Diphtheria Toxin- and GFP-Based Mouse Models of Acquired Hypoparathyroidism and Treatment With a Long-Acting Parathyroid Hormone Analog

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ABSTRACT

Hypoparathyroidism (HP) arises most commonly from parathyroid (PT) gland damage associated with neck surgery, and is typically treated with oral calcium and active vitamin D. Such treatment effectively increases levels of serum calcium (sCa), but also brings risk of hypercalciumia and renal damage. There is thus considerable interest in using PTH or PTH analogs to treat HP. To facilitate study of this disease and the assessment of new treatment options, we developed two mouse models of acquired HP, and used them to assess efficacy of PTH(1–34) as well as a long-acting PTH analog (LA-PTH) in regulating blood calcium levels. In one model, we used PTHcre-iDTR mice in which the diphtheria toxin (DT) receptor (DTR) is selectively expressed in PT glands, such that systemic DT administration selectively ablates parathyroid cells. For the second model, we generated GFP-PT mice in which green fluorescent protein (GFP) is selectively expressed in PT cells, such that parathyroidectomy (PTX) is facilitated by green fluorescence of the PT glands. In the PTHcre-iDTR mice, DT injection (2 × 5 μg/kg, i.p.) resulted in moderate yet consistent reductions in serum PTH and sCa levels. The more severe hypoparathyroid phenotype was observed in GFP-PT mice following GFP-guided PTX surgery. In each model, a single subcutaneous injection of LA-PTH increased sCa levels more effectively and for a longer duration (>24 hours) than did a 10-fold higher dose of PTH(1–34), without causing excessive urinary calcium excretion. These new mouse models thus faithfully replicate two degrees of acquired HP, moderate and severe, and may be useful for assessing potential new modes of therapy. © 2015 American Society for Bone and Mineral Research.

KEY WORDS: ANIMAL MODELS; THERAPEUTICS; DISORDERS OF CALCIUM/PHOSPHATE METABOLISM; CELL/TISSUE SIGNALING-ENDOCRINE PATHWAYS

Introduction

Hypoparathyroidism (HP) is characterized by inadequate or absent secretion of parathyroid hormone (PTH). Lack of PTH leads to hypocalcemia, hyperphosphatemia, and increased renal calcium excretion.[1] In addition, bone turnover is low and bone microarchitecture is abnormal.[2] HP is most often acquired by inadvertent damage or removal of the parathyroid glands during neck surgery.[3]

Conventional therapy for HP consists of oral supplementation of calcium and active vitamin D analogues. Such therapy, however, often fails to keep serum calcium stably within the target range, and can worsen hypercalciumia. Balancing the risk of hypocalcemia with the risk of hypercalciumia is thus a challenging, but important goal for HP therapy.[3] Daily subcutaneous injection of recombinant human (rh) PTH(1–84) has recently been approved by the U.S. Food and Drug Administration (FDA) as adjunct therapy to calcium and vitamin D in patients with HP.[4] Although the approval of rhPTH(1–84) is a major milestone in the development of treatments for the disease, clinical trials did not show lower levels of urinary calcium excretion in the rhPTH(1–84)-treated patients, as compared to placebo-treated controls.[5–7] Novel long-acting PTH analogs and novel modes of delivery of PTH are thus being investigated as potential treatment options for HP.[8–12]

A need to fully understand the mechanisms and consequences of various lines of therapy for HP underlies a demand for more reliable and more accessible animal models of acquired HP. Potential animal models include genetically modified mice carrying either the PTH-null[13] or GCM2-null[14] alleles; however,
Unlike in acquired HP, the most common form of the disease, the HP phenotype in these mice is inherited, and the development of key organs such as the skeleton may be distinctly altered. Furthermore, in patients, HP typically develops acutely, whereas in the available genetic mouse models, the phenotype is chronic. Moreover, some of the mouse models show early lethality and reduced fertility,\textsuperscript{13–15} which limits their use. More widely used are animal models generated by surgical parathyroidectomy (PTX), which is most typically performed in rats,\textsuperscript{16,17} but also in larger mammals.\textsuperscript{18–20} Although the mouse is the most widely used laboratory test animal, it is not generally amenable to PTX surgery, because of the small size of the parathyroid glands and their variable anatomic distribution. As an alternative, thyroparathyroidectomy (TPTX) can be performed in the mouse, but this approach brings the need for thyroid hormone supplementation and the loss of calcitonin made by thyroid glands as added variables.\textsuperscript{21}

To overcome these problems, we established two new mouse models of acquired HP. We thus developed the PTHcre-iDTR mouse, in which diphteria toxin (DT) is used to selectively ablate parathyroid cells, and we also developed the GFP-PTX mouse, in which diphtheria toxin (DT) is used to selectively ablate parathyroid cells and their variable anatomic distribution. As an alternative, thyroparathyroidectomy (TPTX) can be performed in the mouse, but this approach brings the need for thyroid hormone supplementation and the loss of calcitonin made by thyroid glands as added variables.\textsuperscript{21}

To overcome these problems, we established two new mouse models of acquired HP. We thus developed the PTHcre-iDTR mouse, in which diphteria toxin (DT) is used to selectively ablate parathyroid cells, and we also developed the GFP-PTX mouse, in which the parathyroid glands have been surgically removed, a process greatly facilitated by green fluorescence of the glands. We characterize the baseline calcium levels in these two mouse models and use them to assess the efficacy of a long-acting (LA) PTH analog, in comparison to PTH(1–34), as a potential treatment for HP.

**Materials and Methods**

**Experimental animals**

The following mice (catalog numbers are from Jackson Laboratory, Bar Harbor, ME, USA) were obtained: ROSA\textsuperscript{mT/mG} mice (007676),\textsuperscript{22} PTH-Cre mice (005989),\textsuperscript{23} and Cre-inducible DTR transgenic mice (iDTR) mice (007900).\textsuperscript{24} Genotyping was performed on genomic DNA isolated from tails, using previously published protocols. This study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Massachusetts General Hospital (MGH). Male and female mice were used for experiments. Mice were housed in the Center for Comparative Medicine at MGH, at 23°C with 40% humidity in a 12-hour light/dark cycle with free access to water and irradiated diet. Wood bedding and igloo covers were provided for environmental enrichment. Mice were fed a standard chow diet, which contained 1.11% calcium, 0.48% phosphate, and 2.5 IU/g vitamin D; or a low calcium diet (Teklad Diets, Madison, WI, USA) containing 0.005% Ca, 0.6% phosphate, and 0 IU/g vitamin D\textsubscript{3}, as indicated.

**Genome walking to determine the PTH-Cre integration site**

The integration site of the PTH-Cre transgene was determined using the GenomeWalker Universal Kit (Clontech Laboratories, Mountain View, CA, USA). In short, genomic DNA was isolated, digested with the restriction enzymes EcoRV, Dral, or PvuII, and the fragments were purified by phenol-chloroform extraction. The GenomeWalker adaptors were then ligated to the digested fragments. PCR amplification was preformed using a primer to the adaptor sequence and a novel primer, which we designed to the cre portion of the PTH-Cre transgene. A secondary PCR was then performed with nested primers. This yielded a single PCR product that was sequenced and mapped to the mouse genome. With this information, PCR primer pairs were designed to distinguish between mice heterozygous and homozygous for the PTH-Cre transgene. DNA from mice, which were known heterozygous and homozygous for the PTH-Cre transgene by progeny testing, was used to verify the utility of these primers. Based on the integration site we designed PCR primers (forward primer A, CCTGTCAGAGTGGTGAAGA, reverse primer A', TCAGATCAACACACACAGCA, forward primer B, CAGTTGTCTTTAGTTTACTCACTCATC, reverse primer B', GATAATCCGGAACATCCTCGATT) that gave the expected single transgene band in mice homozygous for the PTH-Cre transgene, a single wild-type band in wild-type littermates, and two bands, one wild-type and one transgenic, in mice heterozygous for the PTH-Cre transgene (Supplemental Fig. 2). Mice homozygous for the PTH-Cre transgene were used for subsequent breeding with either homozygous iDTR or homozygous ROSA\textsuperscript{mT/mG} mice. For some DTR experiments, homozygous PTH-Cre-iDTR mice were crossed with homozygous PTH-Cre-mTmG mice to generate PTH-Cre-iDTR-mTmG mice.

**DT injection**

DT (Sigma-Aldrich Corporation, St. Louis, MO, USA) was diluted to 0.1 to 10 μg/mL with saline and injected intraperitoneally (i.p.) in 6-week-old to 7-week-old PTHcre-iDTR mice at 10 mL/kg body weight (final concentration = 1 to 100 μg/kg body weight). For control, the same volume of saline was injected i.p. For repetitive DT injection experiments, the same dose was injected every third day up to five times as indicated. Measurements of serum PTH and ionized calcium (Ca\textsuperscript{2+}) were performed at baseline, and 3 days after the last injection.

**GFP-guided parathyroidectomy**

In the anesthetized and surgically prepared animal, a ventral midline incision was made. Under stereomicroscope and UV illumination, GFP-expressing parathyroid glands were visualized and removed in their entirety using fine surgical forceps and scissors, and the incisions were closed with sutures (ie, GFP-guided parathyroidectomy [GFP-PTX]).

**Biochemical analyses**

For measurements of Ca\textsuperscript{2+}, blood obtained from the tail vein was collected in a capillary tube, and immediately processed using the RapidLab 348 Ca\textsuperscript{2+}/pH analyzer (Siemens, Erlangen, Germany).

For other serum biochemical parameter measurements, blood from the superficial temporal vein was collected into the CAPIJECT T-MG micro collection tube (Terumo Europe N.V., Leuven, Belgium), and centrifuged to yield serum that was stored at –80°C until further analysis. Samples were analyzed for phosphorus using Phosphor C kit (Wako Pure Chemical Industries, Osaka, Japan), for PTH using a mouse PTH 1–84 Elisa kit (Immutopics, San Clemente, CA, USA), and 1,25-dihydroxy vitamin D\textsubscript{3} (1,25(OH)\textsubscript{2}D\textsubscript{3}) using a 1,25-Dihydroxy Vitamin D EIA kit (IDS, Fountain Hills, AZ, USA).

**Histology and immunohistochemical staining**

Three days after the last DT injection, animals were euthanized and dissected under the stereomicroscope. The thyroid and
Parathyroid glands were isolated and removed en bloc, fixed with 4% paraformaldehyde and embedded in paraffin. The long-term effects of DT injection on the parathyroid glands were studied in animals that also carried the ROSA^{mld} transgene for better visualization of parathyroid cells. One month after the last DT injection, mice were euthanized, a larger portion of the neck region around the thyroid removed en bloc, embedded in OCT and snap frozen in liquid nitrogen. Five-micrometer (5-μm) frozen sections were obtained and screened for GFP under a fluorescence microscope. To perform immunohistochemistry using a PTH antibody on sections containing GFP-positive cells, sections were incubated in blocking buffer (5% goat serum) for 1 hour at room temperature, before incubation with mouse anti-human parathyroid hormone (anti-hPTH) antibody (AbD Serotec, Raleigh, NC, USA) in a 1:100 dilution and rabbit anti-GFP antibody (Life Technologies, Grand Island, NY, USA) overnight at 4°C. Sections were stained at room temperature for 2 hours with Alexa Fluor 568/633 goat anti mouse IgG and Alexa Fluor 488 goat anti rabbit IgG antibody (Life Technologies) using a 1:200 dilution. DAPI (Vector Laboratories, Burlingame, CA, USA) was used for nuclear counterstaining.

To evaluate parathyroid cell apoptosis, TUNEL staining was performed on 5-μm paraffin sections of thyroid-parathyroid tissue using In Situ Cell Death Kit (Roche Applied Science, Mannheim, Germany). Antigen retrieval (2.5% proteinase K at 37°C) and staining process were done according to the manufacturer's instructions and DAPI was used for counter-staining. The FITC-labeled TUNEL-positive cells were imaged under fluorescent microscopy.

Peptide injection

Long-acting PTH (LA-PTH) and hPTH(1–34) were synthesized by solid-phase chemistry. LA-PTH contains the hPTH(1–14) sequence modified with Ala^{1,12}, Gln^{10}, Arg^{11}, Trp^{14}, followed by the hPTHrP(15–36) sequence modified with Ala^{18,22} and Lys^{20}. After synthesis, peptides were diluted in vehicle solution (10 mM citric acid/150 mM NaCl/0.05% Tween-80, pH 5.0), to give a stock solution of 1 μmol/L. Stock solution was further diluted to a final working solution of 5 μg/mL body weight on the day of the injection. A single subcutaneous injection of LA-PTH at a dose of 1.5, 3, 5, 10, and 20 nmol/kg, and PTH(1–34) at a dose of 50 nmol/kg was administered to 6-week-old DT injected PTHcre-iDTR mice, and to GFP-PTX mice. Animals in the vehicle control group were injected with saline. Blood and serum samples were collected at 0, 2, 4, 6, 8, 12, 16, 20, 24, 48, 72, 96, and 120 hours after injection.

Statistical analyses

Data were processed using Microsoft Excel 2008 (Microsoft Corp., Redmond, WA, USA) and GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Comparisons of means were performed by using Student’s t test (two-tailed, unequal variances). A p value <0.05 was considered statistically significant. Results are expressed as mean ± SD.

Power calculations to determine minimal group size for the experiments testing PTH analogs indicated that with five mice in each experimental group we would have 95% power to detect a group mean difference of 13% in ionized calcium assuming a standard deviation of 0.08 mmol/L (p<0.05).

**Results**

**PThcre-iDTR mice**

Human diphtheria toxin receptors (DTR) were selectively expressed in parathyroid cells by using mice that carry an iDTR allele. Mice were mated to mice that express the Cre allele under the control of the human 5.5-kb PTH promoter, which is active only in parathyroid cells. In the resulting PTHcre-iDTR mice, parathyroid cells can be ablated simply by systemic injection of diphtheria toxin (DT); all other tissues in the mouse are insensitive to DT. To determine the maximally tolerated dose of DT in mice we injected iDTR mice (without the Cre allele) at DT i.p. at ascending doses. In these mice, the loxP-flanked transcriptional STOP cassette upstream of the DTR gene is intact, and therefore DTR is not expressed. At DT doses ≥75 μg/kg, we observed death in some animals, likely due to nonspecific toxicity (Supplemental Fig. 1). For subsequent experiments in PTHcre-iDTR mice, we therefore chose DT doses ≤50 μg/kg body weight; these doses have also been used by other investigators.

Next, dose-finding studies for the ablation of parathyroid glands were performed in PTHcre-iDTR mice using single i.p. administration of ascending doses of DT. The efficacy was assessed by measuring blood ionized calcium (Ca^{++}) and serum PTH levels 3 days later. A single dose of 5 μg/kg body weight led to hypocalcemia (Ca^{++} = 1.14 ± 0.09 mmol/L) and low PTH levels (PTH = 303 ± 153 pg/mL, limit of detection = 4 pg/mL) compared to vehicle control (Ca^{++} = 2.60 ± 0.05 mmol/L; PTH = 572 ± 164 pg/mL) (Fig. 1A, B). Higher doses of DT did not lead to more severe hypocalcemia. Next, we performed repeated dosing experiments at a fixed dose of 5 μg/kg body weight with two to five doses given at 3-day intervals. After two DT injections given 3 days apart, blood Ca^{++} and serum PTH were lower than after a single injection (Ca^{++} = 1.10 ± 0.07 mmol/L, PTH = 218 ± 156 pg/mL, p < 0.05 versus single injection), and no further decrease was observed with more frequent administration of DT (Fig. 1C, D). Doses lower than 5 μg/kg, even when injected multiple times, showed lower efficacy in reducing blood Ca^{++} and serum PTH (data not shown). Thus we determined that two i.p. injections of DT 3 days apart at 5 μg/kg body weight constitute an optimized dose regimen to induce HP in this mouse model.

To evaluate the degree to which the PTH response is impaired in the DTR HP model, we compared its calcium and PTH to parathyroid-intact mice that were rendered hypocalcemic by dietary means. In parathyroid-intact control animals (PTHcre-iDTR without injecting DT) fed a low-calcium diet for 2 weeks, hypocalcemia was similar to those in the DT model on regular calcium diet (Ca^{++} = 1.18 ± 0.02 versus 1.12 ± 0.07 mmol/L), but PTH levels were almost 10 times higher than in DT-treated mice (Ca^{++} = 218 ± 156 pg/mL, p < 0.05 versus single injection), and no further decrease was observed with more frequent administration of DT (Fig. 1C, D). Doses lower than 5 μg/kg, even when injected multiple times, showed lower efficacy in reducing blood Ca^{++} and serum PTH (data not shown). Thus we determined that two i.p. injections of DT 3 days apart at 5 μg/kg body weight constitute an optimized dose regimen to induce HP in this mouse model.

As expected in HP, serum Pi levels significantly increased after ablating the parathyroid glands with DT (Fig. 20). Total urinary calcium to urinary creatinine (uCa/uCr) did not decrease.
in DT-injected mice compared to control mice, despite lower serum calcium and therefore a lower filtered load of calcium (Fig. 2P). This suggests that the fractional excretion of calcium was increased in DT-injected mice, an observation consistent with HP. The HP phenotype remained stable over 90 days after DT injection (Fig. 2Q).

To investigate the fate of the parathyroid glands after DT injection, we first examined histological sections of the thyroid
Fig. 2. Characterization of PTHcre-iDTR-mTmG mice after DT injection. (A–N) Representative parathyroid gland with surrounding thyroid gland. (A–C) Before DT injection. Intact parathyroid gland with robust PTH staining and no TUNEL-positive cells in the parathyroid region. (D–F) Three days after DT injection (2 i.p. injections at 5 μg/kg 3 days apart). In the parathyroid glands, increased vascularity is observed (D), dramatic decrease in PTH immunoreactivity (E), and abundant apoptotic TUNEL-positive cells (F). (G–N) One month after vehicle or DT injection (2 i.p. injections at 5 μg/kg 3 days apart). In vehicle control, parathyroid glands (G) show strong immunostaining for GFP (H) and PTH (I) staining (using as secondary antibody Alexa Fluor 488 for GFP and 633 Far-red for PTH). In DT injected mice, the normal structure of the parathyroid glands is lost (K), and most GFP and PTH immunoreactivity is no longer detectable. However, a few GFP and PTH double-positive cells (L–N) remained. (O) Serum Pi levels in PTHcre-iDTR mice 3 days after DT or vehicle injection (n = 12, **p < 0.001). (P) Urinary calcium excretion in PTHcre-iDTR mice 3 days after DT or vehicle injection (n = 12). (Q) Serum PTH and blood ionized calcium levels in PTHcre-iDTR mice after DT versus vehicle injection over 3 months. (n = 6 to 8, **p < 0.01, ***p < 0.001).
and parathyroid tissue obtained from mice 3 days after the DT injections. The parathyroid gland structure was grossly intact, but there was an increased number of blood vessels, and almost all PTH immunoreactivity had disappeared (Fig. 2A, B, D, E). In addition, we detected substantial apoptotic cell death in the parathyroid glands by TUNEL staining compared to TUNEL staining that appears negative in normal mice (Fig. 2C, F). Next, we examined the parathyroid gland 1 month after DT injections. Because of the difficulties in locating the ablated glands, we performed DT injection in PTHcre-iDTR-mTmG mice, which carry a ROSA<sup>mt/mG</sup> allele and therefore have green fluorescent parathyroid glands. Although parathyroid glands could no longer be visualized under the fluorescent dissecting microscope one month after DT injection, screening of sections through a larger portion of the neck region around the thyroid under the fluorescent microscope revealed a few dispersed GFP-positive cells. Co-immunostaining with GFP and PTH antibodies showed that the green cells are in fact positive for PTH (Fig. 2L–N). More injections of DT at later time points (1 or 3 months after the two doses of 5 μg/kg DT injection) did not further change blood Ca<sup>2+</sup> and PTH levels of the animals (data not shown).

GFP-PTX mice

For the second mouse model of acquired HP, mice homozygous for the double-fluorescent Cre reporter ROSA<sup>mt/mG</sup> were used. All cells in the ROSA<sup>mt/mG</sup> mouse express membrane-targeted tandem dimer Tomato (tdTomato), whereas in the presence of an expressed Cre allele, tdTomato is silenced, and membrane-targeted GFP is expressed. The ROSA<sup>mt/mG</sup> mice were mated to homozygous PTH-Cre Rosa<sup>Cre</sup>/Rosa<sup>Cre</sup>/ROSAmT/mG that selectively express GFP in the parathyroid glands. Under white light, the two parathyroid glands were indistinguishable from surrounding tissue (Fig. 3A–F), but under UV illumination, they were easily visualized (Fig. 3B, C). GFP-guided surgical removal of the parathyroid glands was thus performed using a stereomicroscope under UV illumination (Fig. 3D–F), to thus generate GFP-PTX mice. Because the GFP greatly facilitated parathyroidectomy, the entire procedure took less than 20 min, and was precise, as the thyroid gland was kept intact. Moreover, overall mortality was low, as the GFP-PTX mice had a survival rate at 30 days similar to that of sham-operated mice (90% versus 95%) (Fig. 3G). Three days after surgery, GFP-PTX mice displayed marked reductions of blood Ca<sup>2+</sup> and serum PTH levels, compared to levels found in sham-operated mice (Ca<sup>2+</sup> = 1.05 ± 0.04 versus 1.30 ± 0.03 mmol/L; PTH = 32 ± 22 versus 580 ± 137 pg/mL) (Fig. 3H, I). As expected, serum Pi was significantly increased in GFP-PTX mice (Fig. 3J). Total urinary calcium excretion did not statistically differ between PTX and sham control mice (Fig. 3K), again suggesting a higher rate of urinary calcium clearance in the absence of PTH. The HP and consequent hypocalcemic phenotype was stable over a 3-month observation period (Fig. 3L). Hypoparathyroid mice of both models were viable and we did not observe seizures.

Treatment with LA-PTH

We then used each of these mouse models of HP to study the calcium- and phosphate-regulating actions of a long-acting PTH analog LA-PTH, as compared to those of unmodified PTH(1–34). LA-PTH was injected subcutaneously at doses of either 1.5, 5, 10, or 20 nmol/kg, and PTH(1–34) at a single dose of 50 nmol/kg. In both GFP-PTX and DT-injected PTHcre-iDTR mice, robust, dose-dependent increases in blood Ca<sup>2+</sup> were observed by 2 hours postinjection of LA-PTH, and at the higher doses, the effects persisted for as long as 72 hours (Fig. 4A, B). At the 5 nmol/kg dose, LA-PTH elevated serum calcium levels in GFP-PTX mice to about the normal range for at least 24 hours. In contrast, PTH(1–34) at 50 nmol/kg induced a similar maximal increase in calcium in GFP-PTX mice, but the response peaked at 2 hours and returned to baseline levels by 8 hours (Fig. 4A, B). LA-PTH also induced a longer-lasting reduction in serum phosphate than did PTH(1–34). Two hours after injection of LA-PTH at doses ranging from 1.5 to 20 nmol/kg, serum Pi levels in both mouse models decreased significantly and remained low for at least 8 hours before returning to baseline levels by 24 hours postinjection. PTH(1–34), by contrast, decreased serum Pi only at the 2-hour time point, as baseline levels were again observed at 4 hours (Fig. 4C, D). Spot-urinary calcium levels were unchanged by injection with LA-PTH at any dose (Fig. 4E, F).

Binding to and activation of the PTH1-receptor by PTH leads to increases in cellular cAMP production. We measured urinary cAMP levels after administration of LA-PTH as a direct readout of PTH1R activation in target tissues. Baseline urinary cAMP levels were lower in GFP-PTX mice than in sham control mice, consistent with the lower circulating PTH levels (Fig. 5A). After LA-PTH injection, urinary cAMP increased and peaked at 2 hours, and the increase was detected for ~16 hours at doses of 10 nmol/kg. In comparison, PTH(1–34) at a dose of 50 nmol/kg increased urinary cAMP only at the 2-hour time point, as the levels returned to baseline by 4 hours (Fig. 5B). The extended urinary cAMP response observed here for LA-PTH in GFP-PTX mice is consistent with that observed for this analog previously in wild-type mice.

At baseline, serum 1,25(OH)<sub>2</sub>D<sub>3</sub> levels in GFP-PTX mice were lower compared to sham control (Fig. 5C). LA-PTH administration led to dose-dependent increases in serum 1,25(OH)<sub>2</sub>D<sub>3</sub>, which at a dose of 10 nmol/kg, lasted for at least 24 hours, whereas PTH(1–34) at a dose of 50 nmol/kg increased 1,25(OH)<sub>2</sub>D<sub>3</sub> levels maximally at 2 hours, and the levels returned to baseline by 12 hours (Fig. 5D).

Discussion

We describe two new mouse models for acquired HP, a disease for which an efficient mouse model was lacking. In the PTHcre-iDTR mice, an inducible human DTR was used for parathyroid gland cell-specific ablation by DT injection. We determined that two i.p. DT injections at 5 μg/kg body weight resulted in efficient ablation of most of the parathyroid cells, leading to mice with chronic HP. By 3 days post–DT injection we observed abundant cell apoptosis in the parathyroid glands by TUNEL staining, and 1 month after injection, parathyroid glands could no longer be identified. The hypoparathyroid phenotype was not particularly severe in this model, as mean blood ionized calcium levels were 1.12 versus 1.26 mmol/L in control animals, yet the low levels were stable over time. One month after the DT ablation, we observed a few dispersed GFP-positive cells in the region of the parathyroid glands. These cells are likely to be remnants of the parathyroid glands that escaped or resisted DT treatment. As shown by the stable persistence of low blood calcium and PTH levels over 3 months, these cells are not able to regenerate the parathyroid glands in this time. We conclude, therefore, that DT treatment is not 100% efficient, and that the residual cells are the most likely source of the detectable PTH in the circulation and
Fig. 3. Hypoparathyroid phenotype of GFP-PTX mice. (A–F) Photographs of the neck region of PTH-Cre;ROSA<sup>mT/mG</sup> mice before and after parathyroidectomy. (A) Under halogen light, parathyroid glands cannot easily be distinguished from the surrounding tissue. (B, C) Under UV illumination, GFP-positive parathyroid glands are easily identified. (D–F) Using fine scissors and forceps, green parathyroid glands were selectively removed. (G) Location of thyroid and parathyroid glands are outlined in red and white, respectively. (G) Survival rate of GFP-PTX mice compared to animals that underwent sham surgery (n = 20). (H–K) Blood Ca<sup>++</sup>, serum PTH, serum Pi, and urinary calcium excretion (uCa/uCr) in GFP-PTX mice 3 days after surgery (n = 10 to 12, ***p < 0.001). (L) Serum PTH and blood ionized calcium levels in GFP-PTX versus sham-operated mice over 3 months (n = 6 to 8, ***p < 0.001).
the milder hypoparathyroid phenotype. Nevertheless, the model is useful as it compares generally to conditions seen in hypoparathyroid patients with mild forms of the disease, for which mean levels of hypocalcemia can vary widely\(^\text{26}\). A noteworthy advantage of this DTR model is that it is simple to induce, as only two i.p. injections of DT are needed to achieve a stable hypoparathyroid state. This enables large numbers of experimental animals to be generated within a few hours, and without the need for surgical procedures.

The second mouse model for acquired HP is based on surgical parathyroidectomy; the surgery is greatly facilitated, however, by expression of GFP specifically in the parathyroid glands. This modification further allows for preservation of the thyroid gland and thus overcomes a potential limitation of TPTX surgery. The
The benefit of the GFP-guided approach is further reflected by the short time it takes to accomplish the procedure (<20 minutes per animal), the high efficiency (95% of mice were hypocalcemic), and the stability of the phenotype. The GFP-PTX mice exhibited a more severe phenotype, with mean ionized calcium levels at 1.05 mmol/L, as compared to 1.12 mmol/L in DT-injected PTHcre-iDTR mice, and 1.30 mmol/L in sham-operated animals. Depending on the desired severity of the phenotype, and on the resources available for surgical procedures, either of these mouse models could be used as an animal model for acquired HP.

Finally, the utility of these models was demonstrated by studying the effects of PTH analog administration on calcium and Pi homeostasis. LA-PTH was thus found to be particularly effective in controlling serum Ca and Pi levels. Studies on the mechanism by which LA-PTH and related long-acting analogs induce prolonged responses in vivo suggest that it does not involve enhanced pharmacokinetics, but rather a unique mode of pseudo-reversible binding to the PTH receptor in target cells, and hence persistent signaling responses, possibly from within internalized cell compartments. We indeed confirmed the persistent cAMP signaling actions of the ligand in the HP mice by measuring urinary cAMP output. This translated into prolonged calcemic and hypophosphatemic responses lasting for over 24 hours, depending on the dose. The analog thus appears to be more efficacious than PTH(1–34) in regulating blood Ca$^{++}$ and Pi levels in at least these animal models of HP.

An increase in renal calcium reabsorption by LA-PTH could potentially mean an important advantage over oral Ca and vitamin D treatment now used by most HP patients. Because of the small size in mice, however, it is technically challenging to precisely quantify rates of urinary calcium excretion, and so such measurements were not performed in the current studies. Further work is therefore needed to understand better how LA-PTH can alter rates of Ca filtration in the kidney, and how these effects might vary with dose and over time of treatment.

**Disclosures**

TG: Chugai: study grant to institution, pending patent on LA-PTH. TW is an employee of Chugai Pharmaceutical Co. Ltd. MM: NPS, Chugai, Amgen: consulting agreements, unrelated to this work. All other authors state that they have no conflicts of interest.

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