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INS2 lineage cell tracking and insulin expression in the related organs of mice

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We tracked lineage map of the Ins2 cells in mice and insulin expression in migration sites of cells. We studied effect of the Wnt/β-catenin signaling pathway on the migration. We studied insulin secretion status in submandibular gland of mice under hyperglycemia stress. Cre/loxp system was used to observe migration sites and timing of the Ins2-cre lineage cells. Immunohistochemistry and immunofluorescence was used to detecte presence of insulin in the Ins2-cre lineage cells. Knockout mice from E9.5 to adulthood was studied to explore role of the Wnt/β-catenin on the migration. Immunofluorescence and the QRT-PCR (Quantitative Real-time Polymerase Chain Reaction) was used to study insulin secretion in submandibular gland under hyperglycemic conditions. Expression sites of the Ins2-cre gene in adult mice decreased compared with postnatal mice, including the pancreas, tongue, submandibular, and brain. In the migration tissues of ins-cre cells, positive insulin expression was detected in the submandibular acinus, vessel element and pancreatic islets. In comparison to wild-type mice, Wnt/β-catenin signaling knockout mice displayed a slight rise of INS2 expression during the neonatal stage, with a notable increase in adulthood, particularly in areas near the oral cavity. Expression of insulin in submandibular gland of mice increased after 6 h of hyperglycemic stimulation ($P < 0.05$). Ins2 lineage cells can migrate to multiple organs in mice, where insulin may expressed. Inhibition or knockout of the Wnt/β-catenin signaling pathway may indirectly enhance the migratory capacity of INS2 cells. Submandibular glands may secrete insulin under stress of maintaining organismal homeostasis. Wnt/β-catenin may be the therapeutic target of diabetes. Submandibular glands may be a new target organ of gene therapy for diabetic patients.

Keywords Insulin, Cre/loxp, Wnt/beta-catenin signaling, Cell lineage tracing, Submandibular gland

Diabetes, a metabolic disorder stemming from pancreatic insulin secretion or dysfunction in pancreatic cells[1], has emerged as one of the three major medical challenges in the modern medicine, with its incidence rate escalating annually. A two-way relationship between oral disease and diabetes exists: diabetes may increase the risk of oral disease, and vice versa, higher blood glucose levels in diabetic patients exacerbate periodontal lesions[2–4]. The severity of periodontal disease incites insulin secretion resistance and further increases the severity of the diabetes[5,6]. Insulin, the sole hormone capable of reducing blood sugar levels, is secreted by endogenous or exogenous substances in pancreatic islet β cells. The insulin gene is divided into two types-Ins1 and Ins2. Ins2, a homologous gene of other mammalian insulin genes[7,8], governs insulin production[9], regulates blood glucose levels[10], and triggers insulin-secreting granule release.

Cell lineage tracing, a biological technique marking certain cells early on, reveals the developmental fate of cells over time by detecting markers[11]. In recent years, the application of inducible recombinase Cre /loxp system has significantly enhanced the accuracy of cell tracking, simplified the tracking methods and steps, and broadened the scope of tracking[12,13]. The labeling method of Cre /loxp system has high accuracy and genetic characteristics. Wicksteed et al. used the Cre/Loxp site recombination system to explore insulin synthesis and secretion mechanisms, revealing cre expression not only in the pancreas but also in the hypothalamus, implicated in some areas of endocrine control[14,15]. Additionally, the choroid plexus serves as a primary expression site of

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Ins2 in the brain[16]. Insulin expresses a few in the tissues such as the thymus, yolk sac, and intestine across various organisms including humans[17]. These findings indicate that the expression of Ins2 is not limited to the β cells of the pancreas and may exist in many tissues and organs. However, there is still a lack of comprehensive and specific research on the overall distribution of the Ins2-cre cell lineage in animals.

Cell development and differentiation hinge on various signaling pathways, with the Wnt/β-catenin signaling pathway pivotal in the development of tissues and organs, regulating cell proliferation, migration, and differentiation. The role of Wnt signaling in pancreatic development and function remains unclear. Some datas show that Wnt4[38]and Wnt5a[39]are involved in the differentiation process of pancreatic β-cells, interestingly, activation of Wnt/β-catenin signaling can promote the dedifferentiation of pancreatic β-cells[40]. In the recent years, the canonical Wnt/β-catenin pathway has emerged as an important player of pancreas organogenesis, β-catenin activation in mouse MPCs has been reported to severely impair pancreas formation, reduce epithelial branching and markedly compromise the differentiation of endocrine and exocrine lineages[41]. Therefore, exploring the Wnt/β-catenin signaling pathway's role in the expression and migration of insulin genes holds significance.

Notably, salivary glands contain insulin-like substances[18–22], which may play an important role in carbohydrate metabolism through exocrine and endocrine mechanisms[23,24]. Radioimmunoassay can detect high concentrations of insulin in numerous mouse and human tissues[25]. Previous studies identified insulin-like substances in the salivary glands of male rats and other mammals[26,27]. In a diabetic mouse model, Patel et al.[26]not only noted reduced pancreas insulin but also decreased levels in the submandibular gland. The submandibular gland, one of the three major salivary glands of human body[29,30], boasts a symmetric bilateral structure, rendering it a potential target for gene therapy[29]. Analogous to the pancreas in glandular structure, its potential insulin secretion warrants investigation.

Materials and methods

Study flowchart

Figure 1 illustrates the study design of the current research. In this study, mice were labeled with the Cre/loxp system to observe distribution of cell lineage expressing insulin gene from postnatal to adult mice and to detect gene expression of insulin in organs. In addition, mice with complete β-catenin knockout were cultivated to explore the changes of migration sites. This experiment also established an animal model of intraperitoneal glucose tolerance test (ipGTT), through which submandibular gland differentiation was induced and insulin secretion was detected.

Animals

A transgenic mouse line carrying Cre was provided by the Sloan Kettering Cancer Center (New York). The ins2 coding sequence was replaced with Cre recombinase cDNA by homologous recombination, enabling Cre expression under the control of the regulatory sequences of ins2. All mice were of a C57BL/6J background and bred in our in-house specified pathogen-free (SPF) animal facility.

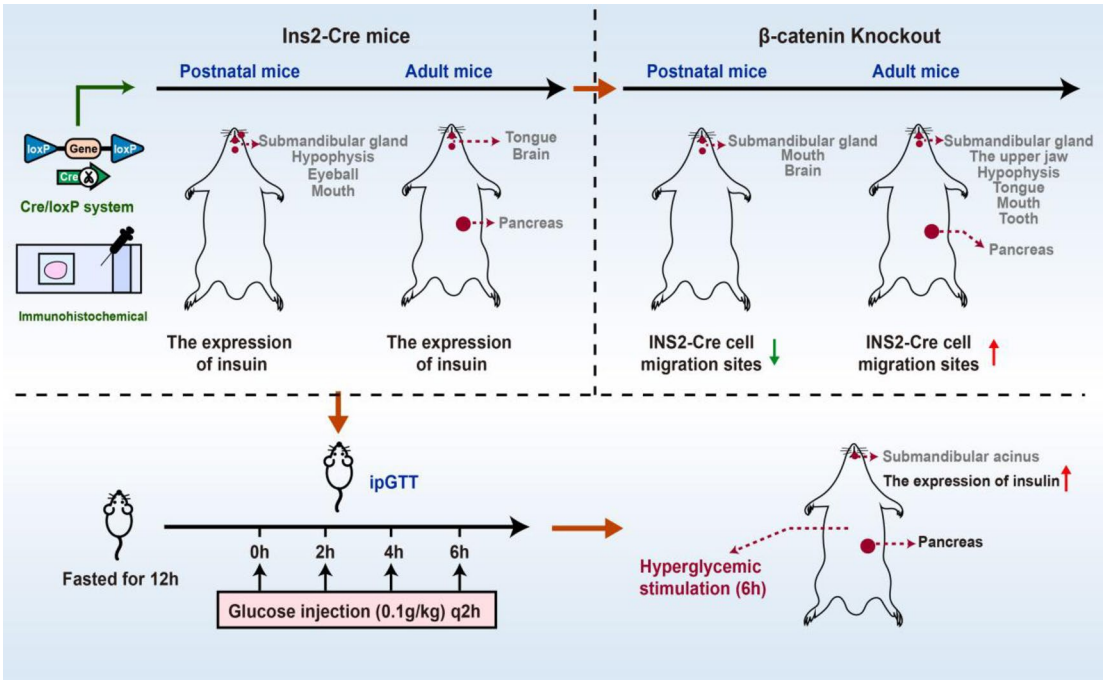


Fig. 1. Schematic diagram of experimental process.

No homozygotes were used in this study. To obtain embryos, timed-pregnant females from the appropriate crosses were euthanized by cervical dislocation following 3% isoflurane anesthesia. Mice were housed under controlled temperature and humidity (20 ± 2 °C, 35 ± 5%) and a 12 h/12 h dark/light cycle. All animals were group-housed (2–4 animals per cage) and had ad libitum access to food and water unless otherwise specified. Each cage housed one male and two females, with weekly changes made during reproductive needs. All animal experiments were approved by the Ethics Committee of Stomatological Hospital Affiliated with Chongqing Medical University (No. 2022 – 163). This study was conducted in accordance with the ARRIVE guidelines and the Guide for the Care and Use of Laboratory Animals.

Genotyping PCR

For genotyping PCR, toes of mice aged 7 days to 10 days were collected in tubes and dissolved in tail lysis buffer (1.87 g KCl, 10 ml 0.5 M Tris-Cl, 255 mg MgCl2-6H2O, 50 mg gelatin, 2.25 ml NP40 and 2.25 ml Tween 20) containing proteinase K (10 mg/ml) at 55°C overnight. After centrifugation at 12000 rpm speed for 10 minutes, supernatant with genotyping DNA were obtained. The dissolved samples were subjected to PCR amplification with primers (see below) Cre-FW, 5'-GAGCATACCTGGAAAATGCTTC-3';Cre-RW,5'-CCGGCAAAACAGG TAGTTATTC-3'. PCR products were separated on a 1% (Ins-Cre) agarose gel in tris, acetic acid, and EDTA (TAE) buffer and visualized post-staining with ethidium bromide.

Fluorescence microscopy

Mice were euthanized during the dark phase by decapitation to collect the tissue samples from postnatal day mice and adult mice were fixed in 4% PFA at 4 °C overnight. After 3 wahses with PBS, tissues were placed on an agar gel for bright-field or fluorescence imaging using an inverted fluorescence microscope(Nikon, Japan). Tissues were embedded in OCT and sectioned into 12 mm thick sections using a −20℃ cryomicrotome. Frozen sections were observed using a Stereotype microscope(Paidiwei, China).

Immunofluorescence staining

Cells on frozen section were washed 3x in PBST and fixed with 4% PFA for 5 min. Subsequently, cells were subjected to antigen repair buffer heating for 25 min at 95℃. And after blocking with 10% fetal bovine serum(FBS), cells were incubated with primary antibodies against insulin overnight at 4 °C. The primary antibodies used in this study were the following: rabbit anti-insulin (1:100; Abcam, USA) and mouse anti-insulin (1:100; Abcam, USA). Slides were then incubated with Alexa Fluor Plus 594 goat anti-mouse or goat anti-rabbit (1:500; Abcam, USA) secondary antibodies for 2 h at room temperature, followed by DAPI counterstaining for nucleus identification. Slides were observed using confocal laser scanning microscope and fluorescence microscope (Nikon Corporation, Japan).

Immunohistochemistry staining

Pancreata and submandibular gland were collected from adult mice, fixed overnight in 4% PFA, dehydrated with ethanol gradient, embedded in paraffin, and sectioned for insulin immunostaining as 2.5. After washing three times with PBS, sections underwent secondary antibody incubation again. The slides were stained with diaminobenzidine (DAB) chromogen. Immunohistochemistry was performed on 4-mm tissue sections, which were deparaffinized in xylene and gradient ethanol.

Images of stained sections were observed using a Inverted Fluorescence Microscope and Graphic Acquisition and Analysis System (Nikon Corporation, Japan) for analysis and photography at room temperature.

Intraperitoneal glucose tolerance Test(ipGTT)

Eight genetically identical Cre/loxP transgenic mice, matched for age, sex, and body weight, were subjected to a 12-hour fasting period in the dark with free access to water but no food. The mice were randomly assigned to four groups (A, B, C, D), with two mice in each group, to perform the intraperitoneal glucose tolerance test (ipGTT) for studying different submandibular gland stress states. The experimental procedures for each group were as follows: group A: Mice were fasted overnight, and on the following day, fasting blood glucose levels were measured. The mice were then euthanized by cervical dislocation, and the submandibular glands were harvested. Half of the tissue was embedded in paraffin for immunofluorescence staining, while the remaining tissue was placed in RNA preservation solution for RT-qPCR analysis. group B: After the fasting period, glucose was administered intraperitoneally at a dose of 0.1 g/kg on the following morning. Blood glucose levels were measured using an Accu-Chek Aviva blood glucose monitor (Roche, Germany). Two hours later, a second intraperitoneal glucose injection was administered, and blood glucose levels were measured again. The tissues were processed as described for Group A. group C: Following the fasting period, glucose was administered intraperitoneally at a dose of 0.1 g/kg at 0 h, 2 h, and 4 h post-fast. Blood glucose levels were measured at each time point, and tissues were processed as described for Group A. group D: After fasting, glucose was administered intraperitoneally at 0 h, 2 h, 4 h, and 6 h post-fast, at a dose of 0.1 g/kg. Blood glucose levels were measured at each time point, and tissues were processed as described for Group A. The experiment was repeated three times, and the average values were used for analysis. The blank control group of mice undergoes no experimental treatment, and their submandibular glands are collected for immunofluorescence staining. The resulting images are used as a baseline for comparison with the experimental groups in fluorescence intensity analysis.

Real time quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted using TRIzol reagent (TIANGEN Biotech, Beijing, China) from submaxillary gland following the manufacturer's protocol (Kangwei century CW0584 RNApure Tissue K it), and treated with ELIASA. cDNA was synthesized using the Takara PrimeScriptTM RT reagent Kit(Dalian Bao, China). RT-qPCR

reactions were conducted using FAST SYBR green and the Viia7 Real Time PCR system (Applied Biosystems). Each sample was run in triplicate and analyzed using the 2-ΔΔCT method with? as the reference gene. Primers used are listed in Sup Table 1.

Immunohistochemistry and histology

Half of the submandibular glands from eight transgenic mice were preserved in RNA preservation solution for RT-qPCR analysis, while the other half sections were stained with Secondary antibodies (validated by the manufacturers for immunofluorescence analyses) and examined using a fluorescence microscope (Nikon Corporation, Japan).

Mouse model of Wnt/β-catenin signal loss

The uterus was stripped from the abdominal cavity of pregnant rats for genotyping PCR. DNA was extracted by splitting amniotic membrane (processing methods refer to 2.2). Embryos were fixed overnight in 4% PFA, dehydrated every 2 h in ethanol gradient, treated with xylene for 30 min, and embedded in paraffin.

The dissolved samples were subjected to PCR amplification with primers (see below): β-catenin-FW, 5'-AAG GTAGAGTGATGAAAGTTGTT-3'; β-catenin-RW, 5'-CACCATGTCCTCTGTCTATTC-3'; Cre-FW,5'-GAGC ATACCTGGAAAATGCTTC-3', and Cre-RW, 5'-CCGGCAAAACAGGTAGTTATTC-3'.

PCR was used to detect β-catenin and cre expression to establish a mouse model with complete loss of wnt/β-catenin signaling. Additionally, the distribution sites of Ins2-cre cells in mouse embryos at different stages was observed using a fluorescence microscope.

Results

Establishment of β-catenin protein knockout mice

Animal models were successfully obtained on the 18th day of embryo (E18.5) and 9th day of embryo (E9.5), and their gene phenotypes were detected by PCR and the expression of β-catenin and cre in embryos were identified(Fig. 2a). PCR was used to assess β-catenin and cre expressions in adult mice, revealing a 300 bp band in the second column for β-catenin and a 400 bp band in the third column of cre. Mice harbored cre recombinase were indicative of complete wnt/β-catenin signal deletion(Fig. 2b).

Lineage cell tracing by Ins2-cre cells

Ins2-cre knock-in mice, generated in prior studies, were identified via PCR to determine the presence of cre recombinase and assess fluorescence distribution in the body. It was found that the expression of fluorescent protein could be observed in mouse embryos from the 9th day of embryo (E9.5) to adulthood. Fluorescent expression was observed in the eyes, somites, dorsal pancreas, and bud primordium of E9.5 mouse embryos(Sup3). It was observed in mouth, brain, and submandibular gland of E18.5 mouse embryos, the migratory localization (Fig. 3a) and expression levels (Fig. 3c) of the INS2 gene demonstrated only a modest elevation in the experimental cohort when compared to the control group (wild-type murine subjects). While the expression sites in adult mice encompassed the pituitary, submandibular gland, teeth, tongue, palate, islets, and mouth, which exhibited significant spatial expansion (Fig. 3b), with notable concentration in the perioral region, concurrent with a pronounced upregulation of transcriptional activity (Fig. 3d). Notably, the survival rate of mice with complete deletion of β-catenin signal was extremely low, comprising solely female mice.

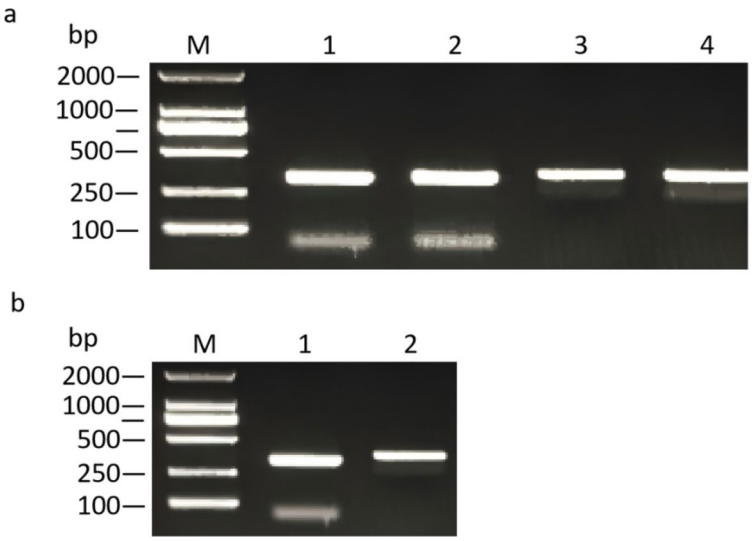


Fig. 2. Identification of mouse embryonic genotypes by PCR (original gels are presented in Supplementary Fig. 2). Lane M: marker; lane 1: strains of β-catenin (E18.5 mouse); lane 2: strains of β-catenin (E9.5 mouse); lane 3: strains of cre (E18.5 mouse); lane 4: strains of cre (E9.5 mouse). (**b**) Identification of adult mouse genotypes by PCR. Lane M: marker; lane 1: strains of β-catenin; lane 2: strains of cre.

Insulin expression in the migration sites

Parallel fixation and embeeding of wild-type mice enabled frozen sectioning to observe the distribution of fluorescent cells, revealing main fluorescence sites in the pancrease, submandibular gland, hypophysis, eyeball, and mouth (Fig. 4a). Quantitative fluorescence intensity analysis demonstrated that INS2 gene expression exhibited an age-dependent decline in both hypophysis and submandibular gland tissues during postnatal development. Concurrently, the pancreatic tissue progressively assumed dominance as the principal site of insulin secretion, suggesting a developmental shift in endocrine organ specialization(Fig. 4b, c).

Immunohistochemical staining showed that pancreatic islets were positively stained, islets were brownish yellow, and nuclei were deeply stained. Submandibular gland acinar cells were positively stained, and nuclei were deeply stained. Immunofluorescence staining showed that islet cells were bright red-dark-dyed, and DAPI staining showed blue-stained granules. Submandibular gland cells were bright red-dark-dyed, and DAPI staining showed blue nucleus Deep dyed granule (Fig. 5) .

Insulin-positive expression was observed in the submandibular acinus, vessel element and pancreatic islets while there was barely no expression detected in the migration sites like eyes, mouth, and brain. This suggested potential insulin secretion in a part of target organs where the insulin gene migrated.

Insulin secretion status of submandibular gland under under hyperglycemia stress

For this study, four different groups were used ($n=8$). The mice in different groups were tested for blood glucose levels post-stimulation, as shown in Fig. 6a. Comparisons with the mice without stimulation revealed statistically differences in blood glucose levels ($P<0.05$) (Fig. 6b; Sup. 1, Table S1). Half of the tissues underwent mRNA isolation and QRT-PCR analysis, while the remainder were subjected to histology. Results showed a significant increase in the expression of insulin-related genes Ins1 and Ins2 in the submandibular gland after 6 h of stimulation (Fig. 6c; Sup. 1, Table S2), with statistically significant differences observed in the stimulation group ($P<0.05$). Submandibular glands extraction from the experimental group followed by immunofluorescence staining on frozen section (as shown in Fig. 6d) reveled strong insulin staining and the bright red cytoplasm in mouse submandibular gland cells after 6 h of stimulation. The above results suggest the presence of insulin expression in the submandibular glands of hyperglycemic mice.

Discussion

In this experiment, transgenic mice generated via the Cre/loxp system in the early stage of the research group were able to mark the expression of the gene and successfully track the cell under the premise of ensuring cell survival by using the bio-tracking technology. This allowed for the comprehensive examination of insulin gene expression lineage cells in adult mice, confirmed by immunohistochemistry and immunofluorescence techniques.

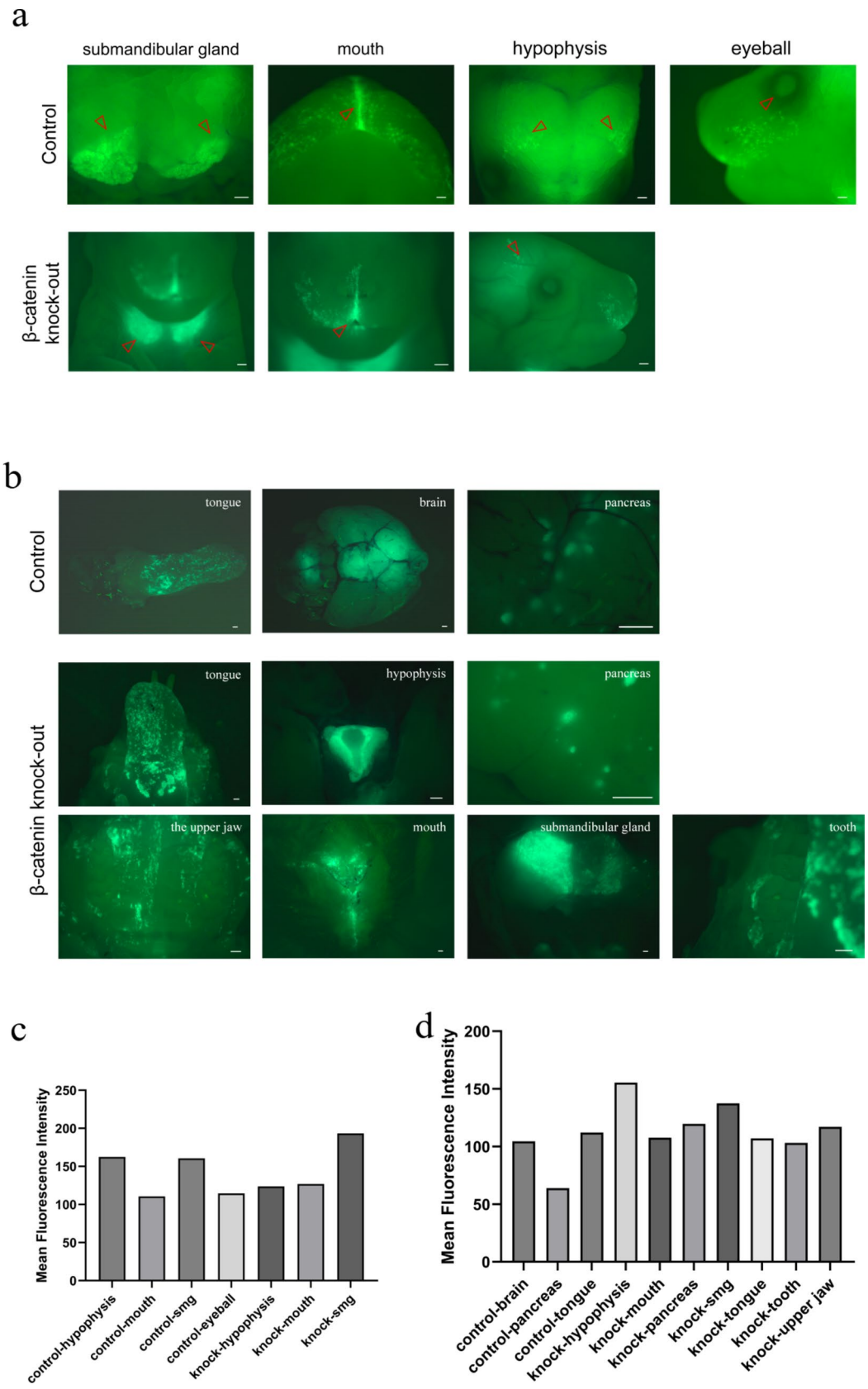
we obtained β-catenin completely knockout mice generated through mating and screening to study the relationships between Wnt signal channels and the migration of Ins2. Our findings demonstrate that neonatal mice with Wnt/β-catenin signaling disruption exhibit slight rise of INS2 expression and migratory capacity compared to wild-type controls, while by adulthood, these mice show a greater number of Ins2-positive cell migration sites and elevated INS2 expression levels.

It have been reported in studies using in parallel two Pdx1-Cre lines to activate β- catenin, demonstrating that the role of this signaling protein in pancreas specification varies depending on the developmental stages[42]. In the early embryogenesis, Wnt/β-catenin signaling activity is critical for the formation of the primitive streak and progression of gastrulation. Subsequently, the inhibition of the pathway is required for proper pancreatic specification during early endoderm development[43]. So far, it seems that the precise spatial and temporal activation of Wnt target genes plays a pivotal role in pancreas development.

However, it's crucial to acknowledge that organism development and gene expression and migration processes are intricate, often involving multiple signaling pathways such as the notch signaling pathway, hh signaling pathway, etc. The regulation of insulin gene expression is not solely reliant on the Wnt/β-catenin signaling pathway. Future research can explore potential cross-control effects of other signaling pathways on their cell migration by inhibiting the expression of a single signaling pathway, thus establishing correlations between signal pathway and gene migration. In subsequent studies, techniques such as real-time quantitative PCR can also be used to detect differences in the expression levels of genes in tissues where insulin genes migrate.

This study included an exploratory experiment wherein an animal model of hyperglycemia stress was established to detect the submandibular gland's latent capacity to respond to stress and secrete insulin. Structurally similar to the pancreas as an exocrine gland, the submandibular gland may exhibit a comparable secretory effect. Since the presence of insulin-like protein was found in cells of submandibular gland (SMG) ducts[31–33], there has been some reports about the compensatory function of submandibular gland for the gene therapy of diabetes mellitus under condition of transplantation[34], vehicle[35], and virus[36]. However, reports on the natural differentiation of SG cells into islet cells are scare. In the evolutionary history of genes expressed in SMGs, lineage-specific adaptations in response to insulin changes has been reported recently[37].

In this experiment, stress state was used to prompt submandibular gland differentiation and induce insulin secretion. Results revealed positive insulin expression in the submandibular gland under hyperglycemia conditions, with significantly higher insulin gene expression compared to the control group, suggesting that the submandibular gland may play a role in insulin synthesis under specific physiological or pathological conditions, particularly in high-glucose environments. This could involve the indirect activation of regulatory factors that promote the expression of insulin genes in the submandibular gland, potentially serving as a compensatory mechanism to help regulate blood glucose levels. These findings provide direct evidence for the use of non-insulin-producing organs, such as the submandibular gland, to express insulin. This approach could be explored



as a potential gene therapy strategy, offering a novel concept for maintaining blood glucose stability in diabetic patients.

The limitation of this experiment is that the role of submandibular gland in insulin secretion is still in the early stages of exploration. Further experiments, such as protein detection and functional validation, are needed

◂**Fig. 3**. Distributions of Ins2-cre cells in the wild-type mice and β-catenin knock-out mice. (**a**) Fluorescence sites observed in the postnatal mice (PND18). control group: submandibular gland, mouth, eyeball and hypophysis; knock-out group: submandibular gland, pancreas, and brain. (**b**) Fluorescence sites observed in the adult mice. control group: tongue, brain, pancreas; knock-out group: tongue, hypophysis, pancreas, the upper jaw, mouth, submandibular gland, tooth (scale bar: 500 μm). (**c**) Analysis of fluorescence intensity at expression sites in postnatal mice. (**c**) Analysis of fluorescence intensity at expression sites in adult mice.

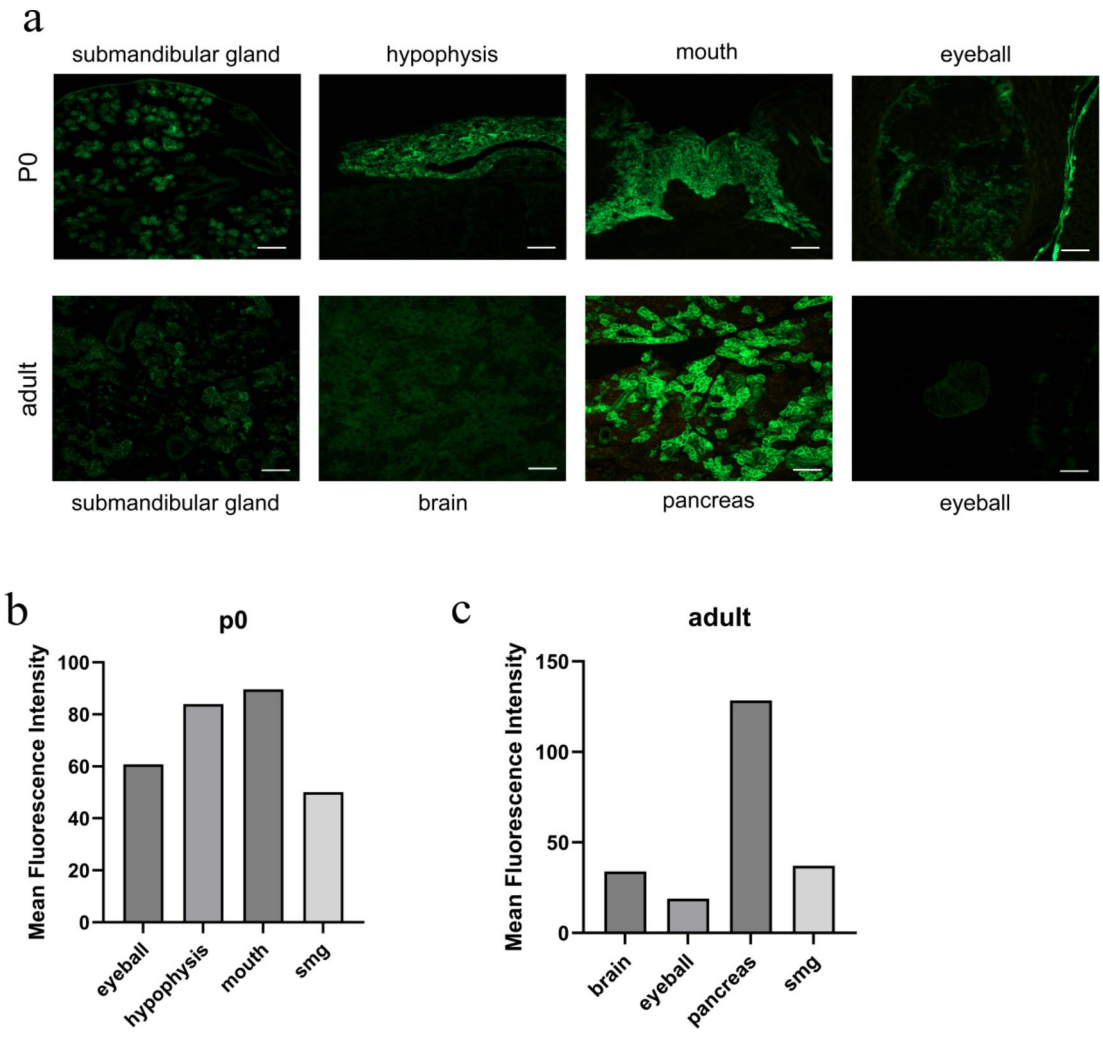


Fig. 4. (**a**) Fluorescence microscopy of tissue specimens obtained from wild-type mice. (**b**) Analysis of fluorescence intensity at expression sites in postnatal mice. (**c**) Analysis of fluorescence intensity at expression sites in adult mice (scale bar: 1 mm).

to confirm whether pathological conditions, such as high glucose stimulation, ultimately lead to an increase in insulin protein levels in the submandibular gland and to understand the associated physiological effects.

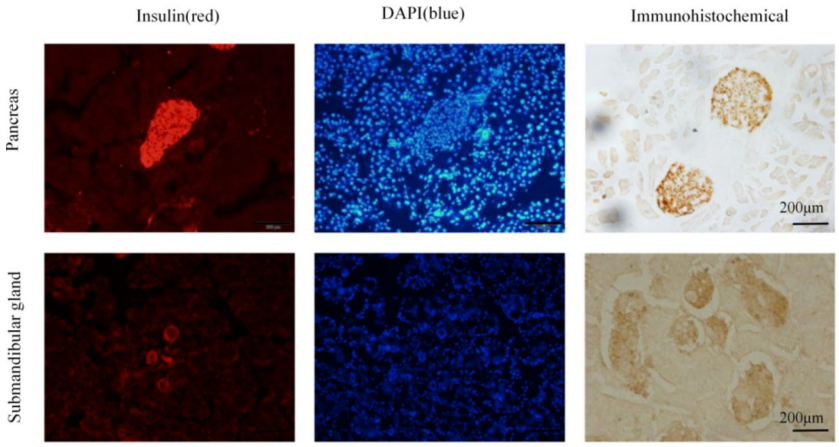


Fig. 5. Immunostaining and immunohistochemical results of the pancreas and submandibular gland (red represents insulin, blue represents DAPI). (scale bar: 200 μm).

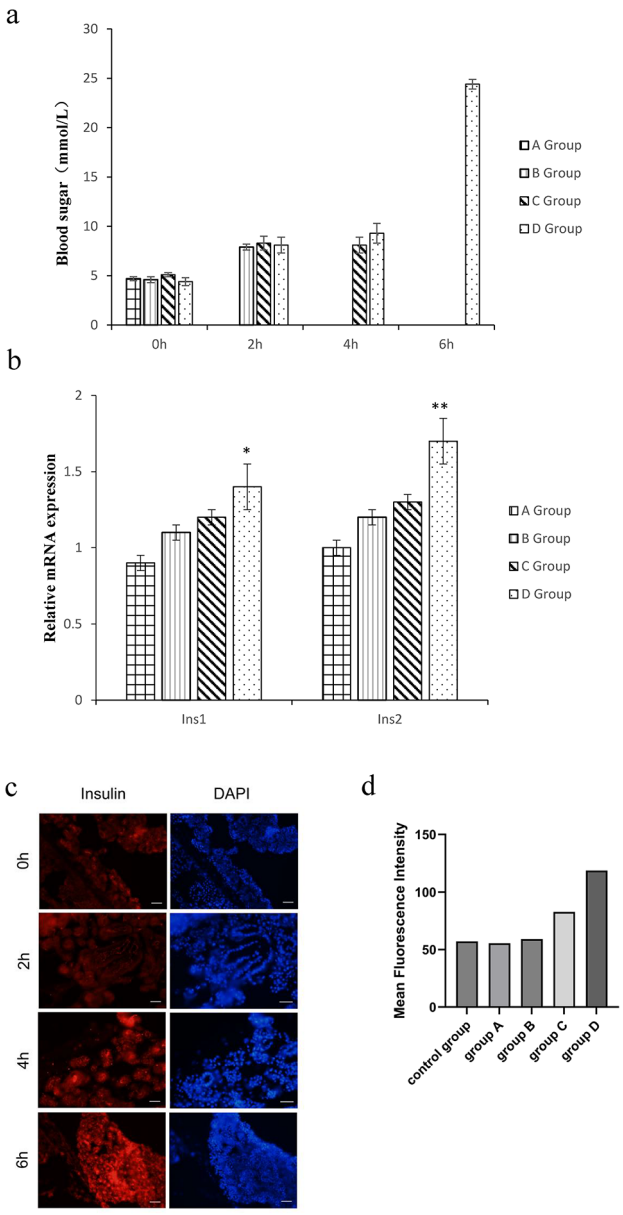


Fig. 6. Insulin secretion status under the condition of high glucose in submandibular gland. (**a**) Blood glucose levels in mice under different stress states (x ± s); (**b**) Relative mRNA expression of insulin1 [$n = 2$;group D, $^{*}P < 0.05$] and insulin2 [$n = 2$; group D, $^{**}P < 0.05$] in submandibular gland under different stress states. (**c**) Immunostaining of submandibular gland stimulated by hyperglycemia after 0, 2, 4, 6 h (red represents insulin, blue represents DAPI). (**d**) Analysis of fluorescence intensity at expression sites in submandibular gland. (scale bar: 200 μm).

Data availability

Data is provided within the manuscript or supplementary information files.

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Declarations

Competing interests
The authors declare no competing interests.

Ethics approval
The study was approved by the Ethics Committee of Stomatological Hospital Affiliated with Chongqing Medical University.

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