Improved regeneration and de novo bone formation in a diabetic zebrafish model treated with paricalcitol and cinacalcet

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ABSTRACT

Bone changes related to diabetes have been well established, but few strategies have been developed to prevent this growing health problem. In our work, we propose to investigate the effects of calcitriol as well as of a vitamin D analog (paricalcitol) and a calcimimetic (cinacalcet), in fin regeneration and de novo mineralization in a zebrafish model of diabetes. Following exposure of diabetic transgenic Tg(ins:nfsb-mCherry) zebrafish to calcitriol, paricalcitol and cinacalcet, caudal fins were amputated to assess their effects on tissue regeneration. Caudal fin mineralized and regenerated areas were quantified by in vivo alizarin red staining. Quantitative real-time PCR was performed using RNA from the vertebral column. Diabetic fish treated with cinacalcet and paricalcitol presented increased regenerated and mineralized areas when compared with non-treated diabetic group, while no significant increase was observed in non-diabetic fish treated with both drugs. Gene expression analysis showed an up-regulation for runt-related transcription factor 2b (runx2b), bone gamma-carboxyglutamic acid-containing protein (bglap), insulin a (insa) and insulin b (insb) and a trend of increase for sp7 transcription factor (sp7) in diabetic groups treated with cinacalcet and paricalcitol. Expression of insra and vdra was up-regulated in both diabetic and nondiabetic fish treated with cinacalcet. In nondiabetic fish treated with paricalcitol and cinacalcet a similar increase in gene expression could be observed but not so pronounced. The increased mineralization and regeneration in diabetic zebrafish treated with cinacalcet and paricalcitol can be explained by increased osteoblastic differentiation and increased insulin expression indicating pro-osteogenic potential of both drugs.
beside not having any impact in regulating glucose levels, this Proins/TNF-α-expressing cells had their origin in bone marrow and then migrated to several parts of the body, initiating diabetic neuropathy.26 Cunha et al.,27 in their experiments with diabetic mice treated with streptozotocin, observed beneficial effects in the secretion of insulin by the tear film of the eye and showed that insulin was locally synthesized in the lachrymal gland.27 With the current body of evidence, it is difficult to determine if extrapancreatic expression of insulin has any effect in glucose homeostasis, or if it serves other undetermined functions.

Zebrafish have been well established as a model for human diseases, spanning a wide range of human pathologies including genetic disorders and physiological processes that are known to be highly conserved throughout vertebrate evolution.28 Recently it was demonstrated, under type 1 diabetic conditions, that fin regeneration was impaired in zebrafish with reduced cell proliferation and increased cell apoptosis.29 We conducted experimental trials to understand if VitD, paricalcitol and cinacalcet, could have beneficial effects on caudal fin regeneration and bone mineralization in adult zebrafish and in operculum development of larvae under type 1 diabetic conditions. To test this hypothesis we used the transgenic Tg(ins:nfsb-mCherry) zebrafish that after being exposed to the prodrug metronidazole becomes hyperglycemic and hipoinsulinemic due to beta-cell ablation, leading to a transient state of diabetes of 10–15 days prior to beta-cell regeneration.30,31 To understand if treatments could induce bone alterations in diabetic conditions, 240 larvae and 145 adults were divided into experimental groups in triplicates and exposed by immersion (larvae) or IP injection (adults) of VitD (0.001 µg/ml) (calcitriol, Sigma-Aldrich), paricalcitol (0.001 µg/ml) (zomplar®, Abbott Laboratories, North Chicago, IL) and cinacalcet (0.05 µg/ml) (mimpara, Amg Europe B.V., Breda, The Netherlands), mimicking the concentrations used in clinical practice. For the control groups of non-diabetic and diabetic fish we used a vehicle solution (citrate buffer 0.05M). Since cinacalcet was found to be lethal to larvae at the concentration used for adults, we have performed treatments with dilutions of the initial concentration at the concentration of 0.005M dissolved in citrate buffer (0.05M). Corresponding control groups were left untreated under the same housing conditions. In addition, we have exposed nontransgenic siblings from a cross between a heterozygous transgenic and a wild type zebrafish, to discard potentially side effects of MET treatment in caudal fin regeneration.

Paricalcitol, cinacalcet and VitD treatments

Seventy-two hours post treatment (hpt) with MET, transgenic zebrafish were screened for loss of fluorescence due to β-cell ablation, as observed by Pisharath et al.30 To understand if treatments could induce bone alterations in diabetic conditions, 240 larvae and 145 adults were divided into experimental groups in triplicates and exposed by immersion (larvae) or IP injection (adults) of VitD (0.001 µg/ml) (calcitriol, Sigma-Aldrich), paricalcitol (0.001 µg/ml) (zomplar®, Abbott Laboratories, North Chicago, IL) and cinacalcet (0.05 µg/ml) (mimpara, Amg Europe B.V., Breda, The Netherlands), mimicking the concentrations used in clinical practice. For the control groups of non-diabetic and diabetic fish we used a vehicle solution (citrate buffer 0.05M). Since cinacalcet was found to be lethal to larvae at the concentration used for adults, we have performed treatments with dilutions of the initial concentration of cinacalcet by 1:10 (0.005 µg/ml), 1:50 (0.001 µg/ml) and 1:100 (0.0005 µg/ml) and included three additional groups of diabetic and nondiabetic larvae treated with cinacalcet.

METHODS

Zebrafish strains and maintenance

The transgenic Tg(ins:nfsb-mCherry) zebrafish line used in our experiments was kindly given by the Laboratory of Molecular Biology and Genetic Engineering, GIGA Research, Liege, Belgium. Transgenic zebrafish were maintained in a recirculating water system (Tecniplast, Buguggiate, Italy). All manipulations were performed by licensed researchers and conducted in accordance with principles and procedures following the guidelines from the Federation of Laboratory Animal Science Associations (FELASA) and in accordance with the EU and national regulations. The Tg(ins:nfsb-mCherry) line generated by Pisharatat el.30 with a Tübingen AB background, contains a construct in which the nfsB gene of E. coli and the florescent protein mCherry are inserted downstream to the promoter region of the insa gene. That bacterial gene encodes a nitroreductase (NTR) enzyme, that converts prodrugs such as metronidazole (MET; Sigma-Aldrich, St. Louis, MO) to cytotoxins. By observing the loss of mCherry fluorescence after MET treatment it is possible to visualize MET dependent β-cell ablation.

Diabetes induction

Larvae at 15 days post fertilization and male and female adults with 1 year old from the Tg(ins:nfsb-mCherry) zebrafish line were anesthetized with tricaine methanesulfonate (Sigma-Aldrich, St. Louis, MO) and exposed to MET either by bath or through intraperitoneal (IP) injection at the concentration of 0.05M dissolved in citrate buffer (0.05M). Corresponding control groups were left untreated under the same housing conditions. In addition, we have exposed nontransgenic siblings from a cross between a heterozygous transgenic and a wild type zebrafish, to discard potentially side effects of MET treatment in caudal fin regeneration.

Staining of mineralized tissue

Larvae (n = 15) were fixed with PFA 4% for 1 hour, washed in PBS and stained in 0.01% alizarin red for 30 minutes. Adults (n = 8) were submitted to live staining in alizarin red at a concentration of 0.01% for 15 minutes prior to observation. Adult regenerated caudal fins and lar

Quantification of operculum mineralized area in larvae

Mineralized area of opercula stained by alizarin red were measured using image J software. Results were normalized...
by dividing operculum area (OA) by total area of the head (HA).

**Quantification of regenerated and mineralized area of adult fin**

Regenerated area was determined by dividing regenerated area (REG) by stump width (STU) and mineralized area was determined by dividing mineralized area (MIN) by mean ray width (MRW) and divided by REG/STU. All quantifications were done using image J software.

**Bone histology and histomorphometry**

The calcified regenerated fins from each of the different groups were transferred to 70% EtOH and processed for dehydration and infiltration on a routine overnight processing schedule. Samples were then embedded in paraffin and sections with 6 μm prepared in a microtome. Before staining, sections were deparaffinized in xylene and dehydrated in an increasing gradient of EtOH. Sections were stained by von Kossa’s as described elsewhere. To determine fin area and thickness, the second, third and fourth hemiray of each fin were measured. Area was assessed by measuring total area of both hemirays and thickness was assessed by four longitudinal measurements of each hemiray. A detailed time course of the different procedures from metronidazole treatment to amputation and data acquisition is shown in Supplementary Figure S1.

**Glucose tolerance test**

To confirm that ablation of β-cells in Tg(ins:nfsb-mCherry) zebrafish led to an increase in glucose blood concentrations, we administrated a solution of glucose at a concentration of 0.1 M or vehicle by IP injection, to two groups of adult zebrafish after 72 hpt with MET. Blood glucose was monitored at 30, 60, 90, 120, 150 and 180 minutes after IP injection with glucose. The glucose levels were measured in 3 Tg(ins:nfsb-mCherry) zebrafish treated with MET and with vehicle at each time point. 6 μl of blood were collected from the caudal aorta, diluted in 2 μl of 2% heparin and rapidly transferred to a blood glucose meter Glucocard MX (Arkray A. Menarini Diagnostics, Florence, Italy). All these procedures were repeated four times with 6–7 specimens by group.

**Total RNA isolation**

Vertebral columns from 6 adults of each group were isolated and pooled in 2 groups (n = 3/each) for RNA purification. The samples were placed in 1 ml of Isol-RNA Lysis Reagent (5 PRIME, Hilden, The Netherlands) and total RNA was purified according to manufacturer’s protocol. RNA quantity and integrity were verified using Experion RNA Analysis Kit (BIO-RAD, Hercules, CA).

**Quantitative real-time polymerase chain reaction (qPCR)**

Reverse transcription of 1 μg of total RNA was performed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Life Technologies, Carlsbad, CA) according to the manufacturer’s protocol. Semi quantitative qPCR was performed using iQ SYBR Green Supermix (Life Technologies), on an CFX96 Touch Real-Time PCR Detection System (BIO-RAD, Hercules, CA), conducted for 45 cycles of 5 seconds at 95°C and 15 seconds at 60°C each. The primer sequences of elongation factor 1alpha (ef1-alpha), 18S ribosomal rna (18S), runt-related transcription factor 2 b (runx2b), bone gamma-carboxyglutamic acid-containing protein (bglap), sp7 transcription factor (sp7), parathyroid hormone receptor a (pth1ra) and insulin receptor a (insra) were listed in Table 1. All gene expression data were normalized against the mean of the gene expression levels of housekeeping genes observed by qPCR, a RT-PCR reaction was performed and the identity of the amplicons confirmed by sequencing. Gene expression results are the mean of two different experiments.

**Statistical analysis**

All statistical analyses were performed using Stata Statistical Software and data was evaluated using the one-way ANOVA followed by Bonferroni multiple comparisons test with p < 0.05 considered statistically significant. Results are presented as means ± standard deviation of the mean (SD).

**RESULTS**

As previously described, Tg(ins:nfsb-mCherry) presented loss of fluorescence in the region of the pancreas after 72 hpi with MET, confirming β-cell ablation (Supplementary Figure S2). To confirm that Tg(ins:nfsb-mCherry) exposed

### Table 1. List of primers. Sequences presented in 5’ to 3’ orientation

| Forward | Reverse |
|---------|---------|
| ef1a    | TGGAGAAGGGAACACTGGC |
| 18s     | ACCACCAACATCGAGAAAA |
| 18s     | GCCCTGGCTTATTTGACT |
| runx2   | GCACGGAGAGGACTGAGG |
| runx2   | AGGGCCACACCTTAAAGCC |
| bglap   | CAAACTCAGACTCGCGATC |
| bglap   | AGCAACATCCCGGTCCAGACCAT |
| sp7     | TTTCAGACAGGACCTTCG |
| sp7     | GCAATCGAAGAGACTTC |
| pth1ra  | GTTTTCGCTATGTGCTTCG |
| vdra    | GTCCAACAGTCTTCAGTCT |
| vdra    | AGTGTGACCCGCCTTAGTG |
| insb    | CTCGTCTACAGGAAAGG |
| insb    | GAGGAGAAGACTGGGAT |
| insa    | CATTTCTGCCCTGCTTTC |
| insra   | TGCCCCGTTAGTGGCTTACA |
| insra   | TCTACAGGGAGAAACAGGC |
| insra   | AGAGATAAGATCGCTCGT |

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to MET could lead to a state of onset of diabetes, a glucose tolerance test was performed to measure how well animals are able to break down glucose, or sugar. We found that glucose concentrations in the plasma of fish treated with MET were significantly higher than in fish treated with vehicle only, with these differences being highly significant at 90, 120, 150 and 180 minutes after IP injection (Figure 1).

An analysis of the mineralized area of the operculum (Figure 2A and B) of larvae from the D group revealed a significant reduction when compared with nondiabetic samples. However, the mineralized area of the operculum was significantly increased when diabetic larvae were treated with paricalcitol, cinacalcet and ViTD compared with untreated diabetic larvae. Nondiabetic larvae treated with the three different treatments showed a tendency for an increase in mineralization compared with untreated nondiabetic larvae, although not significantly different due to individual variability (Figure 2C).

Following β-cell ablation and regeneration we have quantified the regenerated and mineralized areas (Figure 3A).

Figure 1. Transgenic zebrafish Tg(ins:nfsb-mCherry) 72 hours post induction of diabetes have increased blood glucose concentrations at 30, 60, 90, 120, 150, 180 minutes, after intraperitoneal injection with glucose *p < 0.05; **p < 0.001.

Figure 2. (A) Measurements of operculum mineralized areas; (B) Size of the operculum was obtained by dividing the operculum area by total head area; (C) 15-day-old diabetic larvae have reduced operculum mineralized area when compared with nondiabetic, but treatment with paricalcitol and cinacalcet at 0.005 μg/ml, 0.001 μg/ml and 0.0005 μg/ml were found to increase the mineralized area compared with diabetic larvae. Bars with different superscript letters indicate significant differences (p < 0.05). Operculum area (OA), total area of the head (HA). ND, nondiabetic; D, diabetic; D CC 1:10, D CC 1:50, D CC 1:100, ND CC 1:10, ND CC 1:50, ND CC 1:100, diabetic and nondiabetic groups treated with cinacalcet at concentrations 10, 50 and 100 times lower than the used in clinical practice; D PCT and ND PCT, diabetic and nondiabetic treated with paricalcitol; D VitD and ND VitD, diabetic and nondiabetic treated with vitamin D. [Color figure can be viewed at wileyonlinelibrary.com]
Diabetic zebrafish adults had a statistically significant impairment of fin regeneration when compared with nondiabetics. Furthermore, the diabetic and non-diabetic groups treated with paricalcitol and cinacalcet presented a significant increase in regenerated area when compared with diabetic fish, but a not significant increase in regeneration when compared with nondiabetics (Figure 3B). In nondiabetic treated groups, the increase in regenerated area was not significant. The regenerated area of the diabetic and nondiabetic zebrafish groups treated with VitD did not present any significant differences compared with respective control groups (Figure 3B). Wild type (WT) fish treated with MET showed no differences to nondiabetics or to control WT fish exposed to vehicle.

Quantification of the mineralized area showed that groups treated with paricalcitol and cinacalcet had no differences when compared with untreated nondiabetic fish while the diabetic fish showed a significantly reduced mineralization when compared with nondiabetic and with diabetic paricalcitol and cinacalcet treated groups, while VitD treated groups showed no differences relative to the other treated groups (Figure 3C). Nontransgenic siblings treated with MET or vehicle (WT, WT + MET) showed no alterations both in regenerated and mineralized areas when compared with the ND group (Figure 3B and C).

Histology of rays confirmed previous results, showing increased ray area and thickness (Figure 4A) in the regenerated caudal fins of diabetic fish treated with paricalcitol and cinacalcet when compared with untreated diabetic fish, while nondiabetic treated groups showed no significant increase (Figure 4B and C).

Figure 3. (A) Measurements of regenerated (top row) and mineralized (bottom row) areas in nondiabetic (ND) and diabetic (D) zebrafish adults treated with vitamin D (VitD), paricalcitol (PCT) and cinacalcet (CC) and in control wild type AB (WT) zebrafish or treated with metronidazole (MET); (B) Diabetic group had decreased fin regenerated area compared with non-diabetic and to diabetic treated with paricalcitol and cinacalcet; (C) Diabetic group had decreased fin mineralized area compared with nondiabetic and to diabetic treated with paricalcitol and cinacalcet. Bars with different superscript letters indicate significant differences (p < 0.05). Regenerated area (REG), stump width (STU), mineralized area (MIN), mean ray width (MRW). [Color figure can be viewed at wileyonlinelibrary.com]
Analysis of gene expression levels showed no significant differences relative to the expression of pthra when comparing nondiabetic and diabetic fish (Figure 5A). The vdra expression, showed a statistically significant increase in the cinacalcet treated groups compared with all other groups (Figure 5B). insra expression was found to be significantly down-regulated in all diabetic groups (p < 0.5) compared with nondiabetic, but the diabetic fish treated with cinacalcet showed a lower reduction in expression, with statistically higher values compared with diabetic, paricalcitol and VitD treated fish, while nondiabetic cinacalcet treated group showed increased expression compared with all other groups (Figure 5C). sp7 expression showed a trend of reduction in all diabetic fish treated groups compared with nondiabetics, but significant down-regulation was only found in diabetic compared with nondiabetic groups, no differences being observed among nondiabetic groups (Figure 5D). Regarding runx2b, a significant up-regulation could be observed in groups treated with paricalcitol and cinacalcet (Figure 5E). Expression of bglap was down-regulated in diabetic compared with nondiabetic fish while the paricalcitol and cinacalcet diabetic groups showed significant differences compared with untreated diabetic group. In nondiabetic fish, treated versus control groups were not significantly different. Gene expression of insa and insb were found to be up-regulated (p < 0.001) in both diabetic and nondiabetic paricalcitol and cinacalcet treated fish when compared with the other groups (Figure 4G and H).

To demonstrate the extrapancreatic expression of both insa and insb in vertebral column, we performed an RT-PCR using cDNA of liver/pancreas, muscle, kidney, column and cleithrum/operculum from wild type zebrafish, using the same primers used for qPCR. We observed amplification of ins genes in all tissues analyzed. This result was further confirmed by sequencing the PCR amplicons which were confirmed to correspond to insulin a and b isoforms, respectively (Figure 6).

DISCUSSION

This study demonstrated that zebrafish is a suitable model for the study of bone pathologies related to diabetes. Ablation of β-cell, by exposing Tg(ins:nfsb-mCherry) zebrafish to MET, led to loss of mCherry fluorescence in the β-cells at 72 hpi, as already described by previous authors,30,31 and to blood glucose increase, although differences were not as accentuated as previously reported.30,31 Beta-cell ablation was confirmed by loss of mCherry fluorescence after 72 hours of treatment with MET. Transgenic fish that did not present a total loss of fluorescence were removed from further experimental procedures. The injection of glucose in diabetic fish led to an increase in the plasma glucose levels significantly higher than in nondiabetic controls. The levels of blood glucose started to decrease after 120 minutes post treatment, although they remained significantly higher in diabetic fish while in nondiabetics the levels returned to pretreatment levels. The reduction of glucose levels in plasma observed in diabetic fish can be explained by elimination of excess plasma glucose in urine, as previously reported for other species, where after injection with levels from 6.4 mM to 25 mM glucose in tilapia the excretion of excess glucose in the urine was visible after few hours. It was reported that treated fish had a drastic increase in urine glucose levels that were the double of the values measured in plasma.34 It has also been
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shown in rainbow trout that there is a renal regulation of glucose levels in the plasma with the observation that high concentrations of glucose in plasma lead to glycosuria and to elimination in the urine.35 Moreover, it is known that small fish are considered to have lower glucose tolerance than large fish.36

Induction of diabetes in zebrafish caused an impairment in operculum mineralization and bone growth in 15 day larvae, similar to growth retardation observed in diabetes type 1 patients37 and diabetes mice models.38 Treatment with paricalcitol was more efficient than with VitD in promoting an increase in the mineralized area. The concentrations of Cinacalcet in our zebrafish treatments were the same as used in clinical therapies, but were found to be toxic in larvae with a rate of mortality of 100% after 24 hours. However, lower concentrations showed reduced lethality and induced an increase in mineralized area. Regenerated fin areas of adults showed an increase in the diabetic groups treated with paricalcitol and cinacalcet when compared with diabetic group treated with vehicle, while diabetic fish treated with VitD did not present such a marked increase. There are some evidences that vitamin D analogs can have positive regenerative effects after vascular injury, as previously reported for healthy humans, diabetic mice models and conditional knockout of the vitamin D receptor mice.39

In our work, paricalcitol had no effect in vdra expression, but we could detect an up-regulation in the group treated with cinacalcet, in agreement with previous in vitro studies with rat parathyroid glands, demonstrating that class II calcimimetics induce a stimulatory affect in Vдра expression.40 In addition, gene expression results for both insa and insb, which were found to be overexpressed in bone in diabetics treated with paricalcitol or cinacalcet, could help explain this increase in caudal fin regeneration. Vitamin D analogs and calcimimetics have been shown to induce insulin expression and β-cell proliferation and survival,41-43 and our data suggest that this up-regulation of insulin also occurs in bone cells. In the Tg(insa:nsfb-mCherry) zebrafish, it has been described that total pancreas regeneration occurs in 15 days after diabetes induction, but we do not know if this process occurred in a shorter period of time in the paricalcitol and cinacalcet treated groups, which could favor insulin signaling and glucose metabolism. In the cinacalcet treated group we could observe a significant increase in insr suggesting increased insulin signaling. In fact, VitD may have an important role in the treatment of diabetes as identified by Del Pino-Montes et al.,44 who showed that 55% of diabetic rats treated with calcitriol recovered from diabetes.

Insulin expression has also been found to be increased in several tissues under diabetic conditions in both humans and mice,45 but in our study we could not observe such an increase in the nontreated diabetic group, at least in the vertebral column. It has been demonstrated that insulin can be almost ubiquitously expressed in human,46 mouse45 and zebrafish47 although at extremely low levels when compared with pancreatic insulin. Although not well understood, the function of extrapancreatic expression of insulin was associated in some studies to local needs of glucose regulation, specially under diabetic conditions,46,48 but in other reports this phenomenon was related to the development of pathologic conditions.29 Our results demonstrated that paricalcitol and cinacalcet can up-regulate extrapancreatic expression of insulin, including in bony tissues, like demonstrated in the vertebral column of adult zebrafish.

In humans and animal models of diabetes, hyperglycemia leads to accelerated accumulation of advanced glycation end products (AGEs),49 promoting an inflammatory response and increased apoptosis of cells expressing the receptor of AGEs such as osteoblasts.50 In the nontreated diabetic group we could see impairment in osteoblastic activity, since sp7, runx2b and bglap expression were found to be down-regulated compared with nondiabetic fish. In the diabetic groups treated with paricalcitol or cinacalcet, where an increase in mineralized area of the regenerated fin was observed, an up-regulation of runx2b suggests an increase in the process of osteoblastic differentiation, contributing to the process of mineralization. This is in accordance with studies in humans, indicating that VitD effects on osteoblast differentiation are mostly stimulatory and associated with increased RUNX2 expression.51 In addition, in vitro studies with mesenchymal stem cells from human amniotic fluid have correlated calcimimetics with osteogenic differentiation and up-regulation of bone markers including RUNX2.52 The
principal objective of paricalcitol, cinacalcet and VitD in clinical treatment for SH is to reduce parathyroid hormone secretion. We could not observe reduced expression in pthra in all treated groups so no conclusions can be made relatively to the pth regulation of osteoclastic differentiation and bone resorption. In fact, pthra results seem to support the idea that pth pathway was not altered in the treated groups because the VitD treated group did not present such a marked increase in mineralized area as observed in the other two treated groups, while having the same results for pthra. The fact that VitD acts more slowly in exerting its effects, at least when compared with paricalcitol, can be one of the possible explanations for our results. Different pathways related to pth signaling, calcium metabolism or VitD induced osteoblastogenesis can be involved in the increase in bone mineralized and regenerated areas observed in the caudal fin of zebrafish under diabetic conditions treated with paricalcitol and cinacalcet. Up-regulation of insulin and increased osteoblastic differentiation induced by up-regulation of runx2b can help explain our results. Both paricalcitol and cinacalcet were shown to have positive effects in promoting mineral deposition, counteracting bone loss related to diabetes, and may constitute an alternative therapy for prevention of bone related disorders observed in type I diabetes patients.

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REFERENCES
1. King H, Aubert RE, Herman WH. Global burden of diabetes, 1995–2025: prevalence, numerical estimates, and projections. Diabetes Care 1998; 21: 1414–31.
2. Hamann C, Kirschner S, Günther K-P, Hofbauer LC. Bone, sweet bone–osteoporotic fractures in diabetes mellitus. Nat Rev Endocrinol 2012; 8: 297–305.
3. Gehling DJ, Lecka-Czernik B, Ebraheim NA. Orthopaedic complications in diabetes. Bone 2015; 82: 79–92.
4. Andress DL. Adynamic bone in patients with chronic kidney disease. Kidney Int 2008; 73: 1345–54.
5. Elder GJ, Mackun K. 25-Hydroxyvitamin D deficiency and diabetes predict reduced BMD in patients with chronic kidney disease. J Bone Miner Res 2006; 21: 1778–84.
6. Zhao W, Byrne MH, Boyce BF, Krane SM. Bone resorption induced by parathyroid hormone is strikingly diminished in collagenase-resistant mutant mice. J Clin Invest 1999; 103: 517–24.
7. Tsuruta Y, Okano K, Kikuchi K, Tsuruta Y, Akiba T, Nitta K. Effects of cinacalcet on bone mineral density and bone markers in hemodialysis patients with secondary hyperparathyroidism. Clin Exp Nephrol 2013; 17: 120–6.
8. Bergua C, Torregrosa J-V, Fuster D, Gutierrez-Dalmau A, Oppenheimer F, Campistol JM. Effect of cinacalcet on hypercalcemia and bone mineral density in renal transplanted patients with secondary hyperparathyroidism. Transplantation 2008; 86: 413–7.
9. De Schutter TM, Behets GJ, Jung S, Neven E, D’Haese PC, Querfeld U. Restoration of bone mineralization by cinacalcet is associated with a significant reduction in calcitriol-induced vascular calcification in uremic rats. Calcif Tissue Int 2012; 91: 307–15.
10. Coyne D, Acharya M, Qui P, Abboud H, Batlle D, Rosansky S, et al. Paricalcitol capsule for the treatment of secondary hyperparathyroidism in stages 3 and 4 CKD. Am J Kidney Dis 2006; 47: 263–76.
11. Goodman WG. Calcimimetic agents and secondary hyperparathyroidism: treatment and prevention. Nephrol Dial Transplant 2002; 17: 204–7.
12. Coyne DW, Andress DL, Amdahl MJ, Ritz E, de Zeeuw D. Effects of paricalcitol on calcium and phosphate metabolism and markers of bone health in patients with diabetic nephropathy: results of the VITAL study. Nephrol Dial Transplant 2013; 28: 2260–8.
13. Coyne DW, Goldberg S, Faber M, Ghossein C, Sprague SM. A randomized multicenter trial of paricalcitol versus calcitriol for secondary hyperparathyroidism in stages 3–4 CKD. Clin J Am Soc Nephrol 2014; 9: 1620–6.
14. Shoben AB, Rudser KD, de Boer IH, Young B, Kestenbaum B. Association of oral calcitriol with improved survival in nondialyzed CKD. J Am Soc Nephrol 2008; 19: 1615–9.
15. Fishbane S, Shapiro WB, Corry DB, Vicks SL, Roppolo M, Rappaport K, et al. Cinacalcet HCl and concurrent low-dose vitamin D improves treatment of secondary hyperparathyroidism in dialysis patients compared with vitamin D alone: the ACHIEVE study results. Clin J Am Soc Nephrol 2008; 3: 1718–25.
16. Lee Y-T, Ng H-Y, Kuo C-C, Chen T-C, Wu C-S, Chiu TT-Y, et al. Comparison between calcitriol and calcitriol plus low-dose cinacalcet for the treatment of moderate to severe secondary hyperparathyroidism in chronic dialysis patients. Nutrients 2013; 5: 1336–48.
17. Alvarez JA, Ashraf A. Role of vitamin D in insulin secretion and insulin sensitivity for glucose homeostasis. Int J Endocrinol 2010; 2010: 1–18.
18. Jones PM, Kitsou-Mylona I, Gray E, Squires PE, Persaud SJ. Expression and function of the extracellular calcium-sensing receptor in pancreatic beta-cells. Arch Physiol Biochem 2007; 113: 98–103.
19. Krul-Poel YHM, van Wijland H, Stam F, ten Boekel E, Lips P, Simsek S. Study protocol: a randomised placebo-controlled clinical trial to study the effect of vitamin D supplementation on glycemic control in type 2 Diabetes Mellitus SUNNY trial. BMC Endocr Disord 2014; 14: 59.
20. Gagnon C, Daly RM, Carpentier A, Lu ZX, Shore-Lorenti C, Sikaris K, et al. Effects of combined calcium and vitamin D supplementation on insulin secretion, insulin sensitivity and β-cell function in multi-ethnic vitamin D-deficient adults at risk for type 2 diabetes: a pilot randomized, placebo-controlled trial. *PLoS One* 2014; 9: e109607

21. Devaskar SU, Giddings SJ, Rajakumar PA, Carnaghi LR, Menon RK, Zahm DS. Insulin gene expression and insulin synthesis in mammalian neuronal cells. *J Biol Chem* 1994; 269: 8445–54.

22. Pugliese A, Zeller M, Fernandez A, Zalcberg LJ, Bartlett RJ, Ricordi C, et al. The insulin gene is transcribed in the human thymus and transcription levels correlated with allelic variation at the INS VNTR-IDDDM2 susceptibility locus for type 1 diabetes. *Nat Genet* 1997; 15: 293–7.

23. Kojima H, Fujimiyama M, Matsumura K, Younan P, Imaeda H, Maeda M, et al. NeuroD-betacellulin gene therapy induces islet neogenesis in the liver and reverses diabetes in mice. *Nat Med* 2003; 9: 596–603.

24. Kojima H, Fujimiyama M, Matsumura K, Nakahara T, Hara M, Chan L. Extrapancreatic insulin-producing cells in multiple organs in diabetes. *Proc Natl Acad Sci* 2004; 101: 2458–63.

25. Kojima H, Fujimiyama M, Terashima T, Kimura H, Chan L. Extrapancreatic prosinulin/insulin-expressing cells in diabetes mellitus: is history repeating itself? *Endoc J* 2006; 53: 715–22.

26. Chan L, Terashima T, Urabe H, Lin F, Kojima H. Pathogenesis of diabetic neuropathy: had to the bone. *Ann N Y Acad Sci* 2011; 1240: 70–6.

27. Cunha DA, de Alves MC, Stoppiglia LF, Jorge AG, Módulo CM, Carneiro EM, et al. Extra-pancreatic insulin production in RA lachrymal gland after streptozotocin-induced islet beta-cells destruction. *Biochim Biophys Acta* 2007; 1770: 1128–35.

28. Lieschke GJ, Currie PD. Animal models of human disease: zebrafish swim into view. *Nat Rev Genet* 2007; 8: 353–67.

29. Olsen ASA, Sarras MP, Intine RV. Limb regeneration is impaired in an adult zebrafish model of diabetes mellitus. *Wound Repair Regen* 2010; 18: 532–42.

30. Pisharath H, Rhee JM, Swanson MA, Leach SD, Parsons MJ. Targeted ablation of beta cells in the embryonic zebrafish pancreas using *E. coli* nitroreductase. *Mech Dev* 2007; 124: 218–29.

31. Moss JB, Koustubhan P, Greenman M, Parsons MJ, Moss LG. Regeneration of the pancreas in adult zebrafish. *Diabetes* 2009; 58: 1844–51.

32. Westerfield M. The ZEBRAFISH BOOK: a guide for the laboratory use of zebrafish (*Danio rerio*), 5th ed. Eugene: University of Oregon Press, 2007.

33. Gavaia PJ, Simes DC, Ortiz-Delgado JB, Viegas CSB, Pinto JP, Kelsh RN, et al. Osteocalcin and matrix Gla protein in zebrafish (*Danio rerio*) and Senegal sole (*Solea senegalensis*): comparative gene and protein expression during larval development through adulthood. *Gene Expr Patterns* 2006; 6: 637–52.

34. Lin SC, Liou CH, Shiu SY. Renal threshold for urinary glucose excretion by tilapia in response to orally administered carbohydrates and injected glucose. *Fish Physiol Biochem* 2000; 23: 127–32.

35. Bucking C, Wood CM. Renal regulation of plasma glucose in the freshwater rainbow trout. *J Exp Biol* 2005; 208: 2731–9.

36. Tung PH, Shiau SY. Carbohydrate utilization versus body size in tilapia, *Oreochromis niloticus* x *O. aureus*. *Comp Biochem Physiol* 1993; 104A: 585–8.

37. Pham-Short A, Donaghue KC, Ambler G, Chan AK, Craig ME. Coeliac disease in Type 1 diabetes from 1990 to 2009: higher incidence in young children after longer diabetes duration. *Diabet Med* 2012; 29: e286–9.

38. Flanagan SE, De Franco E, Lango Allen H, Zerah M, Abdul-Rasoul MM, Edge JA, et al. Analysis of transcription factors key for mouse pancreatic development establishes NXX2-2 and MNX1 mutations as causes of neonatal diabetes in man. *Cell Metab* 2014; 19: 146–54.

39. Wong MSK, Leisegang MS, Kruse C, Vogel J, Schürmann C, Dehne N, et al. Vitamin D promotes vascular regeneration. *Circulation* 2014; 130: 976–86.

40. Rodriguez ME, Almaden Y, Canadiñas S, Canalejo A, Siendones E, Lopez I, et al. The calcimimetic R-568 increases vitamin D receptor expression in rat parathyroid glands. *Am J Physiol Renal Physiol* 2007; 292: F1390–5.

41. Hills CE, Younis MYG, Bennett J, Siamantouras E, Liu K-K, Squires PE. Calcium-sensing receptor activation increases cell–cell adhesion and β-cell function. *Cell Physiol Biochem* 2012; 30: 575–86.

42. Jayanarayanan S, Anju TR, Smijin S, Paulose CS. Vitamin D3 supplementation increases insulin level by regulating altered IP3 and AMPA receptor expression in the pancreatic islets of streptozotocin-induced diabetic rat. *J Nutr Biochem* 2015; 26: 1041–9.

43. Craig TA, Sommer S, Sussman CR, Grande JP, Kumar R. Expression and regulation of the vitamin D receptor in the zebrafish, *Danio rerio*. *J Bone Miner Res* 2008; 23: 1486–96.

44. Del Pino-Montes J, Benito GE, Fernández-Salazar MP, Covéas R, Calvo JJ, Bouillon R, et al. Calcitriol improves streptozotocin-induced diabetes and recovers bone mineral density in diabetic rats. *Calcif Tissue Int* 2004; 75: 526–32.

45. Chen X, Larson CS, West J, Zhang X, Kaufman DB. In vivo detection of extrapancreatic insulin gene expression in diabetic mice by bioluminescence imaging. *PLoS One* 2010; 5: e9397.

46. Lehner C, Gehwolf R, Wagner A, Resch H, Hirzinger C, Cunha DA, de Alves MC, Stoppiglia LF, Jorge AG, Módulo CM, Carneiro EM, et al. Extra-pancreatic insulin production in RA lachrymal gland after streptozotocin-induced islet beta-cells destruction. *Biochim Biophys Acta* 2007; 1770: 1128–35.

47. Lieschke GJ, Currie PD. Animal models of human disease: zebrafish swim into view. *Nat Rev Genet* 2007; 8: 353–67.

48. Olsen ASA, Sarras MP, Intine RV. Limb regeneration is impaired in an adult zebrafish model of diabetes mellitus. *Wound Repair Regen* 2010; 18: 532–42.

49. Pisharath H, Rhee JM, Swanson MA, Leach SD, Parsons MJ. Targeted ablation of beta cells in the embryonic zebrafish pancreas using *E. coli* nitroreductase. *Mech Dev* 2007; 124: 218–29.

50. Moss JB, Koustubhan P, Greenman M, Parsons MJ, Moss LG. Regeneration of the pancreas in adult zebrafish. *Diabetes* 2009; 58: 1844–51.

51. Westerfield M. The ZEBRAFISH BOOK: a guide for the laboratory use of zebrafish (*Danio rerio*), 5th ed. Eugene: University of Oregon Press, 2007.

52. Gavaia PJ, Simes DC, Ortiz-Delgado JB, Viegas CSB, Pinto JP, Kelsh RN, et al. Osteocalcin and matrix Gla protein in zebrafish (*Danio rerio*) and Senegal sole (*Solea senegalensis*): comparative gene and protein expression during larval development through adulthood. *Gene Expr Patterns* 2006; 6: 637–52.

53. Lin SC, Liou CH, Shiu SY. Renal threshold for urinary glucose excretion by tilapia in response to orally administered carbohydrates and injected glucose. *Fish Physiol Biochem* 2000; 23: 127–32.

54. Bucking C, Wood CM. Renal regulation of plasma glucose in the freshwater rainbow trout. *J Exp Biol* 2005; 208: 2731–9.
human osteoblastic cells: roles of osterix, an osteoblast-related transcription factor. *Matrix Biol* 2006; 25: 47–58.

52. Pipino C, Di Tomo P, Mandatori D, Cianci E, Lanuti P, Cutrona MB, et al. Calcium sensing receptor activation by calcimimetic R-568 in human amniotic fluid mesenchymal stem cells: correlation with osteogenic differentiation. *Stem Cells Dev* 2014; 23: 2959–71.

53. Keller H, Kneissel M. SOST is a target gene for PTH in bone. *Bone* 2005; 37: 148–58.

54. Sprague SM, Llach F, Amdahl M, Taccetta C, Batlle D. Paricalcitol versus calcitriol in the treatment of secondary hyperparathyroidism. *Kidney Int* 2003; 63: 1483–90.

**Supporting Information**

Additional supporting information may be found in the online version of this article at the publisher’s web-site:

**Figure S1.** Detailed timeline of diabetes induction and treatment with experimental drugs during caudal fin regeneration.

**Figure S2.** Confirmation of beta-cell ablation. (A) Metronidazole exposed Tg(ins:nslb-mCherry) zebrafish lose mCherry fluorescence after 72 hours of treatment. (B) Vehicle exposed Tg(ins:nslb-mCherry) zebrafish maintain mCherry fluorescence after treatment.