Autonomous sensing the insulin peptide by an olfactory G protein coupled receptor modulates glucose metabolism

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Abstract

Along with functionally intact insulin, diabetes-associated insulin peptides are secreted by β cells(1-3). By screening the expression and functional characterization of olfactory receptors in pancreatic islets, we identified Olfr109 as the receptor to detect insulin peptides. Engagement of one insulin peptide, insB:9-23, with Olfr109 diminished insulin secretion through Gi-cAMP signalling and promoted macrophage proliferation. Remarkably, Olfr109 deficiency alleviated intra-islet inflammatory responses and improved glucose homeostasis in Akita- and HFD-fed mice. We further determined the binding mode between the insB:9-23 and Olfr109. A pepducin-based Olfr109 antagonist improved glucose homeostasis in diabetic and obese mouse models. Collectively, we found that pancreatic β cells use Olfr109 to autonomously detect self-secreted insulin peptides and this detection arrests insulin secretion and crosstalk with macrophages to increase intra-islet inflammation.

38 Introduction

39 Animals detect and interpret odourant information through a family of G protein-coupled
40 receptors (GPCRs) called olfactory receptors (ORs); ORs constitute one of the largest
41 mammalian gene families, with approximately 370 genes in humans and 800 in mice(4-6). In
42 addition to playing a dominant role in odour detection, several ORs have been identified in
43 organs other than the nasal olfactory epithelium, where they carry out distinct physiological
44 functions, such as mediating sperm chemotaxis, muscle generation, cancer cell growth
45 inhibition, hair growth, hepatic steatosis and adipose tissue lipolysis(6-9). However, the gene
46 expression profiles and functions of ORs in pancreatic islets, where the glucose-regulating
47 hormones insulin and glucagon are secreted, remain largely unknown.

48 Pancreatic islets are the only cellular factories producing insulin, which accounts for
49 approximately 10% of the total protein content in pancreatic β cells. Recent studies showed
50 that not only intact insulin molecules but also insulin peptide fragments are stored in
51 pancreatic β cell vesicles and released into the circulation in response to glucose
52 stimulation(2). Several of these insulin peptide fragments, as well as denatured insulin, act as
53 antigens to trigger islet autoimmunity, which is functionally associated with the incidence of
54 both type 1 diabetes (T1D) and type 2 diabetes(10, 11). Notably, these insulin peptide
55 fragments bind poorly to MHC molecules, with micromolar affinity, in contrast to the
56 relatively low (nanomolar) circulating concentration of insulin peptide in plasma(12, 13).
57 Whether pancreatic β cells have developed a mechanism for detecting these insulin fragments
58 and initiating signalling cascades to modulate the metabolic state remains elusive. Linking
59 these islet secretions with their potential high-affinity receptors (e.g., GPCRs) and examining
60 their corresponding functions will be highly valuable for understanding mechanism regulating
61 islet homeostasis and for developing novel anti-diabetic strategies.

62 In the present study, we identified six ORs with high expression levels in islets. One of
63 these ORs, Olfr109, was expressed in pancreatic islets, as supported by the RNAscope *in situ*
64 hybridization, mRNA levels of GFP-labeled selective pancreatic cell types, as well as specific
65 radio-ligand binding. Olfr109 was the receptor for the endogenous pancreatic peptide
66 insB:9-23 or denatured insulin, modulating insulin secretion from pancreatic islets through
67 Gi-cAMP signalling and manipulating CCL-2 levels to communicate with islet-resident
68 macrophages, which resulted in increased macrophage proliferation. Consistent with these
69 observations, Olfr109 knockout mice fed a high-fat diet (HFD) or on the Akita diabetic mouse
70 background showed improved glucose metabolism, increased insulin secretion and
71 ameliorated inflammatory responses within islets. We characterized the ligand-receptor
72 interaction mode of Olfr109 and further developed a pepducin-based antagonist of Olfr109,
73 which exhibited significant therapeutic potential for diabetes and obesity in mouse models.

74

75 Results

76 Expression of ORs in pancreatic islets

77 ORs are encoded by more than 300 genes in humans and approximately 800 genes in
78 mice. To comprehensively assess human-related ORs with high expression levels in mouse
79 islets, we designed PCR primers specific for all 278 mouse ORs that share more than 80%
80 sequence identity with their closest human homologue and screened the expression levels of
81 the corresponding mRNAs (Fig. S1A and Auxiliary Supplementary Table 1). With a cut-off
82 expression level of more than 1% of the corresponding expression level in nasal tissue, only
83 six ORs were detected in isolated mouse pancreatic islets: Olfr31, Olfr109, Olfr110, Olfr1335,
84 Olfr1420 and Olfr1502 (Fig. 1A-1B and Fig. S1B). We then examined the changes in the
85 expression levels of these receptors under physiological stimulation with high glucose and in
86 mouse models of diabetes or obesity, including streptozotocin (STZ)- or L-Arg-treated mice,
87 Akita mice, nonobese diabetic (NOD) mice and HFD model mice. In response to
88 high-glucose stimulation, the expression level of all six ORs increased significantly in both
89 human and mouse islets (Fig. 1C-1D and Fig. S2A). Intriguingly, in contrast to the differential
90 expression changes in Olfr31, Olfr110, Olfr1335 and Olfr1502 in the different pathogenic
91 models, only Olfr109 exhibited increased expression in all models of diabetes or obesity (Fig.
92 1C-1E and Fig. S2A-S2D).

93 We next preliminarily investigated the functions of these six ORs by overexpressing
94 them in MIN6 pancreatic β cells. Whereas overexpression of all 6 ORs was achieved in MIN
95 6 cells, only Olfr109 and Olfr110 could be detected on the plasma membrane (Fig. S2E-S2F).
96 Specifically, overexpression of Olfr109 but not Olfr110 in MIN6 cells decreased insulin
97 secretion in response to stimulation with high glucose or a combination of high glucose and
98 GLP-1 (Fig. 1F). In addition, decreased expression of the pancreatic β cell proliferation
99 markers mki67, ccnb1, and cdk4 and increased expression of the stress response genes prdx5,
100 sxbp1 and grp78 were observed in cells overexpressing *Olfr109* but not in cells
101 overexpressing Olfr110 (Fig. S2G-S2J). Moreover, the highest expression of *Olfr109* was
102 found in pancreatic islets and spleen compared with all other examined tissues except nasal
103 tissue (Fig. S1B). The specific changes in expression levels in pancreatic islets and the
104 preliminary functional differences observed by overexpression of Olfr109 in pancreatic β cell
105 lines prompted us to explore the potential functions of Olfr109 in depth.

106

107 Improved glucose homeostasis in *Olfr109*-deficient mice in a model of diabetes

108 We used the RNAscope *in situ* hybridization to investigate the expression of *Olfr109* in
109 pancreatic islets and generated the *Olfr109*-deficient (*Olfr109*^{-/-}) mice by the CRISPR-Cas9
110 system to study the *Olfr109* function (Fig 1G, Fig. S3A-S3C). *Olfr109* mRNA was mainly
111 distributed within insulin-expressing cells in the islets of WT mice, but not in those of

112 *Olf109*^{-/-}) mice. This data was consistent with the significant higher mRNA detected in the
113 *Ins2-Cre:GFP*^{f/f} labeled pancreatic β cells than that of *gcg-Cre:GFP*^{f/f} labeled α cells or
114 *sst-cre*^{+/-} *GFP*^{f/f} labeled δ cells (Fig. S3D).

115 To explore the role of *Olf109* in metabolism, we investigated glucose and energy
116 homeostasis in *Olf109*^{-/-} mice. The body weight, energy expenditure and respiratory quotient
117 were similar between *Olf109*^{-/-} mice and wild-type mice (Fig. 1H-1J). We then crossed
118 *Olf109*^{-/-} mice with *Akita* mice, a neonatal model of diabetes mimicking maturity-onset
119 diabetes of the young (MODY) in humans (Fig. S3E)(14, 15). Compared with wild-type mice,
120 *Akita* mice have a significantly decreased weight, exhibit increased energy expenditure in the
121 dark photoperiod and present reduced respiratory quotient values at 2- to 3-months of age.
122 Importantly, knockout of *Olf109* almost completely reversed all of these effects. Moreover,
123 *Olf109* deficiency markedly improved glucose tolerance and glucose-induced plasma insulin
124 levels in *ins2*^{WT/Akita} *Olf109*^{-/-} mice compared with *ins2*^{WT/Akita} mice at 8 weeks (Fig. 1K-1L).
125 Adult *Akita* mice are used as a model of severe insulin-deficient diabetes, with a reduction in
126 insulin content of approximately 80%-90%. Deficiency of *Olf109* significantly compensated
127 for the loss of insulin content in the *Akita* background (Fig. 1M). Consistent with this
128 observation, islets derived from *Ins2*^{WT/Akita} *Olf109*^{-/-} mice showed stronger immunostaining
129 for PDX1 and NKX6.1, two important identity markers of pancreatic β cells than islets
130 derived from *ins2*^{WT/Akita} mice (Fig. S3F-S3G, Auxiliary Supplementary Fig. 1). Notably,
131 recent studies showed that myeloid lineage-derived CD11c⁺ macrophages are specifically
132 expanded inside the islets of HFD-fed mice, which correlated with increased islet
133 inflammation and contributed to impairment of pancreatic β cell functions. Similar to the
134 finding in HFD-fed mice, the population of CD11c⁺ macrophages in the islets of *Akita* mice
135 was markedly increased by approximately 3-fold compared with that in wild-type mice.
136 Knockout of *Olf109* significantly reduced this effect (Fig. 1N). These results indicated that
137 *Olf109* negatively regulated glucose homeostasis in this diabetic model and that deletion of
138 *Olf109* significantly enhanced the relief of diabetic symptoms.

139

140 **Improved glucose homeostasis in *Olf109*-deficient mice in a model of obesity**

141 We then examined the role of *Olf109* in obesity in an HFD-fed model. HFD feeding
142 resulted in greater weight gain in *Olf109*^{-/-} mice than in wild-type mice after 13 weeks of age
143 (Fig. 2A). Consistent with this result, HFD-induced *Olf109*^{-/-} mice showed increased energy
144 expenditure compared with their HFD-induced wild-type littermates at 12 weeks old. The
145 increased energy expenditure of HFD-fed *Olf109*^{-/-} mice may explain their lower weight gain
146 (Fig. 2B). Moreover, HFD-induced *Olf109*^{-/-} mice exhibited increased respiratory quotient
147 values (Fig. 2C), suggesting that glucose utilization was enhanced in mutant mice compared

with wild-type mice under HFD stress, potentially due to the decrease in insulin sensitivity under these conditions (Fig. 2C). Notably, no significant differences in locomotor activity or food intake were observed between *Olfr109*^{-/-} mice and wild-type mice (Fig. S4A-S4C).

At 12 weeks, the white adipose tissue and liver weights were significantly lower in HFD-fed *Olfr109*^{-/-} mice (Fig. S4D-S4E). A significant decrease in the epididymal adipocyte size in the HFD-fed *Olfr109*^{-/-} mice was observed by H&E staining (Fig. S4F). In addition, both the number of F4/80-positive cells in epididymal tissue and the liver steatosis were reduced in *Olfr109*^{-/-} mice compared to their wild-type littermates under HFD stress (Fig. S4G-4H). These decreases were consistent with the decreased insulin resistance of *Olfr109*^{-/-} mice at 13 weeks under HFD stress (Fig. 2D). However, whereas HFD-fed *Olfr109*^{-/-} mice exhibited improved glucose metabolism in the glucose tolerance test (GTT) at a time point as early as 6 weeks (Fig. 2E and Fig. S4I), the difference in insulin resistance between the mutant mice and the wild-type mice was not observed at this early time point (Fig. S4J). These observations indicated that the change in glucose tolerance occurs before the improvement in insulin resistance in HFD-fed *Olfr109*^{-/-} mice. Moreover, whereas the level of plasma GLP-1 did not differ significantly (Fig. S4K), fasting glucose, insulin and leptin levels were lower in HFD-fed *Olfr109*^{-/-} mice (Fig. S4L-S4N). Importantly, the plasma glucose level was decreased and the insulin level was significantly increased after refeeding, which may account for the improved glucose tolerance test results in the HFD-fed *Olfr109*^{-/-} mice (Fig. 2F-2G).

The earlier onset of changes in glucose tolerance test than in the insulin tolerance test (ITT) in HFD-induced *Olfr109*^{-/-} mice and the improvement in insulin secretion after refeeding indicated that *Olfr109* deficiency may enhance glucose homeostasis in HFD-treated mice through regulation of pancreatic islet functions. Consistent with this hypothesis, high glucose-induced insulin secretion was markedly increased under both normal culture and HFD-induced conditions in pancreatic islets isolated from *Olfr109*^{-/-} mice compared to those isolated from their wild-type littermates (Fig. 2H and Fig. S4O). Whereas the size distributions of α and δ cells in islets did not differ significantly between *Olfr109*^{-/-} mice and their wild-type littermates under HFD stress (Fig. S5A-S5B), *Olfr109*^{-/-} mice had more islets with a size between 50-100 μ m than wild-type mice (Fig. S5C-S5D). Moreover, the expansion of intra-islet CD11c⁺ macrophages and level of co-immunostaining of glucagon with insulin were significantly reduced in islets derived from *Olfr109*^{-/-} mice compared with islets derived from their HFD-fed wild-type littermates (Fig. 2I-2J and Auxiliary Supplementary Fig. 2A). These results indicated that *Olfr109* promoted dedifferentiation of pancreatic β cells and obesity-associated islet metaflammation (16).

Identification of the insB:9-23 peptide and denatured insulin as *Olfr109* agonists

185 To gain a preliminary understanding of Olfr109-mediated signalling, we performed
186 quantitative comparative transcriptome analysis in MIN6 cells with or without high-glucose
187 stimulation by knocking down *Olfr109* expression in these cells and comparing them with
188 control cells. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis
189 indicated that genes enriched in metabolic pathways, calcium signalling pathways and
190 pathways regulating stem cell pluripotency showed significant changes depending on *Olfr109*
191 expression (Fig. S6A-6C). In particular, components of the Gs/Gi-cAMP signalling network
192 were also altered (Fig.3A-3B). Selective G protein subtypes are the main downstream
193 transducers mediating OR functions(17-19). Consistent with this role, overexpression of
194 Olfr109 caused notable inhibition of cAMP that was blocked by pertussis toxin (PTX), a
195 Gi/Go inhibitor, indicating constitutive Gi coupling to Olfr109 (Fig. S6D).

196 Suspecting that an endogenous ligand is already present in islet of Langerhans cells, we
197 next stimulated *Olfr109*-overexpressing HEK293 cells with pancreatic islet supernatant and
198 examined its effects on Gs and Gi signaling. Importantly, *Olfr109*-overexpressing cells
199 incubated with islet supernatant collected under high-glucose conditions but not with islet
200 supernatant collected under low-glucose conditions exhibited significant inhibition of
201 forskolin (FSK)-induced cAMP elevation in a concentration-dependent manner (Fig. 3C). In
202 addition, incubation with supernatant collected under high-glucose conditions promoted
203 recruitment of β -arrestin-1, another important regulator of GPCR signal transduction(20-22) ,
204 to YFP-tagged *Olfr109* (Fig. 3D and Fig. S6E). Moreover, supernatant from pancreatic islets
205 derived from diabetic model Akita mice induced significantly higher Olfr109 activity than
206 that derived from wild-type mice in both the cAMP assay and arrestin recruitment assay (Fig.
207 3C-3D). Collectively, these results indicated that an endogenous agonist of *Olfr109* eliciting
208 the activity of both Gi and arrestin is present in pancreatic islet supernatants and that this
209 specific agonist is present at a higher level in the Akita diabetic mouse model.

210 High glucose levels mobilize insulin-containing vesicles to the membrane and finally
211 release them into the intercellular space in pancreatic islets. We therefore fractioned the
212 secretory granules by differential centrifugation. Compared to the fraction obtained by
213 centrifugation at 25000 $\times$ g (the 25k fraction), the fraction obtained by centrifugation at
214 5000 $\times$ g (the 5k fraction) showed an approximately 100-fold increase in potency and a 2-fold
215 increase in Emax, suggesting enrichment of the *Olfr109* agonist in the 5k fraction (Fig.
216 3E-3F). Separation of the soluble 5k fraction with a 3 kD molecular weight cut-off filter
217 indicated that the Olfr109 agonist has a relatively low molecular weight and could be a
218 peptide-like substance (Fig. 3G-3H). We therefore performed mass spectrometry analysis to
219 characterize the side-by-side peptidomes of the 5k fraction from pancreatic islets derived
220 from both Akita and wild-type mice. A total of 40 peptides derived from 12 proteins were
221 found to be enriched in the fraction from Akita mice by more than 2-fold compared with their

222 levels in the fraction from wild-type mice (Table S1, Auxiliary Supplementary Table 2).

223 We then synthesized the top 15 peptides and assessed their ability to activate *Olf109*.
 224 Only the InsB-derived peptides, not the other identified peptides, showed significant cAMP
 225 inhibition and arrestin recruitment activity via *Olf109* (Fig. 3I-3J, Fig. S6I-S6J, and Table
 226 S1). The most efficient InsB peptide, insB9-23, had a measured EC50 of 2.08 ± 0.50 nM (Fig.
 227 3I-3K and Table S2-S3). The ability of insB 9-23 to promote Gi coupling to *Olf109* was
 228 further verified by a G protein dissociation assay (Fig. S6F). Activation of *Olf109* by insB
 229 9-23 was determined to be specific because insB 9-23 showed no cross-activity with *Olf110*
 230 or other receptors with known peptide agonists (Fig. S6G-S6H). Moreover, saturation binding
 231 analysis of 125 I-insB 9-23 confirmed the specific interaction of the ligand with the membrane
 232 fractions of pancreatic islets of wild-type mice, in MIN6 pancreatic β cells and in HEK293
 233 cells overexpressing *Olf109* (B_{max} , $11.01 \pm 0.57 \times 10^3$ cpm and K_d , 3.61 ± 0.03 nM; B_{max} ,
 234 2746.00 ± 92.73 cpm and K_d , 3.24 ± 0.28 nM; and B_{max} , $22.52 \pm 0.42 \times 10^3$ cpm and K_d ,
 235 4.11 ± 0.07 nM, respectively) but not in islets derived from *Olf109*^{-/-} mice or in control
 236 HEK293 cells (Fig. 3L, Fig. S6M-O). Further whole-cell competitive binding assays of
 237 HEK293 cells overexpressing *Olf109* revealed two binding affinities with a K_{Hi} of 2.73 ± 0.07
 238 nM and a K_{Lo} of 109.95 ± 0.15 nM (Fig. 3M). The observed K_{Hi} was similar to the EC50 of
 239 insB 9-23-induced Gi activity via *Olf109*. The specific binding of the OLFR109 by the
 240 125 I-insB 9-23 enabled us to determine the protein levels of OLFR109 in different tissues,
 241 which was correlated well with the mRNA levels of *Olf109* ($R^2=0.90$), further confirming the
 242 specific endogenous expression of OLFR109 in pancreatic islets (Fig. 3N, Fig. S6M).

243 Previous studies have revealed that insB:9-23, which overlaps with the exposed region
 244 of denatured insulin, is a dominant epitope recognized by autoreactive T cells in both NOD
 245 mice and patients with type 1 diabetes(2, 23-25). We therefore measured the response of
 246 *Olf109* to denatured insulin. The EC50 of denatured insulin was 32.4 ± 1.7 nM in the cAMP
 247 assay, and denatured insulin had a higher arrestin bias than insB 9-23 (Fig. S6K-S6L and
 248 Table S2). Considering that the insB 9-23 concentration ranges from 10 pM to 2 nM under
 249 physiological or pathological conditions (2) and that denatured insulin is more enriched in
 250 Akita mice than in wild-type mice and activates *Olf109* more potently, our collective results
 251 demonstrated that both insB 9-23 and denatured insulin are potential endogenous agonists of
 252 *Olf109* in different physiological and pathological processes.

253

254 **Modulation of pancreatic islet function and homeostasis via engagement of insB:9-23** 255 **with Olf109**

256 insB:9-23 is a dominant epitope recognized by T cells in diabetic patients and mouse models.

Free insB 9-23 was found not only to be presented to immune cells but also to circulate to peripheral organs through the circulatory system(2). However, the role of insB 9-23 in signalling has not been characterized. We next investigated the mechanism by which engagement of insB:9-23 with Olfr109 regulates pancreatic islet functions and glucose homeostasis. In primary pancreatic islets, administration of glucose and GLP-1 markedly increased insulin secretion; however, this effect was significantly dampened by incubation with insB:9-23 (Fig. 4A). Whereas overexpression of Olfr109 in primary pancreatic islets greatly reduced the insulin secretion elicited by combination treatment with GLP-1 and high glucose concentrations (Fig. S7A-S7C, Auxiliary Supplementary Fig. 2B), insulin secretion stimulated by high glucose concentrations either alone or in combination with GLP-1 was greatly increased in *Olfr109*^{-/-} islets (Fig. 4A). Importantly, the inhibitory effect of insB:9-23 on insulin secretion was completely abolished in *Olfr109*^{-/-} islets (Fig. 4A). GLP-1 promotes insulin secretion by mobilizing intracellular cAMP in pancreatic β cells(26). Consistent with this observation, we found that insB:9-23 strongly reduced the intracellular cAMP level in islets in response to GLP-1 stimulation and that this reduction was abolished in *Olfr109*^{-/-} islets (Fig. 4B and Fig. S7D).

Moreover, whereas injection of insB:9-23 every other day for seven consecutive days resulted in glucose intolerance, *Olfr109* deficiency reversed this effect (Fig. 4C). We therefore performed transcriptome analysis on islets from both *Olfr109*^{-/-} mice and wild-type mice after insB:9-23 treatment. Importantly, the genes involved in chemokine signalling pathways, including *Ccl2* and *Ccl25*, were highly upregulated in islets treated with insB:9-23 compared with islets from vehicle-treated mice (Fig. 4D). Both chemokines are reported to be involved in macrophage proliferation and infiltration(27, 28). In particular, *Ccl2* has been linked to macrophage proliferation and infiltration in pancreatic islets and plays important roles in islet inflammation and pancreatic β cell dysfunction(29, 30). Notably, the insB:9-23-stimulated increases in chemokine levels were significantly reduced in *Olfr109*^{-/-} mice (Fig. 4E). Consistent with the changes in these chemokine levels, insB:9-23 treatment increased the accumulation of macrophages in pancreatic islets by approximately 3-4-fold, and this increase was significantly reduced in *Olfr109*^{-/-} mice (Fig. 4F and Fig. S7G).

We noted that *Olfr109* was expressed mainly in pancreatic β cells but not in islet-resident macrophages (Fig. 4G). Islets cultured in vitro with supernatants collected from islets of HFD-induced wild-type mice showed markedly increased macrophage proliferation compared with islets cultured with supernatants collected from the corresponding *Olfr109*^{-/-} mice (Fig. 4G-4H), indicating that *Olfr109*-mediated cellular events in islet β cells generate signals to induce macrophage proliferation, which contributed to the increased inflammation in HFD-induced model mice. Notably, not only was the *Ccl2* mRNA level increased in insB:9-23-treated pancreatic islets, the *Ccl2* protein level was augmented in islet supernatant after insB:9-23 treatment, and this increase was diminished by *Olfr109* deficiency (Fig. 4I). We therefore hypothesized that *Olfr109*-mediated insB:9-23 activity caused increased

production of Ccl2, which is the main driving force for the proliferation of pancreatic islet-resident macrophages through its interaction with the chemokine receptor CCR2. Consistent with this hypothesis, administration of INCB3344, a CCR2 antagonist, significantly blocked the promotive effect of insB:9-23 on macrophage proliferation, as indicated by the reduction of both numbers of CD11c-positive intra-islet macrophages and mRNA level of proliferative marker Ki67 (Fig. 4J-K and Fig. S7H). Therefore, engagement of insB:9-23 upregulated the Ccl2-CCR2 chemokine signalling pathway that mediates communication between pancreatic β cells and macrophages, impairing islet homeostasis.

303

304 **Molecular mechanism underlying the engagement of insB:9-23 with Olfr109**

305 The different activities of the various secreted insulin peptides towards Olfr109
306 suggested that recognition of the primary peptide sequence by Olfr109 is an important
307 determinant for ligand-Olfr109 engagement (Fig. 3I-3J and Table S2). To characterize the
308 engagement of insB:9-23 within Olfr109, we performed alanine (Ala) scanning mutagenesis
309 of Olfr109 to alter all residues in the extracellular loop (ECL) and the upper half of the
310 seven-transmembrane bundle (7TM), which have been suggested to form the ligand binding
311 pocket for most solved class A GPCR structures (Fig. 5A). 19 of the 163 examined mutant
312 receptors were not expressed on the surface, even after the amounts of transfected plasmids
313 were adjusted, and were excluded from further screening (Fig. S8). We first performed a
314 concentration-dependent arrestin recruitment assay for Olfr109 mutants as a convenient
315 approach to determine the functional EC₅₀ and E_{max} values. The mutants with severe effects
316 on arrestin function were then subjected to a ligand binding assay to determine their affinity
317 for Olfr109 (Fig. 5A). In total, mutations in 18 residues in the TM bundles and 5 residues in
318 the ECLs significantly impaired arrestin recruitment by Olfr109 in response to insB:9-23
319 engagement (Fig. 5B, Fig. S9A and Table S4). Among these residues, 10 residues in TM1,
320 TM2, TM4 and TM5 and 2 residues in extracellular loop 2 (ECL2) impaired the binding of
321 insB:9-23 with Olfr109 (Fig. 5C and Table S5). Therefore, the ligand binding pocket of
322 Olfr109 interacting with peptide agonists is composed primarily of TM1-TM2, TM4-TM5
323 and ECL2.

324 To further define the hot spot interactions governing the recognition of insB:9-23 by
325 Olfr109, we carried out alanine scanning mutagenesis to characterize the effects on the
326 peptide agonist insB:9-23 and then paired the interactions of the mutants with the key Olfr109
327 residues by comparing the binding of individual peptide mutants with that of the wild-type
328 peptide for all receptor mutations with significant loss of binding ability (Fig. 5A, Auxiliary
329 Supplementary Table 3). For a receptor mutation that severely impaired the response to
330 wild-type insB:9-23 but had much less severe effects on the response to an insB:9-23 peptide
331 with a selective alanine substitution, the receptor mutation and insB:9-23 alanine substitution

could be paired with each other to identify potential hot spot interactions(31). Importantly, the N-terminal residues S9, H10 and L11, as well as V18 in the middle region of insB:9-23, were identified by both arrestin recruitment and ligand binding assays as key residues mediating Olfr109 interactions (Fig. 5D, Fig. S9B and Table S6). The importance of the N-terminal residues was further confirmed by the serial truncation data (Fig. S9C-S9D). Further scanning of pairing between insB:9-23 peptide mutants and the receptor mutants indicated that the specific interactions of S9^{InsB} with P77^{2.59}, H10^{InsB} with F152^{4.55} and Y153^{4.56}, L11^{InsB} with M160^{4.63} and T161^{4.64}, and V18^{InsB} with F166^{ECL2} and F167^{ECL2} are the potential hot spot interactions between Olfr109 and insB:9-23 (Fig. 5E, Table S7) (the superscripts indicate the Ballesteros-Weinstein numbers(32)). Importantly, whereas separate mutation of H10^{InsB} /L11^{InsB} /V18^{InsB} to aspartic acid or mutation of their corresponding putative binding residues F152^{4.55}/T161^{4.64}/F166^{ECL2} to arginine dramatically impaired the binding between the peptide and receptor, complementary mutations of the interacting pairs to build a favored charge interaction effectively restored the binding (Fig. S9E-S9G). Collectively, this mapping of mutational effects coupled with the results of the arrestin recruitment, ligand binding and pairing assays indicated that insB:9-23 binds to Olfr109 via insertion of its N-terminus into the 7TM bundle, with extra interactions provided by engagement of the middle part of the peptide with the ECL2 region of the receptor. Olfr109 specifically recognized insB:9-23 through selective hot spot interactions mediated by the hydrophobic residues contributed by TM2-TM4, as well as ECL2.

More than 170 missense SNPs have been reported in Human database (Fig.5F). Notably, at least five single nucleotide polymorphism (SNP) occurred at the identified potential ligand-binding sites of OR12D3, which is the homologues receptor of OLFR109 in human. Except for Y71F^{2.53}, mutants of C167W^{ECL2}, Y71S^{2.53}, T31S^{1.41}, and T31N^{1.41} significantly impaired both arrestin recruitment and insB:9-23 binding to OR12D3 (Fig. 5G, Fig. S9H). These data not only supported the interacting mode between OLFR109/OR12D3 and insB:9-23, but also indicated potential clinical relevance of these naturally occurring OR12D3 mutations, which might be more resistant toward diabetes development.

360

361 **Pharmacological blockade of Olfr109 by pepducin improved glucose metabolism in** 362 **models of diabetes and obesity**

363 Pepducins are short lipid-coupled peptides derived from the intracellular loops of their
364 cognate receptors; they are effective allosteric regulators of GPCRs and are not only useful
365 for studying the function of a particular GPCR but also have therapeutic potential(33). We
366 designed a series of pepducins according to the Olfr109 sequence and evaluated their ability
367 to modulate Olfr109 activity (Fig. 6A). Three pepducins—o109-i3, o109-i2-5 and
368 o109-i2-6—exhibited dose-dependent inhibition of cAMP accumulation induced by forskolin

via Olfr109, with EC50 values of 811.5 ± 121.3 nM, 574.3 ± 74.5 nM and 236.8 ± 26.2 nM, respectively (Fig. 6B and Fig. S10A-S10D). In particular, o109-i2-2 acted as an Olfr109 antagonist specifically for Gi activity in response to insB:9-23 stimulation, with an EC50 of 210.4 ± 9.2 nM, but did not interact with other olfactory receptors or peptide hormone sensing GPCRs (Fig. 6C, Fig. S10E-10I). Moreover, the decreased insulin secretion and increased endoplasmic reticulum stress elicited by insB:9-23 treatment in pancreatic islets or MIN6 pancreatic β cells were significantly alleviated by o109-i2-2 administration, and these effects were dependent on Olfr109 expression (Fig. 6D-E).

Delighted by the effects of o109-i2-2 on improving islet functions, we then examined the effects of the pepducin Olfr109 antagonist in HFD-induced metabolic obesity and Akita diabetic mouse models. Treatment with o109-i2-2 significantly ameliorated the glucose metabolism disorder in both HFD-fed mice and Akita mice, and this effect was dependent on Olfr109 (Fig. 6F-6G, Fig. S10J-S10K). Therefore, we have developed a pepducin-based Olfr109-specific antagonist, and the effects of this pepducin suggested that pharmacological blockade of Olfr109 improved glucose homeostasis in mouse models of metabolic obesity and diabetes.

385

386 Conclusion

By screening all 278 mouse ORs with considerable homology to all ORs in *Homo sapiens*, we identified six ORs with relatively high expression levels in pancreatic islets that may actively participate in biochemical communications mediating islet functions. The expression of one of these six ORs, Olfr109, was found to be significantly increased in all models of obesity or diabetes. Specific expression of Olfr109 in pancreatic islets were supported by the RNAscope *in situ* hybridization, mRNA levels in GFP-labeled selective pancreatic cell types, radio-ligand binding and using *Olfr109*^{-/-} mice. Interestingly, knockout of Olfr109 in vivo improved glucose homeostasis in both diabetes and obesity models, and overexpression of Olfr109 arrested insulin secretion from pancreatic β cells, suggesting that Olfr109 functions in islets as a potentiator of pathological conditions.

We then identified Olfr109 as a direct receptor for denatured insulin and several insulin peptides derived from “retired insulin”. A pathologically important previous finding is that not only functionally intact insulin in dense core granules but also old insulin granules containing retired insulin are secreted into the bloodstream in response to increased plasma glucose levels(1-3). Considerable amounts of insulin peptides derived from retired insulin are highly associated with diabetes, and recent studies have shown that these insulin peptides can be presented by MHC complexes to trigger immune responses(25). However, whether pancreatic β cells can sense the production of these peptides and other forms of misfolded

insulin remains unknown. Here, we not only presented solid evidence of an interaction between insB:9-23 and Olfr109 using radioligand binding methods and Olfr109 knockout models but also offered a detailed description of the interaction mode between insB:9-23 and Olfr109, thus providing mechanistic insight into the recognition of a peptide ligand by an OR at the molecular level. This knowledge could broadly impact the understanding of ligand recognition by this large family of receptors. Moreover, in response to engagement of insB:9-23 or denatured insulin, Olfr109 coupled to both Gi and β -arrestin-1, decreased the intracellular cAMP concentration to inhibit insulin secretion and promoted CCL2 production in β cells to mediate crosstalk with macrophages, finally facilitating the proliferation of islet-resident macrophages. Recent studies have demonstrated that local proliferation of islet-resident macrophages is significantly enhanced and plays key roles in islet inflammation and diabetes development; however, the underlying mechanism and exact regulation pathway are undefined(16, 34-36). Here, our study presented a new scenario in which a particular signalling pathway governed by ORs in response to endogenous islet peptides modulated crosstalk between pancreatic β cells and macrophages, thus controlling the inflammatory process in pancreatic islets. Thus, Olfr109 may emerge as a pivotal autonomous control point for islet homeostasis via examination of the quality and quantity of peptide products secreted by β cells in an autocrine manner.

The dispensable role of Olfr109 in wild-type mice but negative effects under pathogenic conditions indicated that aberrant amplification of Olfr109 function may adeptly mediate disease-related conditions. By contrast, we have identified that several SNPs in the putative InsB 9:23 binding site of Olfr109 significantly decreased the signaling response downstream of Olfr109. Their clinical relevance awaits further investigation. Moreover, we developed a pepducin-based selective antagonist of Olfr109 that significantly improved glucose homeostasis in both the HFD-induced obesity and diabetic *Akita* mouse models. These findings indicate the new therapeutic potential of antagonizing the pancreatic islet-resident OR Olfr109. Collectively, the results of the present study expand our understanding of insulin biology by demonstrating that an insulin peptide can engage with an autonomous receptor and regulate local inflammation processes that intervene with islet homeostasis. Antagonizing Olfr109 could be a new therapeutic strategy for suppressing islet inflammation to treat obesity and diabetes.

436

437

438 **Figure legends**

439 **Figure 1. Expression of olfactory receptors in mouse islets and the effects of Olfr109**
440 **deficiency on glucose homeostasis in diabetic *Akita* mice**

441 (A) Phylogenetic tree showing the evolutionary relationship of mouse olfactory receptors
 442 (ORs) identified to have relatively high expression levels in pancreatic islets. The ORs
 443 expressed in islets are highlighted in red.
 444 (B) qRT-PCR analysis of mRNA expression levels of six OR genes in pancreatic islets of
 445 wild-type (WT) male mice normalized to their corresponding levels in nasal olfactory
 446 epithelium.
 447 (C) mRNA levels of six mouse OR gene homologues in human islets after stimulation with
 448 low concentration of glucose or high concentration of glucose. Data are from three
 449 independent experiments (n=3).
 450 (D) mRNA levels of six OR genes in islets isolated from 8-week-old WT or Akita mice.
 451 (E) mRNA levels of six OR genes in islets isolated from mice fed a normal chow diet (NCD)
 452 or a high-fat diet (HFD). Data are from three independent experiments (n=3).
 453 (F) Effects of OLFR109 or OLFR110 overexpression in MIN6 pancreatic β cells on insulin
 454 secretion Data are from three independent experiments.
 455 (G) Representative images colorimetric RNAscope in situ hybridization for *Olfr109* (red) in
 456 pancreatic sections of WT and *Olfr109*^{-/-} mice.
 457 (H) Body weight changes in *ins2*^{WT/WT}*Olfr109*^{+/+}, *ins2*^{WT/Akita}*Olfr109*^{+/+}, *ins2*^{WT/WT}*Olfr109*^{-/-},
 458 and *ins2*^{WT/Akita}*Olfr109*^{-/-} mice.
 459 (I-J) Energy expenditure (H) and respiratory quotient (I) values in *ins2*^{WT/WT}*Olfr109*^{+/+},
 460 *ins2*^{WT/Akita}*Olfr109*^{+/+}, *ins2*^{WT/WT}*Olfr109*^{-/-}, and *ins2*^{WT/Akita}*Olfr109*^{-/-} mice, as measured by
 461 indirect calorimetry.
 462 (K-L) Plasma glucose levels (J) and insulin levels (K) in *ins2*^{WT/WT}*Olfr109*^{+/+},
 463 *ins2*^{WT/Akita}*Olfr109*^{+/+}, *ins2*^{WT/WT}*Olfr109*^{-/-}, and *ins2*^{WT/Akita}*Olfr109*^{-/-} mice during the glucose
 464 tolerance test.
 465 (M) Insulin contents of the islets isolated from *ins2*^{WT/WT}*Olfr109*^{+/+}, *ins2*^{WT/Akita}*Olfr109*^{+/+},
 466 *ins2*^{WT/WT}*Olfr109*^{-/-}, and *ins2*^{WT/Akita}*Olfr109*^{-/-} mice. Data are from three independent
 467 experiments (n=3).
 468 (N) Immunostaining (left panel) and quantitative analysis (right panel) of CD11c⁺
 469 macrophages (red) in pancreatic sections from *ins2*^{WT/WT}*Olfr109*^{+/+}, *ins2*^{WT/Akita}*Olfr109*^{+/+},
 470 *ins2*^{WT/WT}*Olfr109*^{-/-}, and *ins2*^{WT/Akita}*Olfr109*^{-/-} mice. Scale bar: 100 μ m. n = 6 mice per group;
 471 4-7 random areas were selected from each islet section, and 10 sections were randomly
 472 selected from each mouse.
 473 The bars indicate the mean $\pm$ SEM values. All data were statistically analysed using one-way
 474 ANOVA with Dunnett's post hoc test.

475

476 **Figure 2. *Olfr109* deficiency improves glucose homeostasis in HFD-fed mice**

477 (A) Body weight changes in WT and *Olfr109*^{-/-} mice fed an NCD or HFD.

478 (B-C) Energy expenditure (B) and respiratory quotient (C) values in WT and *Olf109*^{-/-} mice
 479 fed an NCD or HFD, as measured by indirect calorimetry.
 480 (D-E) Plasma glucose levels in WT and *Olf109*^{-/-} mice fed an NCD or a HFD during the
 481 insulin tolerance test (D) and glucose tolerance test (E).
 482 (F-G) Plasma glucose (F) and insulin levels (G) in WT and *Olf109*^{-/-} mice fed an NCD or a
 483 HFD after refeeding.
 484 (H) Insulin secretion from islets isolated from WT and *Olf109*^{-/-} mice fed an NCD in
 485 response to stimulation with high concentration of glucose. Data are from three independent
 486 experiments (n=3).
 487 (I-J) Immunostaining (I) and quantification (J) of CD11c⁺ macrophages (red) in pancreatic
 488 sections of WT and *Olf109*^{-/-} mice fed an NCD or a HFD. Representative images are shown.
 489 Scale bar: 100 μ m. n = 6 mice per group; 3-5 random areas were selected from each islet
 490 section, and 6-8 sections were randomly selected from each mouse.
 491 (A-J) *P < 0.05; **P < 0.01; ***P < 0.001; ns, no significant difference; *Olf109*^{-/-} mice
 492 compared with WT mice fed the same diet. The bars indicate the mean $\pm$ SEM values. All
 493 data were statistically analysed using one-way ANOVA with Dunnett's post hoc test.

494

495 **Figure 3. Identification of endogenous agonists of OLFR109**

496 (A) Heatmap visualization of representative genes derived from whole transcriptome
 497 RNA-seq analysis of MIN6 cells transfected with si*Olf109* or control siCon.
 498 (B) Gene set enrichment analysis was conducted based on KEGG pathway annotation of the
 499 Gs/Gi-cAMP signalling pathway.
 500 (C-D) Dose-dependent inhibition of forskolin-induced cAMP accumulation (C) and
 501 β -arrestin-1 recruitment to OLFR109 (D) in OLFR109-overexpressing HEK293 cells in
 502 response to incubation with supernatant.
 503 (E-F) Dose-dependent inhibition of forskolin-induced cAMP accumulation (E) and
 504 β -arrestin-1 recruitment to OLFR109 (F) in OLFR109-overexpressing HEK293 cells in
 505 response to incubation with secretory granule fractions .
 506 (G-H) Dose-dependent inhibition of forskolin-induced cAMP accumulation (G) and
 507 β -arrestin-1 recruitment to OLFR109 (H) in OLFR109-overexpressing HEK293 cells in
 508 response to incubation with the 5k secretory granule fraction.
 509 (I-J) Maximum efficacy of cAMP inhibition (I) or β -arrestin-1 recruitment (J) in
 510 OLFR109-overexpressing HEK293 cells in response to the 15 peptides.
 511 (K) Mass spectrum of the insB:9-23 peptide identified in the 5k secretory granule fraction.
 512 (L) Saturation binding of ¹²⁵I-insB:9-23 to pancreatic islet membrane extracts derived from
 513 *Olf109*^{-/-} or WT mice.

(M) A best-fit linear correlation of the mRNA levels of OLFR109 in different tissues measured by qPCR with their protein levels of membrane fractions calculated from radioligand binding of ¹²⁵I-insB:9-23.

(N) Competitive binding curve of insB:9-23 in *Olfr109*-transfected HEK293 cells.

518

519 **Figure 4. InsB:9-23-regulated functions and signalling via OLFR109**

520 (A) Effects of insB:9-23 treatment on insulin secretion in islets derived from WT or *Olfr109*^{-/-}
521 mice. Data are from three independent experiments (n=3).

522 (B) Effects of insB:9-23 treatment on the cAMP concentration in islets derived from WT or
523 *Olfr109*^{-/-} mice. (n=3). Data are from three independent experiments (n=3).

524 (C) Plasma glucose levels during the glucose tolerance test in WT and *Olfr109*^{-/-} mice treated
525 with vehicle control or insB:9-23 .

526 (D) Heatmap visualization of chemokine signalling-related genes derived from whole
527 transcriptome RNA-seq analysis of islets isolated from WT mice.

528 (E) Relative mRNA levels of *Ccl2* and *Ccl25* in the islets isolated from WT or *Olfr109*^{-/-} mice
529 treated with vehicle or insB:9-23.

530 (F) Immunostaining for CD11c (red) in pancreatic sections of WT and *Olfr109*^{-/-} mice treated
531 with insB:9-23 or vehicle control.

532 (G) mRNA levels of *Mertk*, *CD64* and *Olfr109* in macrophages and nonmacrophage cells
533 derived from mouse pancreatic islets normalized to the corresponding expression levels in
534 isolated primary pancreatic β cells. Macrophages were derived from wide-type mice islets by
535 biotinylated mAbs against F4/80 and magnetic nanobeads conjugated to Streptavidin. Primary
536 β cells were derived from *Ins2^{cre}Rosa^{lsl-GFP}* mice islets by fluorescence-activated cell sorting
537 (FACS).

538 (H) mRNA levels of Ki67 in macrophages from mouse pancreatic islets cultured with the
539 supernatants of islets isolated from HFD-fed WT or *Olfr109*^{-/-} mice.

540 (I) Effects of stimulation with insB:9-23 on CCL2 levels in the supernatant of pancreatic islets
541 isolated from WT or *Olfr109*^{-/-} mice.

542 (J) Effects of treatment with the CCR2 antagonist INCB3344 on the proportion of pancreatic
543 islet-resident macrophages in mice.

544 (K) Effects of treatment with the CCR2 antagonist INCB3344 on Ki67 mRNA levels of
545 pancreatic islet-resident macrophages in mice.

546

547 **Figure 5. Alanine scanning and molecular simulation revealing the interaction mode** 548 **between insB:9-23 and OLFR109**

549 (A) Schematic representation of the screening and modelling strategies used to characterize
550 the interaction mode between insB:9-23 and OLFR109.

(B-C) Alanine scanning identified important residues in the OLFR109 receptor that recognize insB:9-23 through a β -arrestin1 recruitment assay (B) and a ligand binding assay (C).
(D) Alanine scanning identified important residues in insB:9-23 that bind to the OLFR109 receptor through the β -arrestin1 recruitment assay (D) and ligand binding assay (E).
(E) Pairing of insB:9-23 mutants with WT OLFR109 and OLFR109 mutants through alanine scanning and a ligand binding assay. The K_i value for the binding of each insB:9-23 mutant to OLFR109 mutants was normalized to the K_i value of the same insB:9-23 mutant binding to WT OLFR109. The receptor mutants that did not show significantly decreased K_i values compared to those of the WT receptor when binding to a specific insB:9-23 mutant are highlighted.
(F) SNPs occurred at the potential ligand-binding sites of OR12D3.
(G) Affinity of insB:9-23 peptide binding to wide-type or selective single nucleotide polymorphisms (SNPs) of OR12D3.

Figure 6. Development of a pepducin-based OLFR109 antagonist that improved glucose metabolism in both diabetic *Akita* mice and HFD-fed mice.

(A) Amino acid sequences of OLFR109 pepducins.
(B) Dose-dependent inhibition of forskolin-induced cAMP accumulation in *Olf109*-transfected HEK293 cells.
(C) The *Olf109*-ICL2-2 (*o109-i2-2*) pepducin induced dose-dependent inhibition of the decrease in cAMP accumulation.
(D) Effects of *o109-i2-2* on insulin secretion from pancreatic islets in response to stimulation with a high glucose concentration alone or in combination with insB:9-23.
(E) Effects of *o109-i2-2* on the mRNA levels of the endoplasmic reticulum stress factors *Prdx5*, *Ptgs1*, *Ptgs2*, and *Sxbl1* in MIN6 pancreatic β cells.
(F) Effects of *o109-i2-2* on plasma glucose levels during the glucose tolerance test in WT or *Olf109*^{-/-} mice fed a HFD for 13 weeks.
(G) Effects of *o109-i2-2* on plasma glucose levels during the glucose tolerance test in ins2^{WT/Akita} *Olf109*^{+/+} and ins2^{WT/Akita} *Olf109*^{-/-} mice.
The bars indicate the mean $\pm$ SD values. All data were statistically analyzed using one-way ANOVA with Dunnett's post hoc test.

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Author Contributions:

665 X.Y. and J.-P.S. conceived and initiated the project. X.Y. and J.-P.S. supervised the overall
666 project design and execution. J.C., Z.Y. and X.-Y. G. performed metabolic and histological
667 studies of mice. J.C., Z.Y., X.-Y. G., R.M., J.-Y.L. Z.-M. T. isolated mice islets and performed
668 *ex vivo* experiments. J.C., M.-X. G., and R.M. performed immunofluorescence studies. J.C.,
669 Z.Y. and X.-Y. G. performed macrophage isolation and chemokine studies. J.C. and X.-Y. G.
670 isolated and lysed β cell granules for mass spectrometry. J.C. and Z.Y. performed radioligand
671 binding assay. X.Y. and J.-P.S. designed the screening assays for dissecting the interaction
672 mode between insB:9-23 and OLFRL109. J.C., X.-Y. G., M.-X. G., and R.M. performed

673 ELISA assay and BRET assay of OLFR109 mutants. X.Y. and J.-P.S. designed pepducins of
 674 OLFR109. J.C., Z.Y., X.-Y. G. and M.-X. G. performed pepducin-based cellular and mice
 675 experiments. Y.-F. X. provided human islets. X.Y., J.-P.S., J.C., and Z.Y. participated in data
 676 analysis and interpretation. F.Y. provided insightful ideas and contributed to structural
 677 modelling. X.Y. and J.-P.S. wrote the manuscript. All of the authors have seen and commented
 678 on the manuscript.
 679

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

A

Alanine Scanning

Functional Assays

Binding Sites

Residue pairing

B

D

F

Change	SNP Number	Amino acid (Codon)
I31N	rs1355948032	I [ATC] → N [AAC]
I31S	rs1355948032	I [ATC] → S [AGC]
Y71F	rs567400895	Y [TAT] → S [TCT]
Y71S	rs567400895	Y [TAT] → F [TTT]
C167W	rs1400769112	C [TGT] → W [TGG]

C

E

G

Figure 6
A