SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
HEALTH CARE ECONOMICS
GENETIC ENGINEERING

Received
3 June 2014

Accepted
8 September 2014

Published
30 September 2014

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Production of recombinant human proinsulin in the milk of transgenic mice

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There is a steady increasing demand for insulin worldwide. Current insulin manufacturing capacities can barely meet this increasing demand. The purpose of this study was to test the feasibility of producing human proinsulin in the milk of transgenic animals. Four lines of transgenic mice harboring a human insulin cDNA with expression driven by the goat β-casein gene promoter were generated. The expression level of human proinsulin in milk was as high as 8.1 g/L. The expression of the transgene was only detected in the mammary gland during lactation, with higher levels at mid-lactation and lower levels at early and late lactation. The blood glucose and insulin levels and the major milk compositions were unchanged, and the transgenic animals had no apparent health defects. The mature insulin derived from the milk proinsulin retained its biological activity. In conclusion, our study provides supporting evidence to explore the production of high levels of human proinsulin in the milk of dairy animals.

Diabetes is a disease characterized by high blood sugar (glucose) levels, which can lead to a number of serious complications, including heart disease, stroke, kidney failure, blindness, nerve damage and foot problem[1,2]. The number of people diagnosed with diabetes has continued to increase worldwide [366 million people had diabetes in 2011; by 2030 this number is projected to rise to 552 million according to the International Diabetes Federation[3]]. Diabetes occurs either when the pancreas does not produce enough insulin (Type I) or when the body cannot effectively use the insulin it produces (Type II)[2,4]. Insulin is used clinically to treat both Type I and Type II diabetes[5,6]. In humans *in vivo*, the single insulin gene (*INS*) is first transcribed and translated to a single chain precursor called preproinsulin [(110 amino acids (aa)] in the β-cells of the islets of Langerhans in the pancreas[7]. The signal peptide (the first 23–24 aa at the N-terminus) is removed during insertion into the endoplasmic reticulum, resulting in proinsulin (86 aa, ~9.5 kDa). Proinsulin consists of three domains: an amino-terminal B chain (30 aa, ~3.4 kDa), a carboxy-terminal A chain (21 aa, ~2.4 kDa) and a connecting C chain (34 aa, ~3.0 kDa)[7,8]. Within the endoplasmic reticulum, proinsulin is cut by neuroendocrine-cell-specific prohormone convertases (PC1 and PC2) to excise the C chain. The remaining B- and A-chains are bound together by disulfide bonds, resulting in the mature form of insulin (~5.8 kDa)[7–9].

Currently, biosynthetic human insulin is manufactured for widespread clinical use employing recombinant DNA technology[10]. In early days, A chain and B chain of human insulin are produced in separate bacterial strains, and, after separate purification, they are joined by air oxidation[11]. At present, clinical insulin and its analogs are primarily produced in yeast as the inactive precursor, proinsulin, which must then undergo enzymatic cleavage of the C chain using trypsin and carboxypeptidase B to obtain full potency[9,10,12]. However, both bacteria and yeast have inherent limitations in productivity and secretion efficiency for the production of high-volume therapeutic insulin[13]. Additionally, the International Diabetes Federation predicts that worldwide, by 2030, one in ten people will suffer from diabetes, giving rise to a large demand for insulin, which is expected to grow from US$12B in 2011 to more than US$32B by 2018[14]. Current insulin production methods are insufficient to meet this rapidly increasing demand.

The production of biopharmaceutical proteins in the mammary glands of genetically modified dairy animals ("dairy pharming") is currently under extensive exploration because it promises to provide high-quality therapeutic medicine for humans at an acceptable cost[15]. The overall objective of this study was to test the technical and health feasibility of producing human proinsulin in the milk of transgenic mice and provide a foundation for the potential scale-up of human proinsulin production in the milk of transgenic dairy animals.

Results

Generation and characterization of the transgenic mice. To generate transgenic mice expressing human proinsulin in milk, we inserted the full length of human insulin cDNA into the mammary gland-specific expression vector pBC1, in which the human insulin cDNA is controlled by the goat β-casein promoter and

flanked by the 5′ and 3′ untranslated sequences of the goat β-casein gene (Fig. 1A). The transgene was linearized from the vector and injected into the fertilized mouse eggs, which were then transferred into recipients. Thirty pups were obtained. Among them, one male (#24) and 3 female (#5, #12, and #15) transgenic founders were identified (Fig. 1B and 1C). The four transgenic founders were mated with wild type mice, and all of them transmitted the transgenes to their offspring. A total of 16 F1 transgenic mice were identified among the 36 offspring.

We used a qPCR technique to estimate the copy number of the transgene in each line of the transgenic mice[16]. The results indicated that the transgene copy numbers in the founders were different, ranging from 7 to 20. Additionally, transgene loss was observed when comparing the transgene copy numbers between the founders and their offspring, in agreement with previously published results[17–19] (Table 1).

Expression of human proinsulin in transgenic mouse tissues. To examine the stage-specific expression of human proinsulin in the mammary gland of the transgenic mice, mammary gland tissues were collected at the pre-pregnancy, pregnancy (pregnant for 16 ~ 18 d), lactation (lactating for 10 ~ 12 d), and involution (5 d after lactation) stages and analyzed for human proinsulin mRNA by qRT-PCR. As shown in Fig. 2A (upper panel), the expression of the human proinsulin transcript increased approximately 40-fold at the late pregnant stage compared to the virgin stage, reaching a peak (~100-fold) at mid-lactation, and became undetectable at the involution stage. This expression pattern is consistent with the endogenous mRNA expression profile of mouse β-casein (Fig. 2A, bottom panel), a major milk protein[20]. In addition, the developmental expression profiles of human proinsulin protein and endogenous mouse β-casein protein in the mammary glands of the transgenic mice were similar to those of their mRNAs (Fig. 2B).

To examine the mammary tissue-specific expression of human proinsulin in the transgenic mice, the kidney, spleen, lung, thymus, salivary gland, ovary, liver, blood, muscle, heart, and mammary glands were collected from the transgenic founders (#5, #12, and #15) at the lactation stage and analyzed for the expression of human proinsulin mRNA by RT-PCR. As shown in Fig. 2C, the human proinsulin transcripts were found only in the mammary gland but not in the other tested tissues of the transgenic mice, except that weak expression was observed in the blood sample of #12. In addition, no human proinsulin mRNA was detected in the mammary gland of the non-transgenic litter mates.

Expression of human proinsulin in the milk of transgenic mice. To examine the presence of human proinsulin in the milk of the transgenic mice, Western blot analysis was performed using an antibody specific for human proinsulin (Fig. 3A). As shown in Fig. 3A, milk samples from #5 (F0), #12 (F0), #15 (F0), and #5′s F1 offspring, #5_1 and #5_2, showed a strong band of human proinsulin at approximately 9.5 kDa, as observed in a commercial recombinant human proinsulin positive control (lanes 1, 2, and 3). Western blotting also showed that the concentrations of human proinsulin in the milk of #5 and its F1 offspring ranged from 1.0 to 3.0 μg/μl based on the amounts of recombinant human proinsulin applied in lanes 1 to 3. No proinsulin band was detected in the milk of the wild-type mice (lanes 7 and 11) or in the transgenic-negative animals (data not shown). The expression of human proinsulin in the transgenic milk was also verified by LC-MS/MS (data not shown).

To quantitatively measure the human proinsulin expression levels in the milk of all the transgenic founder mice and their F1 females at different lactation stages, milk samples were collected at early (3–5 d), mid- (9–11 d), and late (15–17 d) lactation stages and analyzed for human proinsulin by ELISA. As shown in Fig. 3B, the concentrations of human proinsulin in the transgenic milk samples were relatively low at early lactation in all lines but increased at mid-lactation, followed by a decrease at late lactation, except in #12, where the milk at late lactation had the highest level of human proinsulin. Table 1 lists the proinsulin concentrations (ranging from 1.4 to 8.1 μg/μl) in the milk samples of the transgenic mice at mid-lactation. The milk proinsulin concentrations of #5, #5_1, and #5_2 were 1.4, 1.2, and 2.0 μg/μl, respectively, which correlated well with their concentration range determined by the titration in Western blotting (Fig. 3A). Human proinsulin was not detectable in the milk samples from the non-transgenic littermates by ELISA. Notably, the human proinsulin concentrations in the transgenic milk were not correlated with the transgene copy number ($r = 0.39$, $P = 0.71$).

Blood metabolic profiles of the transgenic mice. To test whether human proinsulin was secreted into the blood streams of the transgenic mice, we first measured the blood levels of human insulin in the transgenic mice (3 founders and 6 offspring) at mid-lactation using an ELISA kit (RAB0327, Sigma) specific for human insulin and proinsulin. No human proinsulin or insulin was detected in the blood samples from either the transgenic or the non-transgenic animals. We also used another mouse Ultrasensitive Insulin ELISA kit (Alpco, 80-INSMSU-E10), which has 147% and 0.27% cross-reactivity to human insulin and proinsulin, respectively, in our assay. No differences in blood insulin levels were observed between the transgenic and non-transgenic mice (Fig. 4A), and the detected insulin levels were approximately 5.6 ng/ml for both the transgenic and non-transgenic mice, consistent with the normal physiological plasma insulin levels of mice in a previous study[21].

In addition, the blood glucose levels in these animals were also measured with a glucose meter. No differences in the plasma glucose levels were observed between the transgenic and non-transgenic mice, and the average glucose level was approximately 110 mg/dl for both the transgenic and non-transgenic mice (Fig. 4B).

Major milk compositions in the transgenic mice. Milk samples collected from the transgenic mice at the early, mid-, and late lactation stages were analyzed for their triacylglyceride and total protein levels. In comparison with the milk samples from the WT mice, no significant differences were observed for these compositions at any stages (Table 2).

Bioactivity of proinsulin in transgenic milk. The proinsulin in the transgenic milk was converted to mature insulin via *in vitro* enzymatic digestion with trypsin and carboxypeptidase B. The digested transgenic milk was used to treat CHO cells that over-expressed the human insulin receptor, and then the tyrosine phosphorylation of the insulin receptor in the CHO cells was measured. Commercial insulin, digested non-transgenic milk, and undigested transgenic milk were used as controls. As observed in Fig. 5, both commercial insulin and the digested transgenic milk could phosphorylate the insulin receptor, whereas the undigested transgenic milk and the digested and undigested non-transgenic milk did not show any detectable activity.

Discussion

The mammary gland-specific expression vector pBC1 was used in this study to generate the transgene construct for producing transgenic mice that expressed human proinsulin in milk. In the construct, the full-length human insulin cDNA was flanked by the 2 × β-globin insulator, the goat β-casein promoter and the untranslated exons E1 and E2 in the 5′ region, and the untranslated goat β-casein exons E7, E8, and E9 and the 3′ genomic DNA sequence in the 3′ region. β-Casein is one of the major milk proteins and accounts for approximately 28% of the total milk protein in mice[22] and 37% in goats[23]. Thus, the β-casein promoter has been widely used to drive the high level expression of foreign transgenes in the mammary gland[24]. The other untranslated sequences of the β-casein genes in

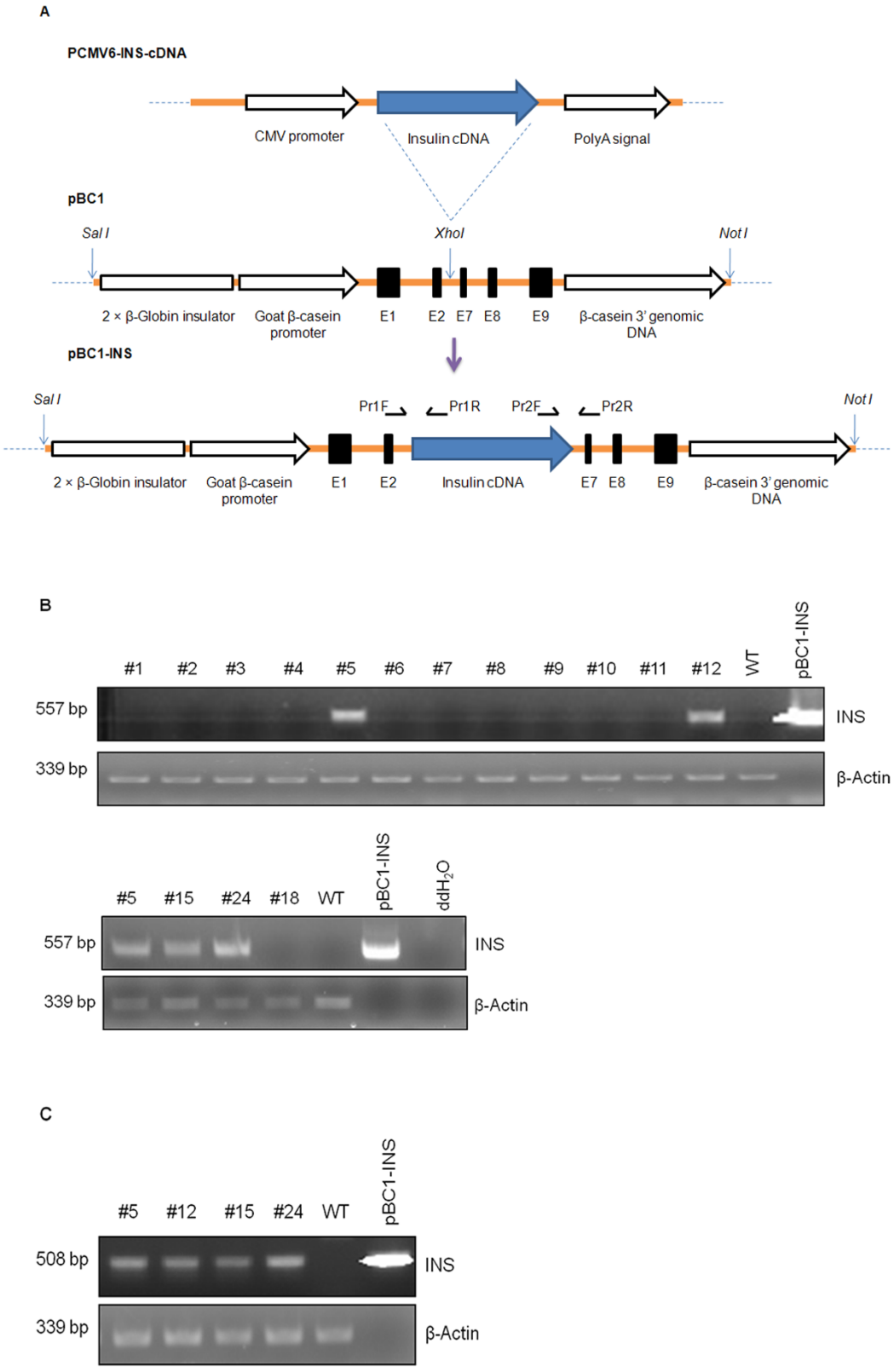

Figure 1 | Transgene construction and the identification of transgenic mice. (A) Schematic representation of the transgene construction. The full length of insulin cDNA in the pCMV6-XL5-INS-cDNA was amplified by PCR and inserted into the pBC1 vector at the *Xho* I site, generating the pBC1-INS construct. Before microinjection, the pBC1-INS construct was excised with *Sal I* and *Not I*. From left to right, the linearized pBC1-INS comprises the 2 × β-globin insulator; the goat β-casein promoter and untranslated exons E1 and E2; human insulin cDNA; untranslated goat β-casein exons E7, E8, and E9; and 3′ genomic DNA. Pr1F, Pr1R, Pr2F, and Pr2R primers were used in PCR for the identification of the transgenic mice. (B&C) Identification of the transgenic mice by PCR using the Pr1 primer pair (B) and Pr2 primer pair (C). Non-transgenic wild-type (WT) mouse DNA was used as a negative control, and the DNA used for microinjection served as a positive control. β-Actin was amplified to show the same amount of DNA used in each PCR reaction.

Table 1 | Transgene copy numbers and human proinsulin expression levels in multiple lines of transgenic mice

Line F0 (gender[a])	Offspring F1 (gender[a])	Transgene copy number	Expression level in milk[b] (μg/μl)
5 (F)		13	1.4
	5_1 (F)	7	1.2
	5_2 (F)	6	2.0
12 (F)		20	7.8
	12_1 (F)	10	7.2
	12_2 (F)	6	8.1
15 (F)		7	4.1
	15_1 (F)	5	2.1
	15_2 (F)	3	1.6
24 (M)		9	NA
	24_1 (F)	5	5.7

[a]F = female, M = male.
[b]Milk samples were collected at mid-lactation, and the concentration of human proinsulin was quantified by ELISA.

the construct are considered to enhance the stability of the mRNA of the transgene in the mammary gland. The effectiveness of this vector and the goat gene sequences in mouse were confirmed in our study by the high levels of human proinsulin in the milk of all transgenic mouse lines. In addition, our study also confirmed the mammary-specific and lactation stage-specific expression of the transgene. The expression of human proinsulin followed the profile of endogenous β-casein in the transgenic mice. Although our RT-PCR results indicated that there was a weak band for the human proinsulin transcript expressed in the blood sample of one of the transgenic mice (#12, Fig. 2C), possibly resulting from a classical position effect or contamination of the sample, we could not detect any human proinsulin protein in the blood of all tested animals by ELISA. It is important to note that our sequencing analysis discovered that the goat β-casein promoter sequence in pBC1 misses 31 bp of the block B sequence of the β-casein proximal promoter (data not shown). This block B sequence has been well-identified to contain the binding sites for the signal transducer and activator of transcription 5 (STAT5) and for the glucocorticoid receptor; it is also essential for the induction of β-casein proximal promoter activity by the lactogenic hormones prolactin and glucocorticoids in *in vitro* analyses[25,26]. Our study indicated that the block B sequence may not be as important in a genomic context as in *in vitro* analysis of the proximal promoter.

Transgene loss during animal passage is a common phenomenon[17–19]. In this study, only approximately half of the transgene copies were transmitted from the transgenic founders to their offspring. Transgenes are exogenous fragments of DNA that are introduced into the genome at random sites, and they are usually concatamerized into a tandem array[17]. Due to a lack of balancing transgenes on the paired chromosome during meiosis, transgenes may become unstable and rearrange, perhaps gradually, causing copy number loss from generation to generation[17–19].

Several lines of evidence indicated that the form of insulin in the transgenic milk was proinsulin rather than mature insulin. First, the proinsulin detected in the milk by Western blotting showed the same size as the commercial proinsulin product (Fig. 3A). Second, no insulin bioactivity was detected in the transgenic milk without enzymatic digestion, even though the concentration of insulin product in the transgenic milk was high, whereas following endopeptidase digestion of the transgenic milk, insulin bioactivity was detected (Fig. 5). These results indicated that the mammary gland could not process the maturation of insulin during the secretion process as the pancreas does. The mammary gland may not express PC1 and PC2, the enzymes required to release peptide C from proinsulin. However, the mammary gland can recognize the insulin signal peptide and secrete the proinsulin protein into milk.

The expression levels of recombinant human proinsulin in the transgenic milk were shown to range from 1.2 to 8.1 μg/μl (Table 1). The expression levels determined by ELISA were in agreement with those titrated by Western blotting. However, the expression levels were not correlated with the transgene copy numbers, suggesting that the expression was still position dependent, although the pBC1 vector was intentionally designed to minimize the position effect of the transgene by incorporating a segment of insulating sequences, namely, the chicken β-globin insulator (Fig. 1A)[27].

The global insulin market is expected to triple by 2018, and current insulin production practices will face rising difficulty in meeting this rapidly rising demand[14]. Currently, almost all clinical insulin is the recombinant protein or "analog" produced in yeast. The secretion level of recombinant proteins in yeast is still at the magnitude of mg/L (approximately 80 mg/L for human proinsulin)[14,18]. To meet the increasing demand for human insulin, our study confirmed that it is feasible to produce high amounts of human proinsulin in the milk of transgenic animals, an emerging biotechnology called "dairy pharming". In this study, the concentrations of human proinsulin in transgenic milk samples ranged from 1.2 to 8.1 μg/μl. Even if the lowest level of human proinsulin (1.2 μg/μl) were expressed in the milk of transgenic goats or cows, the average production by goat (3 kg milk/day) or cow (40 kg milk/day) would produce 3 g or 48 g of proinsulin per day or 0.9 and 13 kg per 9 month lactation, respectively. This rate of production would provide an unlimited human insulin supply to treat diabetes using a limited number of animals.

No health side effects were observed in the transgenic mice. No human proinsulin was detected in the blood of these animals, and the animals had normal blood insulin and glucose levels. In addition, the total levels of milk protein and triacylglycerol were unchanged in these animals.

In conclusion, we successfully generated transgenic mice expressing high levels of human proinsulin in their milk. Our results suggest that the gene construct used in this study is expected to allow a production of insulin at an industrial scale from dairy animals, such as dairy goats and cows.

Methods

Generation of transgenic mice that express human proinsulin in milk. This study was approved by the University of Vermont Animal Care and Use Committee, and all of the animal work and handling was carried out in accordance with institutional polices and federal guidelines. Full-length human INS cDNA was amplified by PCR from the plasmid PCMV6-XL5-INS-cDNA (Origene, Rockville, MD) using the primers pCMV-INS-F and pCMV-INS-R (Table 3). The PCR product (495 bp) was cloned into a commercial mammary gland expression vector (pBC1, Invitrogen, Grand Island, NY) at the Xho I site to generate the pBC1-INS plasmid via blunt cloning using the Quick Blunting and Quick Ligation Kit (New England Biolabs, Ipswich, MA) (Fig. 1A). The pBC1-INS plasmid was digested with the restriction enzymes *Not I* and *Sal I* to release a 16.3 kb linear DNA fragment that contained human insulin cDNA flanked by the goat β-casein gene promoter and 3′ and 5′ untranslated sequences (Fig. 1A). The released transgene DNA fragment was then purified by agarose gel electrophoresis and electro-elution before being microinjected into the pronuclei of fertilized C57BL/6 oocytes to generate transgenic mice at the Transgenic Mouse Facility of the University of Vermont following standard procedures. The mice were maintained on a C57BL/6 background.

Screening of transgenic animals by PCR. Genomic DNA was isolated from the tail tips of 2- to 3-week-old mice. The identification of transgenic mice carrying the pBC1-INS transgene construct was carried out by PCR with two pairs of primers: Pr1F, Pr1R, Pr2F, and Pr2R (Table 3). Primers Pr1F and Pr1R were complementary to the 5′-flanking sequence of the goat β-casein gene and to the human INS cDNA, respectively, whereas Pr2F and Pr2R were complementary to the human INS cDNA and the 3′-flanking sequence of the goat β-casein gene, respectively (Fig. 1A). To verify that the same amount of genomic DNA was used in each PCR reaction, mouse β-actin genome DNA was also amplified with mβActin-F1 and mβActin-F1 (Table 3). The PCR conditions were as follows: an initial denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 68°C for 1 min. The final PCR products were visualized by electrophoresis in a 3% agarose gel in Tris-acetate-EDTA (TAE, 40 mM Tris, 20 mM acetate, and 1 mM EDTA, pH 7.6) buffer.

Evaluation of the transgene copy number by real-time PCR (qPCR). The transgene copy number in the transgenic mice was determined by qPCR as described previously[16] with primers designed for a single copy control gene (lymphotoxin B

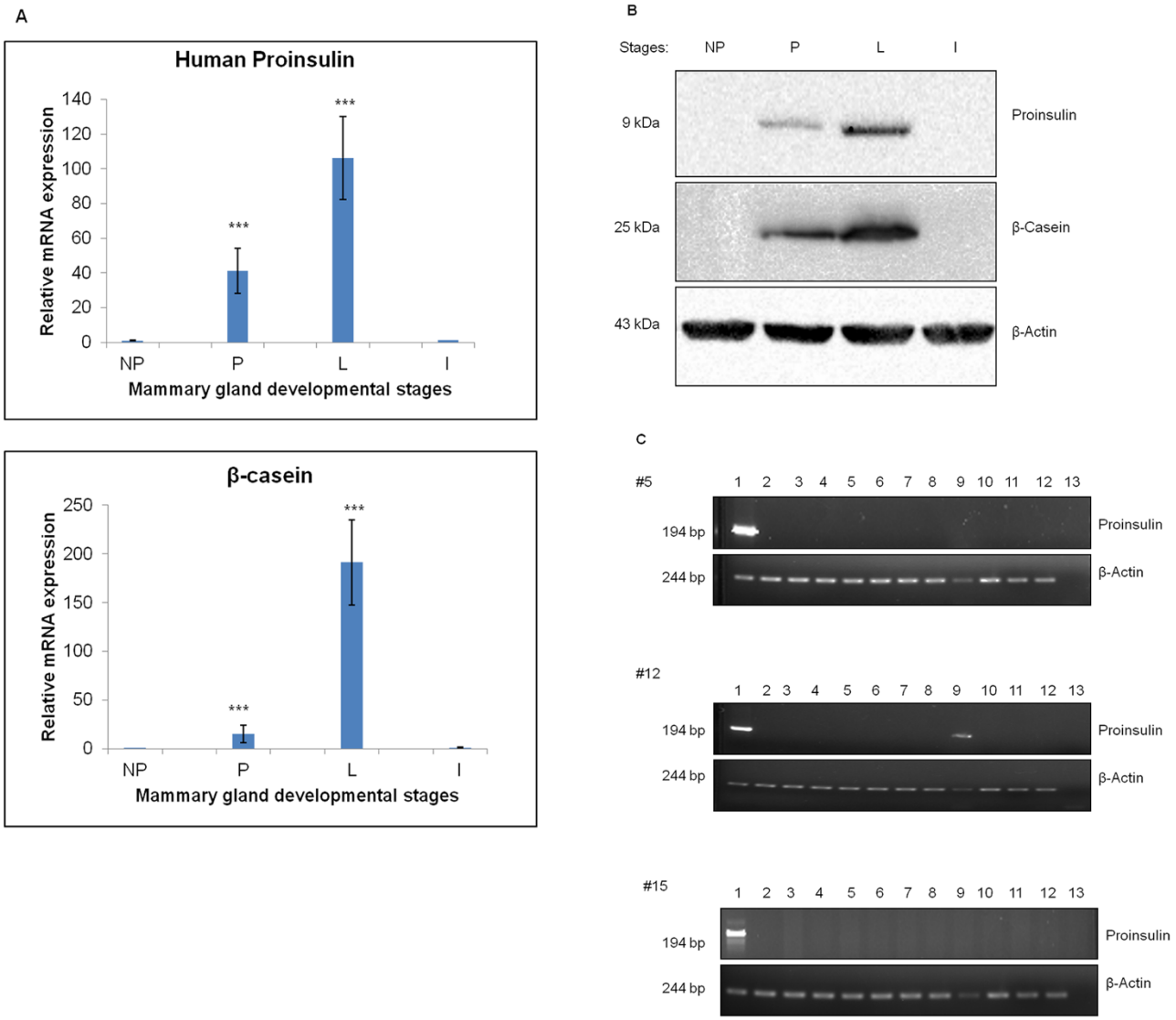

Figure 2 | Expression of the human insulin transgene in transgenic mouse tissues. (A) qRT-PCR analysis of human proinsulin transcripts in the mammary gland tissues of transgenic F1 mice at the virgin (NP), pregnancy (P), lactation (L), and involution (I) stages (upper panel). β-Actin was used as an internal control. Three mice in each stage were included for the analysis. The data are expressed as the mean ± SE. ***, $P < 0.001$ when compared to the NP group. The endogenous β-casein gene expression was measured as a positive control (bottom panel). (B) Western blot analysis of the protein expression of human proinsulin and endogenous β-casein in mammary gland tissues at the different developmental stages depicted in (A). The same amount of protein was applied in each lane, and each lane used the mammary tissue sample pooled from three transgenic mice at the same stage. β-Actin was used as a loading control. (C) RT-PCR analysis of human proinsulin transcripts in various tissues of transgenic females #5, #12, and #15 at the mid-lactation stage. 1, mammary gland; 2, kidney, 3, spleen; 4, lung; 5, thymus; 6, salivary gland; 7, ovary; 8; liver, 9, blood; 10, muscle; 11, heart; 12, mammary gland from non-transgenic mice; 13, ddH_20. β-Actin was used as a loading control.

gene: Ltb) and the pBC1-INS transgene. The primer sequences for Ltb have been described previously[16], and the primer sequences for the pBC1-INS were pBC1-INS-F and pBC1-INS-R (Table 3). The PCR products for Ltb were linked to those of pBC1-INS, and the resulting Ltb-pBC1-INS DNA fragment was cloned into the pCR-Blunt II-TOPO vector (Invitrogen) and transformed to TOP10 Competent Cells (Invitrogen). Subsequently, the Ltb-pBC1-INS plasmid was isolated and used as a calibration sample with a known pBC1-INS/Ltb ratio (1 : 1). qPCR assays were performed on a CFX96 Real-Time PCR system (BioRad, CA) using a 20 μl reaction mixture containing 10 μl SsoFast EvaGreen supermix (Bio-Rad, Hercules, CA), 500 nM forward and reverse primers, and 20 ng of genomic DNA. The PCR cycling conditions were as follows: one cycle at 98°C for 2 min and 40 cycles of 98°C for 5 s and 65°C for 5 s. Using the $2^{-\Delta\Delta Ct}$ method[28], the relative copy number of the transgene was determined with respect to the calibration sample.

Blood glucose level measurement. Blood was obtained by nicking the lateral tail vein using a sterile scalpel blade and immediately measured with a FreeStyle Lite Blood Glucose Monitoring System (FreeStyle, Alameda, CA). The measurements were carried out at 9 to 10 am, and no pre-fasting was performed.

Western blotting. Mammary gland tissues were collected from transgenic mice at pre-pregnancy, pregnancy (pregnant for 16 ~ 18 d), mid-lactation (lactating for 10 ~ 12 d), and involution (5 d after lactation) time points. The total protein was extracted from mammary gland tissue that had been homogenized in NP40 lysis buffer [50 mM Tris (pH 7.4), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1% Nonidet P40, proteinase inhibitor cocktail (Sigma, St. Louis, MO), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] using a Dounce homogenizer. The homogenate was then vigorously rocked at 4°C for 30 min, followed by a 10 min centrifugation at 4°C. The supernatant was saved, and the protein concentrations were determined using the Microplate BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). The protein extracts were boiled for 5 min in 1 × Laemmli sample buffer [62.5 mM Tris-Cl (pH 6.8), 2.5% SDS, 0.002% bromophenol blue, 10% glycerol, and 710 mM β-mercaptoethanol]. Equal amounts of protein from each developmental stage were then analyzed via Western blotting with specific antibodies against human proinsulin (Abcam, Cambridge, MA), as described previously[29].

Milk and blood sample collection and ELISA. Milk samples from the transgenic mice and non-transgenic mice were collected during early (3–5 d), middle (9–11 d),

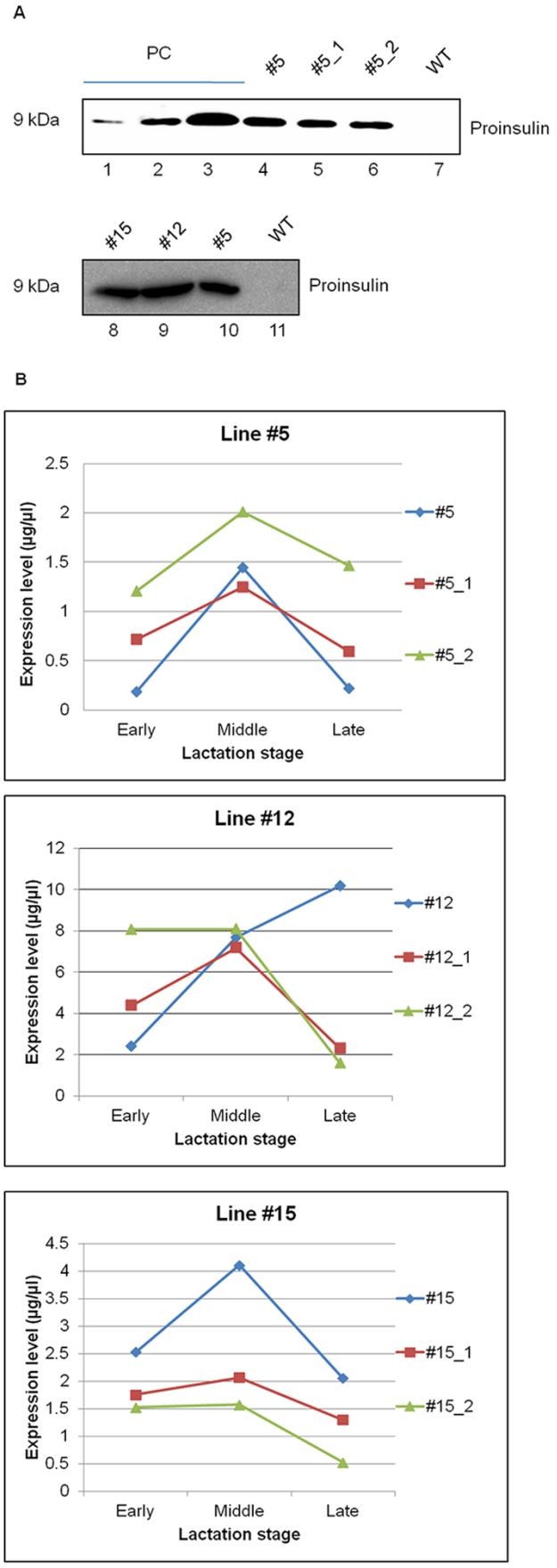

Figure 3 | Detection of human proinsulin in the milk of transgenic mice. (A) Western blot analysis of human proinsulin in milk samples from transgenic mice. Commercial recombinant human proinsulin at concentrations of 0.5, 1.0, and 3 μg/μl (PC, lanes 1–3, respectively) was used as a positive control. Lanes 4, 5, and 6 are milk samples from transgenic founder #5 and its F1 offspring, #5_1 and #5_2, respectively. Lanes 8 to 10 are the milk of #15, #12, and #5, respectively. Milk from wild-type (WT) mouse (lanes 7 & 11) was used as a negative control. The same volume of milk samples was loaded in each well. (B) Expression profiles of human proinsulin in the milk of transgenic mice throughout lactation. Milk samples from three transgenic lines were collected at early, mid-, and late lactation and measured for human proinsulin concentrations by ELISA. The presence of proinsulin was barely detectable in the corresponding non-transgenic littermates (negative controls).

and late (15–17 d) lactation using a Medela Freestyle pump (McHenry, IL). Just prior to milking, 5 I.U. of oxytocin was injected intraperitoneally to the mice. The milk samples were defatted by centrifugation at 4°C for 15 min at 10,000 *g*. The resulting skim milk was diluted one million fold, and the human proinsulin concentrations were then determined with a Human Insulin ELISA Kit (RAB0327) from Sigma according to the manufacturer's instructions. Tail blood samples (40 μl) were drained into heparinized microhematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA), transferred to centrifuge tubes, and centrifuged at 4°C and 2,000 *g* for 10 min. The resulting supernatant (plasma) was saved and used for ELISA analysis. Two different ELISA kits were used: 1) a Human Insulin ELISA Kit (RAB0327, Sigma), which is specifically used to measure human insulin and proinsulin; and 2) a mouse Ultrasensitive Insulin ELISA kit (80-INSMSU-E10, Alpco, Salem, NH), which has 147% and 0.27% cross-reactivity against human insulin and proinsulin, respectively.

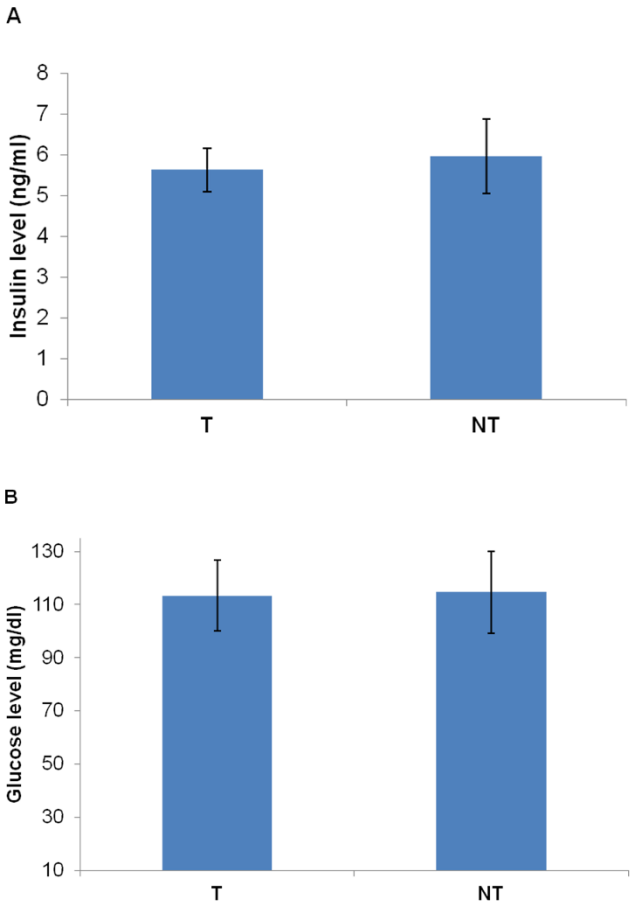

Figure 4 | Blood insulin and glucose levels in transgenic mice at mid-lactation. (A) Blood insulin concentrations in transgenic (T) and non-transgenic (NT) mice at mid-lactation as analyzed by ELISA. (B) Blood glucose concentrations in transgenic (T) and non-transgenic (NT) mice at mid-lactation. Nine transgenic-positive mice and nine transgenic-negative littermates in 3 transgenic lines were analyzed. The data are expressed as mean ± SE.

Table 2 | Total protein and triacylglyceride levels of milk from transgenic and wild-type mice during different lactation stages

		Transgenic (n = 6)	Wild-type (n = 6)	P value (*t* test)
Protein (g/L)	Early lactation	102.5 ± 5.1	100.8 ± 6.4	0.541
	Mid-lactation	109.0 ± 8.4	106.9 ± 8.9	0.636
	Late Lactation	99.2 ± 4.5	97.5 ± 7.6	0.483
Triacylglycerides (g/L)	Early lactation	335.7 ± 39.5	345.8 ± 42.1	0.604
	Mid-lactation	320.6 ± 33.1	309.1 ± 30.0	0.538
	Late Lactation	301.9 ± 30.9	290.8 ± 23.1	0.371

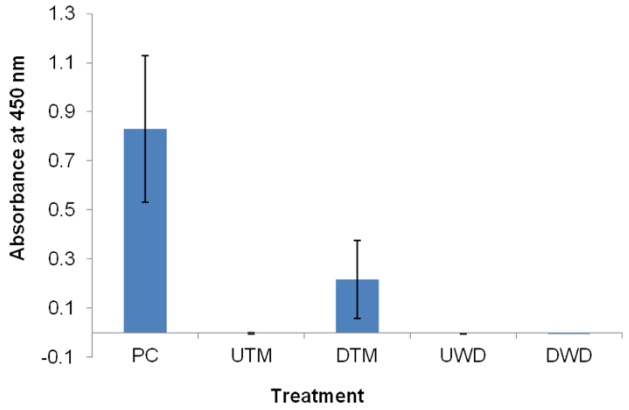

Figure 5 | Insulin receptor autophosphorylation in CHO cells treated with enzymatically digested transgenic milk. The proinsulin (100 μg) in transgenic milk was converted to mature insulin via in vitro enzymatic digestion with trypsin and carboxypeptidase B (DTM) and then used to treat CHO cells that over-expressed the human insulin receptor. After 1 h of treatment, the cells were lysed, and 20 μg of protein lysate was used in ELISA assays for tyrosine phosphorylation. Commercial insulin (PC), undigested transgenic milk (UTM), and digested (DWD) and undigested (UWD) non-transgenic milk were used as controls. Three experiments were repeated, and representative results are shown here. In the first two experiments, the transgenic milk samples from #5 (F0) and its offspring, #5_1(F1) and #5_2 (F1), were combined to obtain 100 μg of proinsulin. In the third experiment, the transgenic milk samples from #12 (F0) and its offspring, #12_1 (F1), were combined.

RT-PCR and qRT-PCR. Total RNA was isolated from various tissues (mammary gland, kidney, spleen, lung, thymus, salivary gland, ovary, liver, blood, muscle, and heart) of the transgenic and non-transgenic mice using Trizol reagent (Invitrogen) and digested by RNase-free DNase I (Invitrogen). A total of 5 μg of each DNase I-treated RNA sample was used to synthesize the first-strand cDNA using the Reverse SuperScript II reverse transcriptase (Invitrogen) and oligo (dT) primer per the manufacturer's protocol. The primers for amplification of the human proinsulin transcript were ProINS-F and ProINS-R (Table 3). The primers for mouse β-actin were mβActin-F2 and mβActin-R2 (Table 3). An initial reaction of 5 min at 95°C was followed by 32 cycles (28 cycles for β-actin) of 30 s denaturation at 95°C, 30 s of annealing at 62°C, and 1 min of extension at 68°C. A total of 10 μl of each PCR reaction was resolved on 1% agarose gels via electrophoresis. For qRT-PCR, the reactions were performed in duplicate in a 20-μl volume containing 10 μl of SsoFast EvaGreen Supermix (Bio-Rad), 500 nM forward and reverse primers (1 μl each), and 8 μl of diluted cDNA (corresponding to 25 ng of reverse-transcribed total RNA). The relative expression of the proinsulin gene was normalized to β-actin and calculated by the $2^{-\Delta\Delta Ct}$ method[29].

Milk composition analyses. The milk samples were diluted 5-fold with distilled water. The milk triacylglyceride concentration was measured by a Colorimetric Assay Kit provided by Cayman Chemical (Ann Arbor, MI), and the milk protein concentration was measured using a BCA Protein Assay Kit provided by Pierce (Rockford, IL).

Liquid chromatography tandem mass spectrometry (LC-MS/MS). The defatted milk proteins were electrophoretically separated using a 20% SDS-PAGE gel, and then stained with coomassie blue (Pierce, Rockford, IL). The band with size of 10 kDa was cut and sent to the Proteomics Facility at University of Vermont, where the gel pieces were digested and the extracted peptides were subjected to LC-MS/MS using a Linear Ion Trap (LTQ)-orbitrap Hybrid Mass Spectrometer (Thermo Electron, San Jose, CA).

Conversion of proinsulin to mature insulin. The conversion of the proinsulin in the transgenic milk to insulin was carried out by proteolysis using trypsin (Roche, Indianapolis, IN) and carboxypeptidase B (Roche), as described previously[30]. Specifically, wild-type (WT) milk and transgenic milk were digested with trypsin and carboxypeptidase B with proinsulin to enzyme ratios of 300 : 1 (w/w) and 600 : 1 (w/w), respectively. The amount of transgenic milk added to the reaction was calculated based on the proinsulin concentration determined by ELISA, and WT milk containing the same amount of protein as the transgenic milk was used. The digestion was carried out in a buffer (pH 7.5) with 0.1 M Tris-HCl and 1 mM $MgCl_2$ at 37°C for 1 h.

Assay of insulin receptor autophosphorylation. Assays for the autocatalytic activity of the insulin receptor were performed as described[31,32]. Chinese hamster ovary (CHO) cells were maintained in Ham's F-12 medium supplemented with 10% fetal bovine serum plus 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 25 μg/ml of Fungizone. Before transfection, CHO cells were seeded at 0.1×10^6 cells/well in 12-well plates and grown in a humidified incubator at 37°C and 5% CO_2 overnight to 70–80% confluence. Then, the cells were transfected with 1.25 μg/well of human insulin receptor expression plasmid (#24049, Addgene, Cambridge MA) using Lipofectamine (Invitrogen) according to the manufacturer's instructions. After transfection, the cells were made quiescent by serum starvation for 12 h, then treated with 1 μM commercial insulin (Sigma), with digested and undigested transgenic milk (with equivalent insulin concentration as the commercial insulin used), as well as with digested and undigested WT milk samples (with the same protein concentration as the transgenic milk used) for 1 h. The CHO cells were then lysed in 50 mM Tris (pH 7.4) containing 130 mM NaCl, 5 mM EDTA, 1.0% Nonidet P-40, 1 × proteinase inhibitor cocktail (Sigma), 100 mM NaF, 50 mM β-glycerophosphate, and 100 μM Na_3VO_4. Equal amounts of cell lysates (20 μg of protein) were applied to 96-well ELISA and analyzed for tyrosine phosphorylation of the insulin receptor using the Phospho-IR ELISA Kit (Millipore, Billerica, MA).

Statistical analysis. All statistical analyses were carried out using JMP statistical software (SAS, Cary, NC). The comparisons between two groups were performed

Table 3 | Sequences of primers used in this study

Forward	Sequence (5′-3′)	Reverse	Sequence (5′-3′)
pCMV-INS-F	GGCCGCGAATTCGGCCATT	pCMV-INS-R	TTGTTGGTTCAAGGGCTTTATTC
mβActin-F1	TAGACTTCGAGCAGGAGATG	mβActin-R1	CCACCAGACAGCACTGTGTT
Pr1F	ACCAGGGATCAAACCTGCAC	Pr1R	ACGCTTCTGCAGGGACCCCT
Pr2F	TTGTGAACCAACACCTGTGC	Pr2R	TGCTGAGAATCATTAATCTCAGC
ProINS-F	CAACACCTGTGCGGCTCACA	ProINS-R	CACAATGCCACGCTTCTGCA
mβActin-F2	TAGACTTCGAGCAGGAGATG	mβActin-R2	CCACCAGACAGCACTGTGTT
mCSN2-F	AGAGGATGTGCTCCAGGCTA	mCSN2-R	TAAGGAGGGGCATCTGTTTG
pBC1-INS-F	CAGGAATCGCGGATCCTC	pBC1-INS-R	CCATGGCAGAAGGACAGTGAT

using the *t*-test, and the comparisons between more than two groups were analyzed with one-way ANOVA followed by post hoc Dunnett's multiple comparison test.

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Acknowledgments

We are grateful to Dr. Mercedes Rincon and Mr. John Dodge at the University of Vermont Transgenic Facility for their service in producing the transgenic mice. We thank the staff of the Small Animal Facility of the University of Vermont for the excellent animal care. We also thank Dr. Frederick Stanley for providing the human insulin receptor plasmid. This work was supported by a Hatch grant (VT-H01508) from the USDA National Institute of Food and Agriculture (to F.Q.Z.).

Author contributions

F.-Q.Z. planned, designed, and oversaw the whole project. X.Q. designed and performed the experiments, wrote the main manuscript text, and prepared the figures. Y.N. and J.K. performed experiments. All the authors discussed the results and reviewed the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Qian, X., Kraft, J., Ni, Y. & Zhao, F.-Q. Production of recombinant human proinsulin in the milk of transgenic mice. *Sci. Rep.* **4**, 6465; DOI:10.1038/srep06465 (2014).