary data

Supplementary Table 1: Primer sequences used in cloning and qPCR analysis in this work.

Supplementary Table 2: GenBank accession numbers of the sequences used in the phylogenetic analysis.

Supplementary Table 3: Amino acid identities (top right) and similarities (bottom left) of Nk-lysins/Granulysin from vertebrate species. The accession numbers of the protein sequences are also included in the Supplementary data Table 2.

Supplementary Figure 1: Expression of different cell markers in the cell populations from zebrafish kidney.

Supplementary Figure 2: SVCV nucleoprotein (N) gene expression during the viral challenge experiments in zebrafish larvae (A) and in kidney from adult zebrafish (B).

figure 1.tif
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figure 3.tif
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A

Gallus gallus	--MAAIVTIALGLAVVAVTEPPEDCHRDLEAGSHNEQWHLIGDSSAURDAEEDAMSPKQIN--	SPVSLVKNVQIVQDQFQEDAINGLNHS
Coturnix japonica	--MAAIVTIANMAVAVVTEPPEDCHRDLEAGSHNEQWHLIGDSSAURDAEEDAMSPKQIN--	SPVSLVKNVQIVQDQFQEDAINGLNHS
Scophthalmus maximus	NETSSVLLVCLVTCVHTVHGRSLEINIDQGEVIVAIS-----	VEAGELPQNDGADGALNVRVQVHNPVTSQVSKLHIVCH
Paralichthys olivaceus	NETSSVLLVCLVTCVHTVHGRSLEINIDQGEVIVAIS-----	VEAGELPQNDGADGALNVRVQVHNPVTSQVSKLHIVCH
Cynoglossus semilaevis	NETSSVLLVCLVTCVHTVHGRSLEINIDQGEVIVAIS-----	VEAGELPQNDGADGALNVRVQVHNPVTSQVSKLHIVCH
Salmo salar	NETSSVLLVCLVTCVHTVHGRSLEINIDQGEVIVAIS-----	VEAGELPQNDGADGALNVRVQVHNPVTSQVSKLHIVCH
Danio rerio H&C	NETSSVLLVCLVTCVHTVHGRSLEINIDQGEVIVAIS-----	VEAGELPQNDGADGALNVRVQVHNPVTSQVSKLHIVCH
Danio rerio H&C1d	NETSSVLLVCLVTCVHTVHGRSLEINIDQGEVIVAIS-----	VEAGELPQNDGADGALNVRVQVHNPVTSQVSKLHIVCH
Ictalurus punctatus H&C1	NETSSVLLVCLVTCVHTVHGRSLEINIDQGEVIVAIS-----	VEAGELPQNDGADGALNVRVQVHNPVTSQVSKLHIVCH
Ictalurus punctatus H&C2	NETSSVLLVCLVTCVHTVHGRSLEINIDQGEVIVAIS-----	VEAGELPQNDGADGALNVRVQVHNPVTSQVSKLHIVCH
Ictalurus punctatus H&C3	NETSSVLLVCLVTCVHTVHGRSLEINIDQGEVIVAIS-----	VEAGELPQNDGADGALNVRVQVHNPVTSQVSKLHIVCH
Danio rerio H&C1a	NETSSVLLVCLVTCVHTVHGRSLEINIDQGEVIVAIS-----	VEAGELPQNDGADGALNVRVQVHNPVTSQVSKLHIVCH
Danio rerio H&C1b	NETSSVLLVCLVTCVHTVHGRSLEINIDQGEVIVAIS-----	VEAGELPQNDGADGALNVRVQVHNPVTSQVSKLHIVCH
Sus scrofa	NETSSVLLVCLVTCVHTVHGRSLEINIDQGEVIVAIS-----	VEAGELPQNDGADGALNVRVQVHNPVTSQVSKLHIVCH
Equus caballus	NETSSVLLVCLVTCVHTVHGRSLEINIDQGEVIVAIS-----	VEAGELPQNDGADGALNVRVQVHNPVTSQVSKLHIVCH
Bos taurus	NETSSVLLVCLVTCVHTVHGRSLEINIDQGEVIVAIS-----	VEAGELPQNDGADGALNVRVQVHNPVTSQVSKLHIVCH
Homo sapiens	NETSSVLLVCLVTCVHTVHGRSLEINIDQGEVIVAIS-----	VEAGELPQNDGADGALNVRVQVHNPVTSQVSKLHIVCH

Gallus gallus	TGR-RGRSIFQQLIKLQQLSDALQDQDPRVA--TTLOI--	NO-----
Coturnix japonica	TGR-RGRSIFQQLIKLQQLSDALQDQDPRVA--TTLOI--	NO-----
Scophthalmus maximus	EIG-LLKSLDGFVTHKDELVELSTTCOVRTI--	MTGAKSPKQSHLLFTPKQESQTEIIEYS
Paralichthys olivaceus	EIG-LLKSLDGFVTHKDELVELSTTCOVRTI--	MTGAKSPKQSHLLFTPKQESQTEIIEYS
Cynoglossus semilaevis	EIG-LLKSLDGFVTHKDELVELSTTCOVRTI--	MTGAKSPKQSHLLFTPKQESQTEIIEYS
Salmo salar	EIG-LLKSLDGFVTHKDELVELSTTCOVRTI--	MTGAKSPKQSHLLFTPKQESQTEIIEYS
Danio rerio H&C	EIG-LLKSLDGFVTHKDELVELSTTCOVRTI--	MTGAKSPKQSHLLFTPKQESQTEIIEYS
Danio rerio H&C1d	EIG-LLKSLDGFVTHKDELVELSTTCOVRTI--	MTGAKSPKQSHLLFTPKQESQTEIIEYS
Ictalurus punctatus H&C1	EIG-LLKSLDGFVTHKDELVELSTTCOVRTI--	MTGAKSPKQSHLLFTPKQESQTEIIEYS
Ictalurus punctatus H&C2	EIG-LLKSLDGFVTHKDELVELSTTCOVRTI--	MTGAKSPKQSHLLFTPKQESQTEIIEYS
Ictalurus punctatus H&C3	EIG-LLKSLDGFVTHKDELVELSTTCOVRTI--	MTGAKSPKQSHLLFTPKQESQTEIIEYS
Danio rerio H&C1a	EIG-LLKSLDGFVTHKDELVELSTTCOVRTI--	MTGAKSPKQSHLLFTPKQESQTEIIEYS
Danio rerio H&C1b	EIG-LLKSLDGFVTHKDELVELSTTCOVRTI--	MTGAKSPKQSHLLFTPKQESQTEIIEYS
Sus scrofa	EIG-LLKSLDGFVTHKDELVELSTTCOVRTI--	MTGAKSPKQSHLLFTPKQESQTEIIEYS
Equus caballus	EIG-LLKSLDGFVTHKDELVELSTTCOVRTI--	MTGAKSPKQSHLLFTPKQESQTEIIEYS
Bos taurus	EIG-LLKSLDGFVTHKDELVELSTTCOVRTI--	MTGAKSPKQSHLLFTPKQESQTEIIEYS
Homo sapiens	EIG-LLKSLDGFVTHKDELVELSTTCOVRTI--	MTGAKSPKQSHLLFTPKQESQTEIIEYS

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