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Hyperinsulinemia induces insulin resistance and immune suppression via Ptpn6/Shp1 in zebrafish

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Abstract

Type 2 diabetes, obesity, and metabolic syndrome are pathologies where insulin resistance plays a central role, and that affect a large population worldwide. These pathologies are usually associated with a dysregulation of insulin secretion leading to a chronic exposure of the tissues to high insulin levels (i.e. hyperinsulinemia), which diminishes the concentration of key downstream elements, causing insulin resistance. The complexity of the study of insulin resistance arises from the heterogeneity of the metabolic states where it is observed. To contribute to the understanding of the mechanisms triggering insulin resistance, we have developed a zebrafish model to study insulin metabolism and its associated disorders. Zebrafish larvae appeared to be sensitive to human recombinant insulin, becoming insulin-resistant when exposed to a high dose of the hormone. Moreover RNA-seq-based transcriptomic profiling of these larvae revealed a strong downregulation of a number of immune-relevant genes as a consequence of the exposure to hyperinsulinemia. Interestingly, as an exception, the negative immune modulator protein tyrosine phosphatase nonreceptor type 6 (*ptpn6*) appeared to be upregulated in insulin-resistant larvae. Knockdown of *ptpn6* was found to counteract the observed downregulation of the immune system and insulin signaling pathway caused by hyperinsulinemia. These results indicate that *ptpn6* is a mediator of the metabolic switch between insulin-sensitive and insulin-resistant states. Our zebrafish model for hyperinsulinemia has therefore demonstrated its suitability for discovery of novel regulators of insulin resistance. In addition, our data will be very useful in further studies of the function of immunological determinants in a non-obese model system.

Key Words
- hyperinsulinemia
- insulin resistance
- immune suppression
- ptpn6
- zebrafish

Journal of Endocrinology
(2014) **222**, 229–241

Introduction

Metabolic disorders (e.g. diabetes, obesity, and metabolic syndrome) have over the past three decades reached pandemic dimensions, affecting a large number of the world population (Wild & Byrne 2006, Lin & Sun 2010). Insulin resistance is a common aspect of a number of these metabolic pathologies, such as type 2 diabetes, and also a powerful parameter for predicting the incidence of cardiovascular diseases and cancer (Shanik *et al.* 2008). Hyperinsulinemia is the hallmark of insulin resistance in mammals. For instance, a twofold increase in plasma insulin levels can induce insulin resistance in mice, resembling the pattern in insulin-resistant patients with pathological obesity or type 2

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diabetes who show similar increases in insulin levels (Shanik *et al*. 2008). As insulin resistance is common to a number of metabolic diseases of which the relationships are still poorly understood, it is difficult to study with respect to causal relationships and genetic factors that are involved. Current models used for the study of insulin resistance range from mutant rodents to obese monkeys (Shafrir 2010). Although these models reproduce the human situation with regard to obesity and insulin resistance, there still appear to be many conflicting results compared with the human situation, mainly due to the heterogeneity of the metabolic states where insulin resistance is observed (Harano *et al*. 2002, Kahn 2003, Steinberger & Daniels 2003).

The zebrafish (*Danio rerio*) is emerging as a model for deciphering the mechanisms underlying pathologies caused by an altered metabolism (Seth *et al*. 2013). Principles of energy expenditure and metabolism are evolutionarily conserved in metazoans (Schlegel & Stainier 2007). In addition, zebrafish larvae at 4 days post fertilization (dpf) have developed many functional organs (Elo *et al*. 2007), with the advantages inherent to the zebrafish larvae system as presented in many review papers (Lieschke & Currie 2007, Ali *et al*. 2011, Liu & Leach 2011, Meijer & Spaink 2011, Santoriello & Zon 2012, Driessen *et al*. 2013, Seth *et al*. 2013). We therefore set out to develop a non-obese zebrafish model for the study of insulin metabolism and its associated disorders. In this study, we report on the finding that 4 dpf zebrafish larvae are sensitive to human insulin and become insulin-resistant when treated with high doses of insulin. Moreover, by profiling the transcriptomic response of insulin-sensitive and insulin-resistant larvae, we further characterize the effects of hyperinsulinemia on the metabolism. At the transcriptome level, we observed a general downregulation of immune-relevant genes potentially mediated by protein tyrosine phosphatase nonreceptor type 6 (*ptpn6*).

PTPN6 (also known as SHP1) is expressed mainly in hematopoietic cells (Yi *et al*. 1992) and has been described as an important negative regulator of immune signaling pathways (An *et al*. 2008, Croker *et al*. 2008). In addition, it has been reported that PTPN6 may be involved in the development of insulin resistance and non-alcoholic fatty liver diseases in obesity (Xu *et al*. 2012, 2014*a*). Pioneering studies performed by Dubois *et al*. (2006) showed that PTPN6-deficient mice exhibited improved insulin sensitivity and glucose tolerance. Further studies by this group demonstrated that hepatocyte-specific *Ptpn6* knockout ($Ptpn6^{H\text{-}KO}$) mice were protected from hepatic insulin resistance and hepatocellular damage caused by diet-induced obesity (Xu *et al*. 2012, 2014*a*).

In this study, we made use of a previously described test system for Ptpn6 function in zebrafish, using reverse genetic approaches (Kanwal *et al*. 2013). We showed that knockdown of *ptpn6* in zebrafish larvae interferes with the effects caused by hyperinsulinemia. In conclusion, using this non-obese vertebrate model, we were able to demonstrate that *ptpn6* expression is stimulated by hyperinsulinemia, and that *ptpn6* knockdown regulates key elements of the insulin pathway and also the leptin signaling pathway.

Materials and methods

Fish maintenance

WT zebrafish of the AB/TL strain were handled in compliance with the Local Animal Welfare legislation and maintained as described previously (Kimmel *et al*. 1995, Westerfield 2000). Embryos were grown at 28.5 °C in egg water (60 μg/ml Ocean Salts). During PBS/insulin injections, fish were kept under anesthesia in egg water containing 0.02% buffered 3-aminobenzoic acid ethyl ester (Sigma–Aldrich).

Morpholino injections

For knockdown of *ptpn6*, a morpholino oligonucleotide (Gene Tools, LLC, Philomath, OR, USA) targeting *ptpn6*, kindly donated by Dr A H Meijer, was injected (Kanwal *et al*. 2013). The morpholino (5′-ACTCATTCCTTACCC-GATGCGGAGC-3′) was diluted to a concentration of 0.08 mM in 1× Danieau's buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, and 5.0 mM HEPES (pH 7.6)) and 1 nl was injected at the one-cell stage using a Femtojet injector (Eppendorf, Hamburg, Germany). Specificity of the morpholino was confirmed phenotypically and by RT-PCR (Supplementary Fig. 1, see section on supplementary data given at the end of this article).

Insulin injections

To inject PBS and human recombinant insulin (Sigma–Aldrich), 1 nl was injected into the caudal aorta of 4 dpf zebrafish larvae using a glass capillary (Supplementary Fig. 2, see section on supplementary data given at the end of this article).

qPCR analysis

RNA was isolated using TRIzol (Life Technologies). RNA samples were treated with RQ1 DNAse (Promega) and reverse

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transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories B.V.), according to the manufacturers' protocols. For the quantification of mRNA expression, qPCR was carried out using iQ SYBR Green Supermix (Bio-Rad Laboratories B.V.). The reactions were run in a iCycler Thermal Cycler (Bio-Rad Laboratories B.V.) under the following conditions: 2 min at 50 °C, 8 min at 95 °C, followed by 40 cycles of 15 s denaturation at 95 °C and 30 s at the corresponding melting temperatures, and a final melting curve of 81 cycles from 55 to 95 °C (0.5 °C increments for every 10 s). mRNA expression levels were normalized against the expression of *ppial* as a housekeeping gene. Primer sequences are presented in Supplementary Table 7, see section on supplementary data given at the end of this article.

Glucose measurements

Glucose measurements were done using a fluorescence-based enzymatic detection kit (Biovision, Inc., Mountain View, CA, USA) as described previously (Jurczyk *et al.* 2011).

RNA deep sequencing (RNA-seq)

Ten larvae per condition were homogenized in 1 ml of TRIzol reagent (Life Technologies), and total RNA was extracted according to the manufacturer's instructions. RNA samples were treated with DNaseI (Life Technologies) to remove residual genomic DNA. RNA integrity was analyzed by Lab-on-a-chip analysis (Agilent, Amstelveen, The Netherlands). The average RNA integrity (RIN) value of the RNA samples was 9.7, with a minimum of 9.5. A total of 2 μg of RNA was used to make RNA-seq libraries using the Illumina TruSeq RNA Sample Preparation Kit v2 (Illumina, Inc., San Diego, CA, USA). The manufacturer's instructions were followed with the exception of two modifications. In the adapter ligation step, 1 μl, instead of 2.5 μl, adaptor was used. In the library size-selection step, the library fragments were isolated with a double Ampure XP purification with a 0.7× beads to library ratio (Beckman Coulter, Woerden, The Netherlands). The resulting mRNA-seq library was sequenced using an Illumina HiSeq2500 Instrument (Illumina, Inc.) according to the manufacturer's instructions with a read length of 2×50 nucleotides. Image analysis and base-calling were done by the Illumina HCS version 2.0.12. Data analysis was performed using Genetiles Software (www.genetiles.com, W J Veneman, J de Sonneville, K J van der Kolk, A Ordas, Z Al-Ars, A H Meijer and H P Spaink 2014, unpublished). False discovery rate (FDR)-adjusted *P* values were calculated based on the algorithm of Benjamini & Hochberg (1995). The raw RNA-seq data have been deposited in the NCBI GEO database under accession number GSE55836.

Western blot analysis

Whole, homogenized zebrafish larvae were prepared in lysis buffer ($n=15$/sample; 25 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium-deoxycholate, and 0.1% SDS) with protease inhibitor (Roche Applied Sciences). The lysates were centrifuged at 12 000 ***g*** for 10 min at 4 °C and supernatants stored. Supernatants were incubated at 95 °C for 10 min. The protein concentration was determined using a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). The extracted protein samples were subjected to SDS–PAGE and transferred onto nitrocellulose membranes (Bio-Rad). The membranes were blocked with 3% BSA in Tris-buffered saline with 0.1% Tween 20 for 1 h at room temperature. The membranes were then incubated with antibodies recognizing phosphorylated AKT (Cell Signaling Technology, Beverly, MA, USA; no. 9271, phospho-Akt Ser 473), total AKT (Cell Signaling, no. 9272), and Pan-actin (Cell Signaling, no. 4968) as a loading control overnight. After washing with Tris-buffered saline with 0.1% Tween 20, the membranes were incubated with the appropriate HRP-conjugated secondary antibodies (GE Healthcare, Chalfont St Giles UK) for 1 h and washed again. Immunoreactivity was detected by chemiluminescence (GE Healthcare). Finally, the bands were quantified by densitometry using ImageJ Software (National Institutes of Health, Bethesda, MD, USA). The bands were quantified by densitometry using ImageJ 64 Software (National Institutes of Health).

Results

Zebrafish that received human insulin showed inhibition of gluconeogenesis and transient hypoglycemia

In order to study whether zebrafish are sensitive to human insulin, we analyzed phosphoenolpyruvate carboxykinase 1 (*pck1*) expression levels because the transcription of this gene is well known to be inhibited by insulin (O'Brien & Granner 1990). For instance in mammalian systems, *pck1* RNA levels are decreased after 20–40 min of insulin stimulation (Sasaki *et al.* 1984, O'Brien & Granner 1990). Zebrafish larvae at 4 dpf received injections of 1 nl of 100 nM human recombinant insulin into the caudal aorta (Supplementary Fig. 2A). qPCR analysis of *pck1* expression was carried out in zebrafish larvae 0.5, 1, 2, and 4 h after injection (hpi) (Fig. 1A). The observed results confirmed that *pck1* expression was significantly reduced in the presence of insulin at all the time points

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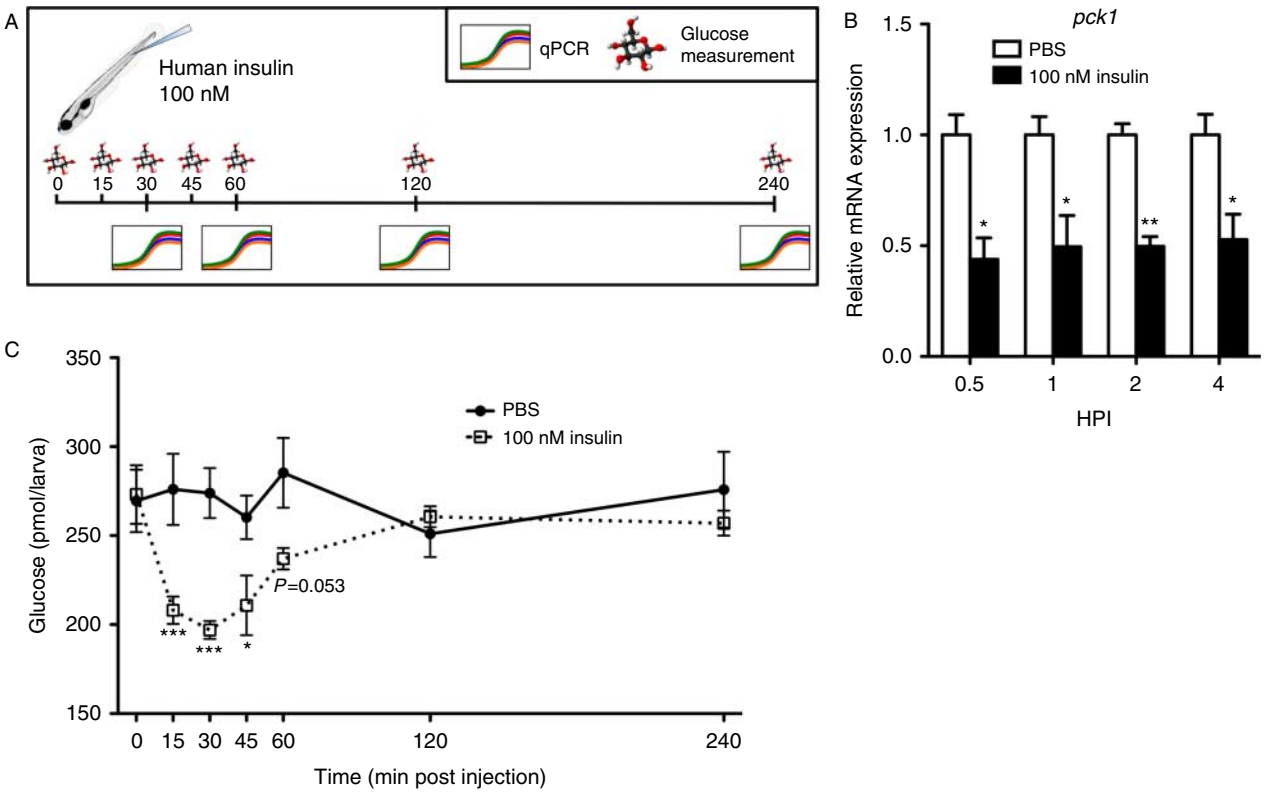

Figure 1
Zebrafish larvae are sensitive to human insulin. (A) Outline of the experiment. Zebrafish larvae received injections of 100 nM human recombinant insulin into the caudal aorta at 4 dpf. Control larvae received injections of PBS. Samples for glucose measurements were taken 0, 15, 30, 45, 60, 120, and 240 min after injection. The samples for qPCR analysis were taken at 0.5, 1, 2, and 4 hpi. Values on the scale are in minutes. (B) Expression of *pck1* was analyzed by qPCR 0.5, 1, 2, and 4 hpi. RNA was extracted from larvae that had received injections of PBS or 100 nM insulin. Data (mean ± S.E.M.) are combined from four biological replicates ($n = 10$ larvae/group) and expressed relative to their respective PBS control, which is defined as 1. (C) Glucose dynamics were studied from 0 to 240 min after 100 nM insulin administration and compared with results for larvae that received injections of PBS. Data (mean ± S.E.M.) are combined from five biological replicates ($n = 10$ larvae/group). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-14-0178.

studied (Fig. 1B). Next, to further confirm if insulin injection exerts the hypoglycemic effect observed in mammals, we carried out a time course experiment for measuring glucose levels of zebrafish larvae that received injections of insulin (Fig. 1A). Our results showed that, as in mammals, insulin administration leads to a fast reduction in free glucose levels from 15 to 45 min post injection, restoring normoglycemia from 2 hpi and onwards (Fig. 1C). Unfortunately, we failed to detect significant changes in Akt phosphorylation on the residue S473 30 min after injection, probably due to the fact that the analysis was performed at a whole-embryo level losing in this way sensitivity (Supplementary Fig. 2B).

Zebrafish larvae treated with a high dose of human insulin develop insulin resistance

The prolonged downregulation observed in *pck1* expression, up to 4 hpi, together with the fact that larvae injected with 100 nM insulin may experience supra-physiological concentrations of the hormone, led us to propose the hypothesis that the larvae that received injections of insulin may be developing hyperinsulinemia-derived insulin resistance. To test this hypothesis, zebrafish larvae were administered human insulin as described earlier and at 4 hpi the same larvae received a second dose of insulin (Fig. 2A), while control individuals received injections of PBS at the same time points. Interestingly, after the injection of the second dose of insulin, zebrafish larvae exhibited transient hyperglycemia rather than the expected hypoglycemia (Fig. 2B). In addition, to confirm whether this is a dose-dependent effect, we carried out qPCR analysis of *pck1* expression of larvae that had received injections of 1 nl of 10 nM, 100 nM, or 1 μM human insulin at 4 hpi. Our results indicated that at 10 nM, *pck1* expression was not significantly reduced compared with results for control embryos, while embryos that received injections of 100 nM and 1 μM insulin

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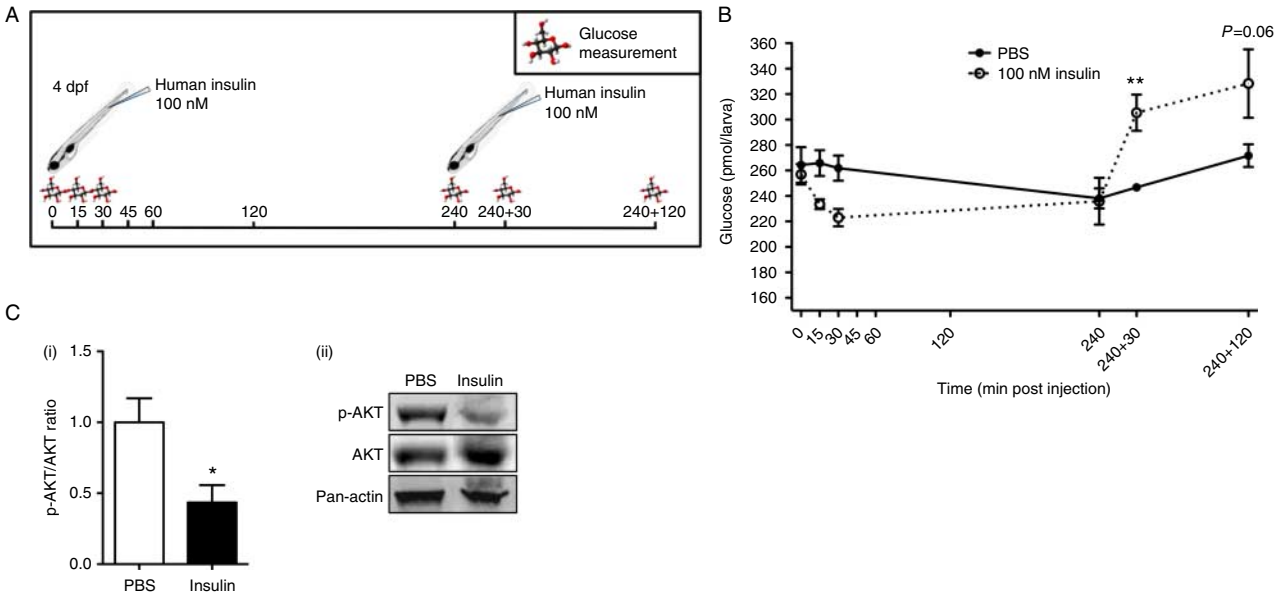

Figure 2

Zebrafish larvae treated with a high dose of insulin lose insulin sensitivity. (A) Outline of the experiment. Zebrafish larvae received injections of 100 nM human recombinant insulin into the caudal aorta at 4 dpf and a second injection 4 hpi (240 min). Control larvae received injections of PBS. Samples for glucose measurements were taken 0, 15, 30, and 240 min after the first injection and 30 and 120 min after the second injection. Values on the scale are in minutes. (B) Glucose dynamics were studied 0, 15, 30, and 240 min after administration of 100 nM insulin to confirm the effect of the first injection. A second injection with the same dose of insulin was performed 240 min after the first dose and glucose levels were analyzed 30 and 120 min later. Larvae that had received injections of PBS were used as controls and exhibited no significant changes in glucose levels during the experiment. Data (mean±S.E.M.) are combined from four biological replicates ($n=5$ larvae/group). (C) Western blotting analysis of whole larvae at 4 hpi of PBS or 100 nM insulin was carried out as indicated in 'Materials and methods' section. Data (mean±S.E.M.) are combined from four biological replicates ($n=15$ larvae/group) and expressed relative to their respective PBS control, which is defined as 1 (i). A picture of a representative immunoblot is shown (ii). *$P<0.05$ and **$P<0.01$. A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-14-0178.

presented a significant downregulation of *pck1* expression (Supplementary Fig. 3, see section on supplementary data given at the end of this article).

Next, to further confirm that zebrafish larvae at 4 hpi experienced insulin resistance, we analyzed Akt activity by measuring phosphorylation levels on residue S473. In agreement with the previous observations, larvae that received injections of 100 nM insulin at 4 hpi exhibited a significant reduction in Akt phosphorylation (Fig. 2C).

Overall, these results indicate that administration of high doses of human insulin to zebrafish larvae results in a loss of insulin sensitivity.

Transcriptomic profiling shows that key genes for the insulin metabolism and immune system are downregulated in hyperinsulinemic zebrafish larvae

To better characterize how the shift from an insulin-sensitive state to an insulin-resistant state may affect zebrafish larvae, we carried out an RNA-seq-based transcriptome analysis of zebrafish larvae that received injections of insulin at 0.5 and 4 hpi (Fig. 3A). Larvae that received injections of PBS were used as controls and RNA was isolated from ten individuals per condition. To analyze the data, we used significance cutoffs at 1.5-fold change at $P<0.05$ (FDR adjusted P value). As expected, samples at 0.5 hpi showed a reduced number of differentially expressed genes (DEGs) compared with samples at 4 hpi, with 630 (202 upregulated and 428 downregulated) and 4187 (1639 upregulated and 2548 downregulated) DEGs respectively. Next, to further study the annotated DEGs at 0.5 and 4 hpi, we carried out a gene ontology (GO) analysis for functional classification (Supplementary Tables 1 and 2, see section on supplementary data given at the end of this article). Study of GO biological processes at 0.5 hpi revealed a significant enrichment in functional categories involved in metabolism (e.g. response to hormone, response to nutrient levels, gluconeogenesis, and glucose metabolic process), ion transport (e.g. potassium ion transport and monovalent inorganic cation transport), lipid biosynthetic process, and rhythmic process (Fig. 3). Analysis of GO-biological process at 4 hpi showed enrichment in categories related to carbohydrate/insulin

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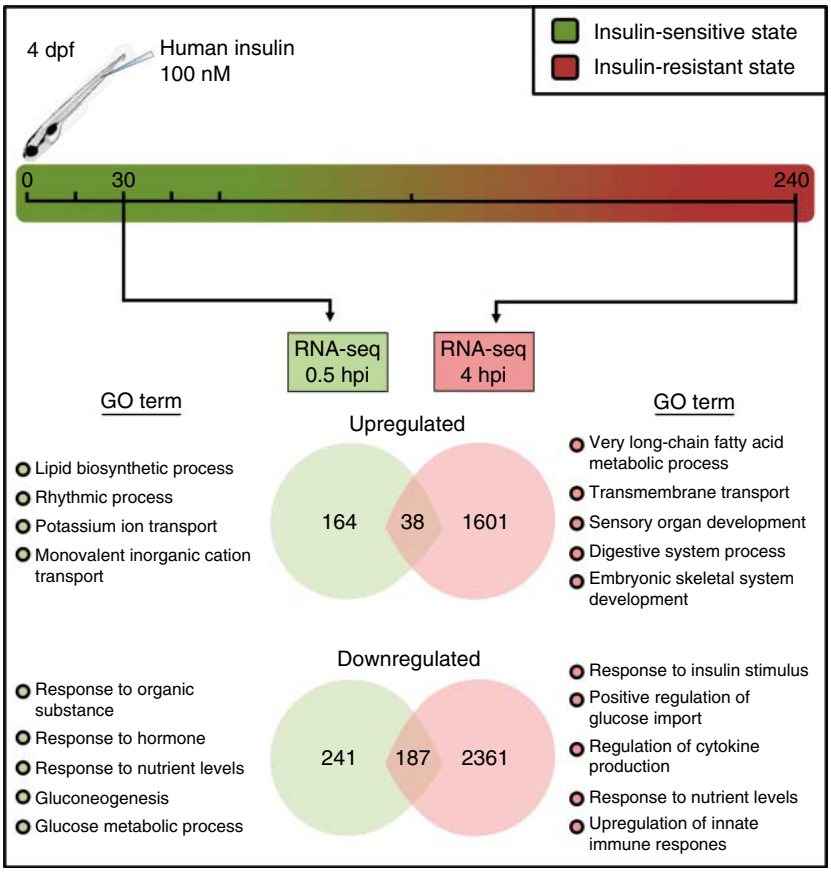

Figure 3

Transcriptome analysis and comparison of zebrafish larvae in an insulin-sensitive state and insulin-resistant state. Zebrafish larvae received injections of 100 nM human recombinant insulin at into the caudal aorta at 4 dpf. Control larvae received injections of PBS. RNA-seq was carried out on RNA samples extracted from pools of ten larvae per condition and a total of four samples per condition were analyzed. Significance cutoffs were set at fold change $>|1.5|$ and $P<0.05$. Venn diagrams show the overlap between DEGs after 0.5 and 4 hpi. The number of DEG is indicated in the Venn diagrams and selected GO terms for biological processes are indicated next to the appropriate condition. A detailed overview of the data is shown in Supplementary Tables 1–7. A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-14-0178.

metabolism (e.g. response to insulin stimulus, positive regulation of glucose import, and response to nutrient levels), innate immune response (e.g. regulation of cytokine production and upregulation of innate immune response), organ development (e.g. sensory organ development and embryonic skeletal system development), fatty acid metabolic process, digestive system process, and transmembrane transport (Fig. 3).

Interestingly, larvae at 4 hpi showed downregulation of insulin metabolism, confirming our previous observations, and also appeared to experience a general downregulation of immune-related processes (Fig. 3). Among DEGs involved in the insulin signaling pathway, all the genes known to play a central role in this pathway (*insr*, *irs1*, *irs2*, *pik3cb*, and *pdk1*) appeared to be downregulated at 4 hpi (Fig. 4). In addition, to further understand the effects on the innate immune system, we analyzed the expression of genes coding for important cytokines and for signal mediators and transcription regulators previously demonstrated to be relevant to the immune response (van der Vaart *et al.* 2012, Meijer *et al.* 2014). Strikingly, our data showed that all the genes studied in this context were downregulated at 4 hpi, except *ptpn6*, *cxcr3.1*, *cxcr4b*, and *irf5* (Fig. 4). Furthermore, other genes known to have a relevant role during the defense response in zebrafish showed similar effects (Fig. 4). Finally, we also studied genes important for various metabolic processes: genes involved in the leptin signaling pathway, gluconeogenesis and lipid metabolism. Overall, the observed effect on the expression of these genes confirmed the effect of insulin at 0.5 hpi, for instance on *per2* and *pck1* which are known to be rapidly regulated by insulin in mammals (O'Brien & Granner 1990, Tahara *et al.* 2011). The data also provide further evidence of the loss of insulin sensitivity at 4 hpi, as observed with *lepb*, *lepr*, *gck*,

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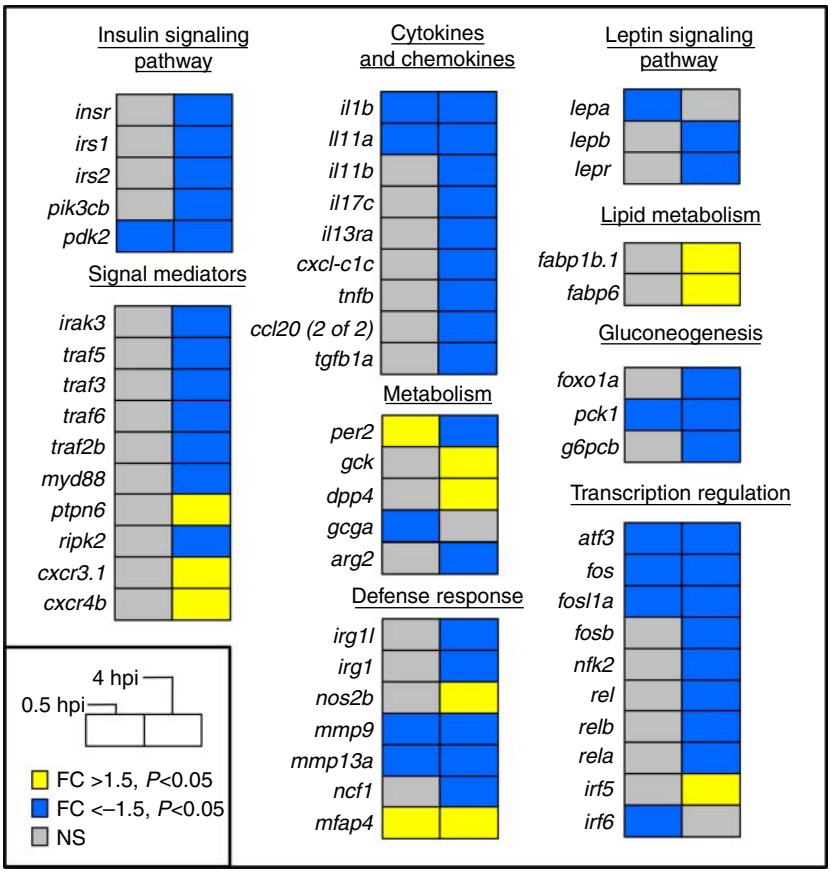

Figure 4

Transcriptomic profile of the switch from an insulin-sensitive state to an insulin-resistant state confirms the downregulation of insulin and leptin signaling pathways and reveals strong downregulation of immune-relevant genes. For each gene, the left box corresponds to the changes observed at 0.5 hpi and the right indicates changes observed at 4 hpi. Blue boxes represent downregulation, yellow boxes represent upregulation, and grey boxes indicate that no significant changes were observed. Significance cutoffs were set at fold change (FC) $>|1.5|$ and $P<0.05$. A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-14-0178.

and *dpp4* reported to be dysregulated in insulin-resistant states (Wang *et al*. 2001, Jiang *et al*. 2011, Sell *et al*. 2013). In addition, the expression of *il1b*, *cxcl-c1c*, *lepb*, *lepr*, and *pck1* was analyzed by qPCR to validate the RNA-seq data, the same effects on the expression of all the analyzed genes were detected by both techniques at 4 hpi of insulin (Supplementary Fig. 4, see section on supplementary data given at the end of this article).

Altogether, our data indicate that administration of a high dose of insulin inactivates the transcription of genes of importance for the immune system and downregulates the insulin signaling pathway and other metabolic genes. Therefore these results resemble gene expression patterns observed in insulin-resistant states in mammalian models (Ginsberg *et al*. 1976, Martin *et al*. 1983, Araki *et al*. 1994, Tamemoto *et al*. 1994, Patti & Kahn 1996, Withers *et al*. 1998, Boura-Halfon & Zick 2009).

Knockdown of *ptpn6* prevents the downregulation of insulin and leptin signaling pathways and immune-relevant genes in hyperinsulinemic larvae

One of the interesting observations from our transcriptomic profiling was that, despite the general immune suppression observed at the gene expression level, one of the few genes significantly upregulated at 4 hpi was *ptpn6*. This gene is a well-known immune modulator playing a critical role as a negative regulator of the immune response (An *et al*. 2008, Croker *et al*. 2008). Importantly, PTPN6 has been described as a modulator of insulin signaling (Dubois *et al*. 2006), and recent findings have demonstrated its importance in the development of insulin resistance and non-alcoholic fatty liver diseases in diet-induced obesity (Xu *et al*. 2014*a*,*b*). Recently, Kanwal *et al*. (2013) have described a zebrafish model for *ptpn6* deficiency, in which

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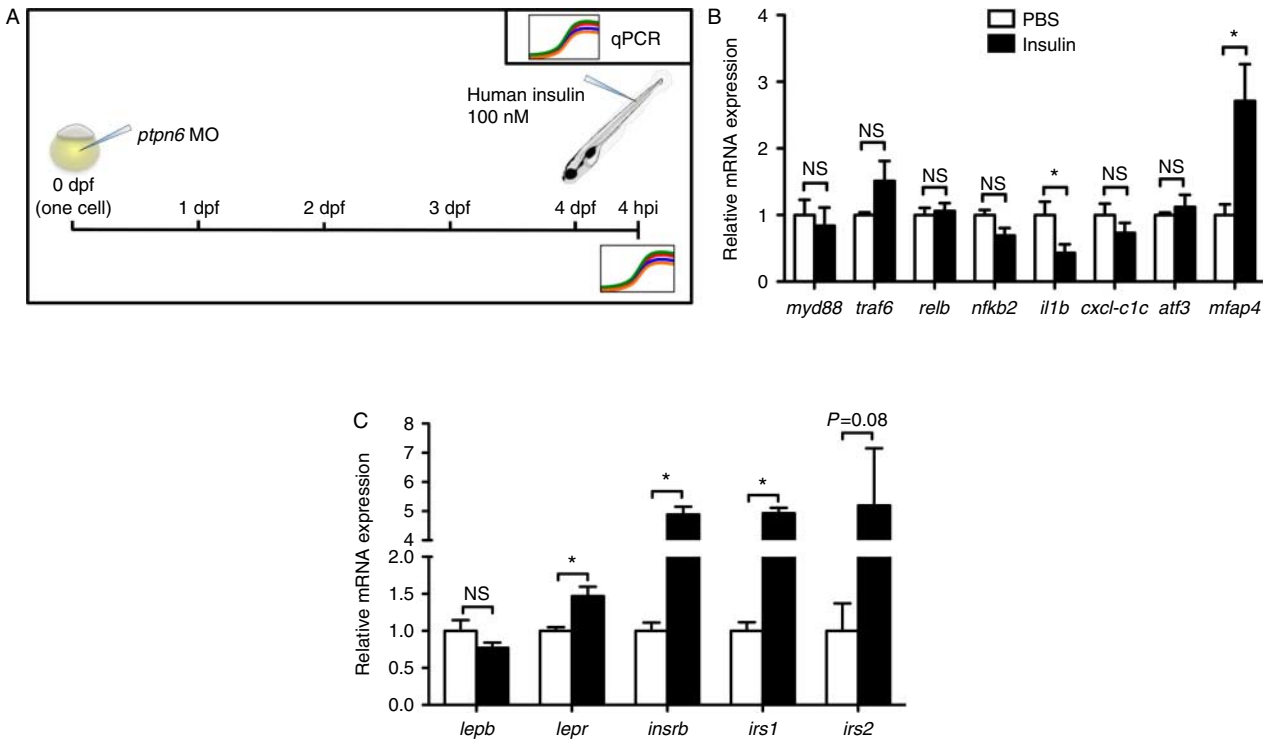

Figure 5

ptpn6 abrogation interferes with the transcriptional effect of the hyperinsulinemia. (A) Outline of the experiment. Zebrafish larvae at the one-cell stage received injections of *ptpn6* MO and morphant larvae at 4 dpf received injections of 100 nM human insulin or PBS for the controls. Expression of pro-inflammatory genes (B) and genes of the leptin and insulin signaling pathways (C) in *ptpn6* morphants was analyzed by qPCR at 4 hpi. RNA was extracted from larvae that had received injections of PBS or 100 nM insulin. Data (mean±S.E.M.) are combined from four biological replicates ($n=5$ larvae/group) and expressed relative to their respective PBS control, which is defined as 1. *$P<0.05$. NS, no significant differences. A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-14-0178.

they observed that abrogation of this gene causes hyperactivation of the innate immune system, indicating that the piscine ortholog of *ptpn6* may play the same role as its mammalian counterpart. In view of this, we proposed the hypothesis that long exposure to high plasma levels of insulin may stimulate *ptpn6* expression, which in turn led to a dampening of the immune system and, at least partially, to the observed insulin-resistant phenotype. To test this hypothesis, we injected 100 nM insulin intravenously at 4 dpf in larvae that had previously received injections of the *ptpn6* morpholino (Fig. 5A) which has been recently shown to efficiently abrogate *ptpn6* expression (Kanwal *et al.* 2013).

At 4 hpi we analyzed (by qPCR) RNA expression of immune relevant genes, such as *myd88*, *traf6*, *relb*, *nfkb2*, *il1b*, *cxcl-c1c*, *atf3*, and *mfap4*, as well as genes important for leptin and insulin signaling pathways, namely *lepb*, *lepr*, *insr*, *irs1*, and *irs2*. Our results (Fig. 5B) show that the immune genes analyzed were not affected by insulin in the *ptpn6*-deficient larvae, except in the case of *il1b* and *mfap4* where the same effects as detected by RNA-seq from WT larvae was observed. In addition, our data indicates that *ptpn6* abrogation prevents the downregulation of all the metabolic genes analyzed, while the expression of *lepr*, *insrb*, and *irs1* was significantly increased (Fig. 5C). Moreover, analysis of basal expression levels of *il1b*, *cxcl-clc*, *lepb*, and *lepr* in *ptpn6* morphants showed that *il1b* and *lepr* basal expression levels were similar to those of control embryos, while *lepb* and *cxcl-c1c* appeared to be significantly higher (Supplementary Fig. 5, see section on supplementary data given at the end of this article). We cannot exclude the possibility that the *ptpn6* abrogation may prevent insulin resistance and immune suppression due to a basal overexpression of some genes (e.g. *lepb* and *cxcl-clc*). Future in-depth studies would be needed to address this complex question.

Interestingly, a time course experiment (Fig. 6A) demonstrated that *ptpn6* upregulation was not observed at earlier time points (Fig. 6B). Next, to further study whether *ptpn6* morphants exhibit improved insulin sensitivity, we injected a second dose of insulin 4 hpi of the first dose and glucose levels were measured (Fig. 6A). Our results indicated that *ptpn6* morphants presented a

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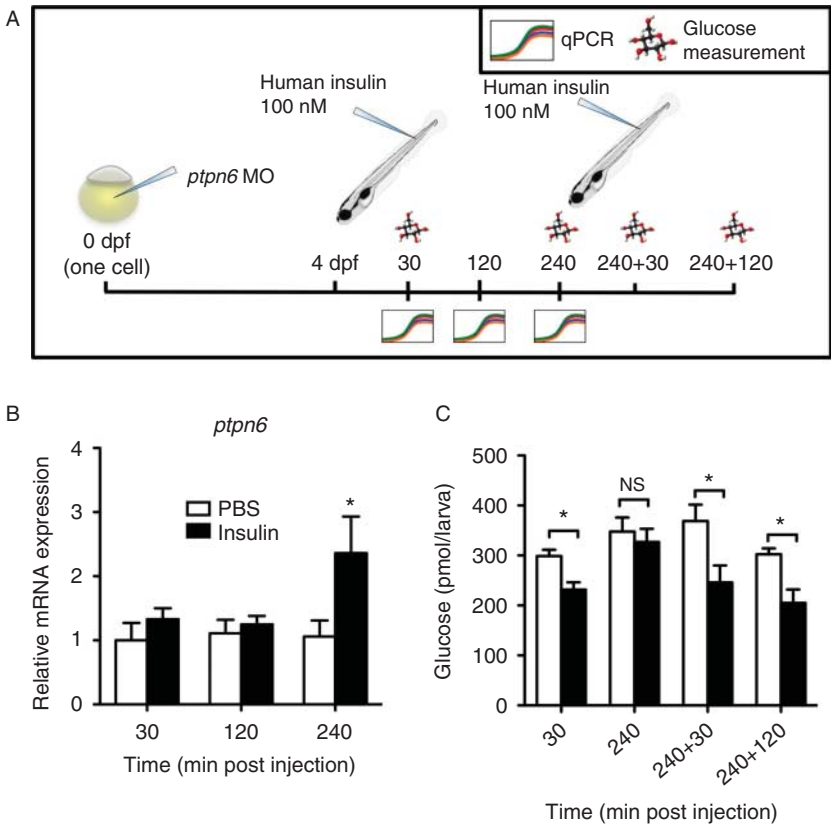

Figure 6

ptpn6 abrogation prevents the loss of insulin sensitivity caused by the hyperinsulinemia. (A) Outline of the experiment. Zebrafish larvae received injections of *ptpn6* MO at the one-cell stage and morphant larvae at 4 dpf received injections of 100 nM human insulin or PBS for the controls and received a second injection 240 min after the first injection. Values on the scale are in minutes. (B) *ptpn6* expression in control embryos was analyzed by qPCR 30, 120, and 240 min after the first insulin injection. RNA was extracted from larvae that had received injections of PBS or 100 nM insulin. Data (mean±S.E.M.) are combined from four biological replicates ($n=3$ larvae/group) and expressed relative to their respective PBS control, which is defined as 1. (C) Glucose dynamics were studied 30 and 240 min after administration of 100 nM insulin to confirm the effect of the first injection. A second injection with the same dose of insulin was administered 240 min after the first dose and glucose levels were analyzed 30 and 120 min later. Larvae that had received injections of PBS were used as controls and exhibited no significant changes in glucose levels during the experiment. Data (mean±S.E.M.) are combined from four biological replicates ($n=5$ larvae/group) *$P<0.05$. NS, no significant differences. A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-14-0178.

significant reduction in glucose levels 30 and 120 min after the second injection, confirming that abrogation of *ptpn6* improves the glycemic control of the larvae, therefore contributing to the prevention of the development of insulin resistance (Fig. 6 C).

Discussion

In the present report, we describe a non-obese animal model for the study of hyperinsulinemia-derived insulin resistance. Using a newly developed method to administer human insulin intravenously into developed zebrafish, we demonstrate not only that human insulin is functional in zebrafish, but also that these zebrafish larvae become insulin-resistant when high concentrations of human insulin are administered by injection. Our findings indicate that this effect is mediated by stimulation of *ptpn6* expression in the hyperinsulinemic scenario, leading concomitantly to immune suppression. Moreover, abrogation of *ptpn6* was found to counteract the effects of high plasma levels of insulin on the transcription of key genes of the insulin and leptin signaling pathways as well as of the innate immune response.

After injection of 100 nM human insulin into zebrafish larvae at 4 dpf, *pck1* transcription was rapidly inhibited and free glucose levels were significantly reduced at 15 min post injection, reaching normoglycemia again at 1 hpi, mimicking the effect in mammals (Sasaki *et al.* 1984, Barthel & Schmoll 2003, Seino *et al.* 2011). Next, the strong *pck1* downregulation observed at 4 hpi led us to propose the hypothesis that larvae that had received injections of insulin

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could be experiencing insulin resistance caused by high plasma levels of insulin. In mammals, it is well established that insulin concentrations in the blood oscillate between post-absorptive periods, increasing again 4 h after each meal (Polonsky *et al.* 1988). In our case, a second insulin injection 4 h after the first injection did not cause transient hypoglycemia as observed after the first injection, instead we observed that these larvae showed transient hyperglycemia, consistent with other models of insulin resistance that present higher levels of plasma glucose (Shafrir 2010). However, it is worth mentioning that, despite not being statistically significant, at 2 hpi of the second insulin dose, glucose levels remained higher than in larvae that had received injections of PBS. This observation seems to contradict the observed downregulation of *pck1* and *g6pc* at 4 hpi, as these genes have been typically described as mediating a rate-limiting step of gluconeogenesis. In consequence, hyperglycemic states in pathologies characterized by poor glycemic control (e.g. type 2 diabetes, obesity, and metabolic syndrome) have been thought to be associated with upregulation of both *pck1* and *g6pc* expression. However, recent observations have challenged this paradigm. Samuel *et al.* (2009) demonstrated that expression of PEPCK and G6Pc was unaltered during fasting hyperglycemia using rodent models for diabetes. Moreover, in the same study, the authors showed that patients with type 2 diabetes did not exhibit any changes in hepatic PEPCK nor in G6Pc expression (Samuel *et al.* 2009). In addition, recently it has been shown that *Pepck* mutant mice exhibiting a 90% reduction in PEPCK protein levels only exhibit a 40% reduction in gluconeogenic flux (Burgess *et al.* 2007). Altogether, previous studies and the present data highlight the complex regulation of gluconeogenesis and indicate that further research focused on other less studied gluconeogenic genes (e.g. pyruvate carboxylase) should be carried out.

It is known that hyperinsulinemia can lead to insulin resistance in mammals by downregulating the mediators of the insulin signaling pathway (Kahn & Flier 2000), as shown by a number of *in vivo* studies where administration of high doses of insulin led to insulin resistance (Kobayashi & Olefsky 1978, Martin *et al.* 1983, Rizza *et al.* 1985). Supporting this notion, our data indicated that the transcription of genes coding for the key mediators of the insulin signaling pathway was inhibited, coinciding with the loss of insulin sensitivity and a decrease in Akt phosphorylation which is associated with insulin-resistant states (Tomas *et al.* 2002). Moreover, at this stage hyperinsulinemic larvae appeared to be immunosuppressed at the transcriptional level, whereas the only immune modulator showing an increased expression was *ptpn6*. In mammals and zebrafish, *ptpn6* has been reported to be a negative regulator of the immune response (Zhang *et al.* 2000, Tsui *et al.* 2006, Pao *et al.* 2007, An *et al.* 2008, Croker *et al.* 2008, Lorenz 2009, Kanwal *et al.* 2013) and a key determinant of development of insulin resistance and non-alcoholic fatty liver diseases (Xu *et al.* 2012, 2014*a*). We found that knocking down of *ptpn6* interfered with the inhibitory effect observed on key transcription factors and signal mediators of the NF-κB pathway as well as on various cytokines in larvae that had received injections of insulin. It is worth mentioning that, although *il1b* expression was reduced after insulin injection also in *ptpn6* knocked-down larvae, this is consistent with the observation that *il1b* expression was altered in *ptpn6* morphants at 5 dpf and onwards, but not at 4 dpf (Kanwal *et al.* 2013). Besides this, we also observed that some genes identified as playing a role in innate immunity, such as *mfap4*, *nos2b*, *cxcr3.1*, *cxcr4b*, and *irf5*, were upregulated at 4 hpi, and after *ptpn6* abrogation *mfap4* was still induced by insulin. We cannot rule out the possibility that other immune modulators involved in particular tissue-specific innate immune processes are under-represented in our data set because in our model we focussed on major changes at the whole-embryo level. Future studies on dissected organs or FACS-sorted cells using tissue-specific labeled transgenic lines will be helpful to improve our understanding of which mechanisms contribute to the development of this immune dysregulation.

Studying the effect of *ptpn6* abrogation on the insulin signaling pathway, we observed that morphant larvae administered insulin exhibited an increase in the expression of *insrb*, *irs1*, and *irs2* (though not significant in the case of *irs2*). These results are in agreement with data from *Ptpn6*-deficient (*Ptpn6*$^{me\text{-}v/me\text{-}v}$) mice, which displayed

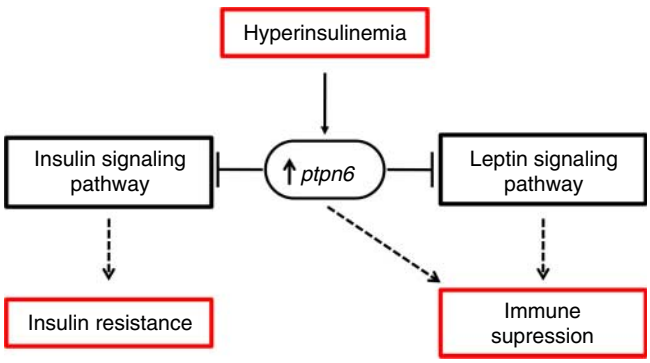

Figure 7
Model for hyperinsulinemia-induced immune suppression and insulin resistance via *ptpn6*. A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-14-0178.

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increased tyrosine phosphorylation of INSR, IRS1, and IRS2 and an enhanced insulin sensitivity (Dubois *et al.* 2006). Supporting this notion, *ptpn6* morphants showed improved glycemic control, exhibiting hypoglycemia up to 2 h after administration of a second dose of insulin. This is consistent with the previous observations and confirms the central role of *ptpn6* in mediating hyperinsulinemia-derived insulin resistance in zebrafish.

In addition, key members of the leptin signaling pathway, namely *lepb*, are known to be regulated by nutritional factors in zebrafish as in its mammalian ortholog (Gorissen *et al.* 2009), and *lepr* appeared to be downregulated in insulin-resistant larvae while *ptpn6* abrogation was found to prevent this transcriptional inhibition.

Interestingly, it has been observed that stimulation of PTPN6 by insulin leads to JAK2 dephosphorylation, therefore interfering with the leptin signaling pathway (Kellerer *et al.* 2001). With these observations in mind, and knowing that animal models for insulin resistance with mutations in leptin or leptin receptor genes are immuno-suppressed it is tempting to propose the hypothesis that hyperinsulinemia may downregulate the insulin signaling pathway leading to insulin resistance via induction of *ptpn6*. Our results also indicate that the observed immuno-suppression may be due to a combination of factors, of which *ptpn6* is exerting its role as a negative immune regulator by inhibiting the leptin signaling pathway that eventually contributes to the observed immune dysregulation (Fig. 7).

These observations raise an interesting point for discussion. Leptin-deficient rodents used as models for insulin resistance are hyperinsulinemic, insulin-resistant, and immuno-compromised (Meade *et al.* 1979, Chandra 1980, Zhang *et al.* 1994, Lord *et al.* 1998, Howard *et al.* 1999). Importantly, these observations are, in part, opposite to the human scenario where obese individuals that develop insulin resistance exhibit high leptin levels and an induction of inflammation (Gregor & Hotamisligil 2011). To circumvent this, high-fat-diet-induced obese mice are also used as a model as they develop insulin resistance and hyperinsulinemia, but still there are some discrepancies with the human situation. One of the major issues we face when studying insulin resistance is that this is a state associated with a number of pathophysiologies, and in consequence a multifactorial disease, making it difficult to study using animal models. In this light the main contribution of our model to the existing ones is that it complements them as it allows the study of insulin resistance in a non-obese animal, helping in this way to clarify the mechanisms by which insulin exerts its effect and more importantly how this can trigger insulin resistance.

In summary, in the present study, we provide a new tool for the study of insulin metabolism and insulin resistance. Taking advantage of the zebrafish model, in which we can easily obtain and handle large numbers of non-obese individuals with fully functional organs, we have demonstrated that zebrafish larvae are sensitive to human insulin. Importantly, we showed how i.v. injection of a high dose of insulin leads to insulin resistance and immune suppression. Moreover, we showed that modulating *ptpn6* expression interferes with the effects caused by hyperinsulinemia, preventing the downregulation of insulin and leptin signaling pathways and blocking the immune dysregulation. As mentioned by Kanwal *et al.* (2013), *ptpn6* regulation of transcription and its relation with immune suppression via the glucocorticoid pathway is still poorly understood. Future studies will be needed to elucidate the even more complex role that *ptpn6* plays regulating the transcription of the metabolic and immune signaling pathways mentioned in this study.

In conclusion, we present a new hyperinsulinemia animal model that complements the existing rodent models, allowing the study of insulin resistance in a non-obese state, and propose *ptpn6* as a key mediator triggering hyperinsulinemia-derived insulin resistance and immune suppression. This important connection between insulin regulation and the immune system can now be studied further in the various infection and cancer models that have been recently developed for zebrafish.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-14-0178.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
R M-J was supported by a Marie Curie fellowship as Experienced Researcher in the EU Initial Training Network FishForPharma (PITN-GA-2011-289209). S Y was supported by a grant from the China Scholarship Council.

Author contribution statement
R M-J conceived the study, designed and performed experiments, analyzed the data, and wrote the paper. S J-R designed and performed experiments. S Y designed and performed experiments. H P S designed experiments, supervised the work, and wrote the paper. All authors assisted with editing of the manuscript and approved the final version.

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DOI: 10.1530/JOE-14-0178

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Acknowledgements

The authors thank Dr Annemarie H Meijer for the gift of the *ptpn6* morpholino. They deeply express their indebtedness to Drs Josep V Planas, Annemarie H Meijer, and Anna-Pavlina Haramis for critical reading of the manuscript. They thank Davy de Witt, Ulrike Nehrdich, and Laura van Hulst for fish caretaking and Hulya Ozupek, Drs Hans Jansen, and Ron Dirks (ZF-Screens B.V.) for assistance with RNA-seq analysis. They also want to thank Dr Jan de Sonneville and Kees-Jan van der Kolk for making the Genetiles Software available before publication and to Vanesa Jiménez-Amilburu for her assistance with the western blotting analysis.

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Received in final form 30 May 2014
Accepted 5 June 2014
Accepted Preprint published online 5 June 2014

http://joe.endocrinology-journals.org
DOI: 10.1530/JOE-14-0178

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