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Production and sorting of transgenic, modified human parathyroid hormone in vivo in rat salivary glands

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ABSTRACT

Polarized salivary epithelial cells can sort secretory proteins towards either the basolateral or apical pole. Transgenic human parathyroid hormone (hPTH) exclusively sorts apically in rat submandibular glands. To help understand this specific process we modified the hPTH cDNA sequence and delivered the cDNAs to glands in vivo using adenoviral (Ad) vectors. The Ad vectors encoded: (1) the native form of hPTH (Ad.pre-pro-hPTH1–84), (2) the native sequence, but with the pro-segment deleted (Ad.pre-hPTH1–84), and (3) a sequence containing the pre-segment followed by the first 34 amino acids of hPTH (Ad.pre-hPTH1–34). hPTH production and sorting were studied after two days. All constructs were effectively transcribed in targeted glands. However, the pre-hPTH1–84 modification led to reduced hPTH secretion and production, while no immunoreactive hPTH resulted from pre-hPTH1–34 cDNA infusion. The pre-hPTH1–84 modification had no effect on apical sorting. These in vivo results show that the signal responsible for hPTH's apical sorting does not reside in the pro-segment and that deleting both the pro-segment and the carboxyl-terminal region severely impairs post-translational processing of hPTH.

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Introduction

Secretory proteins produced in salivary gland epithelial cells can be sorted to either, or both, the basolateral and the apical pole [1]. The mechanisms underlying such sorting differences are largely unknown [2,3]. It is likely, however, these cells distinguish the sorting pathway to be taken for each individual protein by an interaction of structural signals on the secretory protein with the cellular sorting machinery. To study these mechanisms in vivo, we have used direct salivary gene transfer and specific transgenic model proteins, such as human parathyroid hormone (hPTH) [4]. PTH is a regulated secretory pathway (RSP) protein, essential for maintaining serum calcium levels [5–7]. Physiologically, hPTH is translated as a pre-pro protein. The pre-sequence is a 25 residue N-terminal signal sequence necessary for efficient transport into the endoplasmatic reticulum (ER) [8,9]. This signal sequence is rapidly [10] cleaved off co-translationally in the rough ER and the resultant pro-hPTH peptide is moved to the Golgi apparatus [11]. In the trans-Golgi network, the six amino acid pro-sequence N-terminal extension is proteolytically removed [12] and the mature hPTH is stored as an 84-amino acid single chain polypeptide in secretory granules. The first 34 amino acids alone are sufficient to raise serum calcium levels [13]. When hPTH is expressed as a transgene in rat submandibular glands, it is very specifically secreted apically into saliva [4]. It is unknown whether any specific domain of the hPTH protein is responsible for this sorting behavior. To assess if the pro-sequence and the C-terminal amino acids (35–84) have any effect on hPTH sorting and production, we have constructed three serotype 5 adenoviral (Ad5) vectors encoding either of three different cDNA sequences (Ad.pre-pro-hPTH1–84, Ad.pre-hPTH1–84 and Ad.pre-hPTH1–34) and tested them in vivo after delivery to rat submandibular glands.

Methods

Animals. Male Wistar rats ($n = 6$/treatment group) were obtained from Harlan Sprague–Dawley (Walkersville, MD) at 7 weeks of age. They were acclimatized for 1 week before the start of experiments, and normal water and food were provided ad libitum. All animal procedures were approved by the Animal Care and Use Committee of the National Institute of Dental and Craniofacial Research and by the Biosafety Committee of the National Institutes of Health.

Construction of recombinant adenoviral vectors. The Ad5 vector expressing pre-pro-hPTH1–84 was cloned, produced and purified as previously described [4]. Both the pre-hPTH1–84 and the pre-hPTH1–34 cDNA were created by gene synthesis (EZBiolabs, Westfield, IN) and cloned into a pUC57 shuttle plasmid. After enzymatic digestion of the pUC57 carrier plasmid, the obtained

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pre-hPTH1–84 and pre-hPTH1–34 cDNAs were directionally cloned into the Ad5 expression shuttle plasmid pACCMV-pLpA [4]. All three sequences were confirmed by automated sequencing. hPTH expression from each plasmid was confirmed in vitro after transfection of 293 cells. After that, E1 deleted recombinant Ad5 vectors were generated as previously described [14]. Vectors were amplified in 293 cells and the crude viral lysates purified by two rounds of CsCl gradient centrifugation [15]. Vector particle (vp) titers were measured by real-time quantitative polymerase chain reaction (PCR), with primers designed to amplify part of the CMV promoter region [4].

Transduction of salivary glands in vivo. Rats were anesthetized, submandibular glands cannulated and vectors delivered, as previously reported [4,16]. Vectors (5×10^9 vp/gland) were suspended in 200 μl of saline and delivered to both Wharton's ducts by retrograde instillation. After infusion, syringes were left in place for 15 min to prevent backflow of fluid. Saline administration to glands was used as control [4]. Forty-eight hours later, the animals were re-anesthetized and given a subcutaneous injection of pilocarpine (0.5 mg/kg in saline) to stimulate salivary flow. Saliva was collected as described [4] and snap frozen. Animals were euthanized in a CO_2 chamber and, after death was confirmed by bilateral thoracotomy, blood was collected via the vena cava, serum separated and stored at −80 °C. Submandibular glands were excised, cleaned, frozen and stored at −80 °C.

Measurement of transgene transcription by reverse transcriptase PCR. One half of each gland was homogenized and mRNA was extracted using the RNeasy kit from Qiagen (Valencia, CA) according to the manufacturers' instructions. RNA quality was confirmed by gel electrophoresis and quantified by spectrophotometry. Using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), cDNA was synthesized from 1 μg mRNA according to the instruction manual and the reaction mix was diluted 1:1 with TE buffer to a total volume of 40 μl. For reverse transcriptase PCR, 2 μl of cDNA solution was amplified using Taq Platinum DNA polymerase (Invitrogen, Carlsbad, CA) using the following primer sets: PTH1FWR (5′-CGG ATGGGAAATCTGTTAAGAAGAG-3′) and PTH1REV (5′-CATCAGCTTT GTCTGCCTCTCCAA-3′) resulting in a 247 bp PCR product using Ad.-pre-pro-hPTH1–84 as a positive control; PTH2FWR (5′-CGGAT GGGTCTGTGAGTGAAATAC-3′) and PTH1REV producing a 229 bp PCR product using Ad.pre-hPTH1–84 as a positive control; and PTH2FWR and PTH2REV (5′-TGTGCACATCCTGCAGCTTCTTAC-3′) giving a 106 bp PCR product using Ad.pre-hPTH1–34 as a positive control (see Fig. 2). Amplification was then performed in a DNA Thermal Cycler 480 (Perkin-Elmer Cetus, Foster City, CA) as follows: 5 min at 95 °C followed by 40 cycles of 1 min at 68 °C, 2 min at 72 °C, 1 min at 94 °C, respectively, followed by a final extension phase at 72 °C for 10 min. The PCR products were analyzed by standard agarose gel electrophoresis on a 1% agarose gel containing ethidium bromide for UV-assisted visualization of the amplicons.

Detection of intact hPTH protein. Human PTH1–84 and hPTH1–34 were measured using an ELISA kit from MD Biosciences (St. Paul, MN) and Alpco Diagnostics (Salem, NH), respectively, following the manufacturer's instructions. Each sample was measured twice and in duplicate. The serum-to-saliva distribution ratio for hPTH was calculated as described previously [4,17]. For hPTH determinations in gland protein extracts, half of each gland was homogenized in 1× RIPA buffer (Thermo Scientific, Rockford, IL), incubated for 1 h on ice and centrifuged at 12,000 rpm for 5 min. Total protein concentration was determined in supernatants by the Bio-Rad protein assay and 1 μg total extracted gland protein from each sample was used for the ELISA.

Statistical analysis. Differences in means between groups were determined by the Kruskal–Wallis test, followed by a Mann–Whitney *U* rank-sum test. $p < 0.05$ was considered statistically significant. All analyses were done using SPSS version 15.0 (SPSS, Chicago, IL). Data are expressed as means ± SEM.

Results

Adenoviral constructs

Fig. 1 schematically displays the three Ad5 vectors constructed. The first contains the full-length pre-pro-hPTH1–84 cDNA, leading to production of the native hPTH (Fig. 1A). This cDNA consists of the signal sequence (pre), a pro-sequence, the biologically active N-terminal sequence (amino acids 1–34) and the C-terminal sequence (amino acids 35–84). In the second construct, the DNA coding for the pro-portion was deleted, keeping the signal sequence followed by amino acids 1–84 (Fig. 1B). For the third construct, the DNA coding for amino acids 35–84 was also removed (Fig. 1C).

Transduction and transgene transcription in salivary glands in vivo

To assess if all vectors transduced salivary glands and led to transcription of the three different hPTH sequences encoded, glands were removed two days after vector infusion, mRNA was isolated, and cDNA prepared. To distinguish the different transcripts in vivo, specific primer sets were designed (Fig. 2A), each

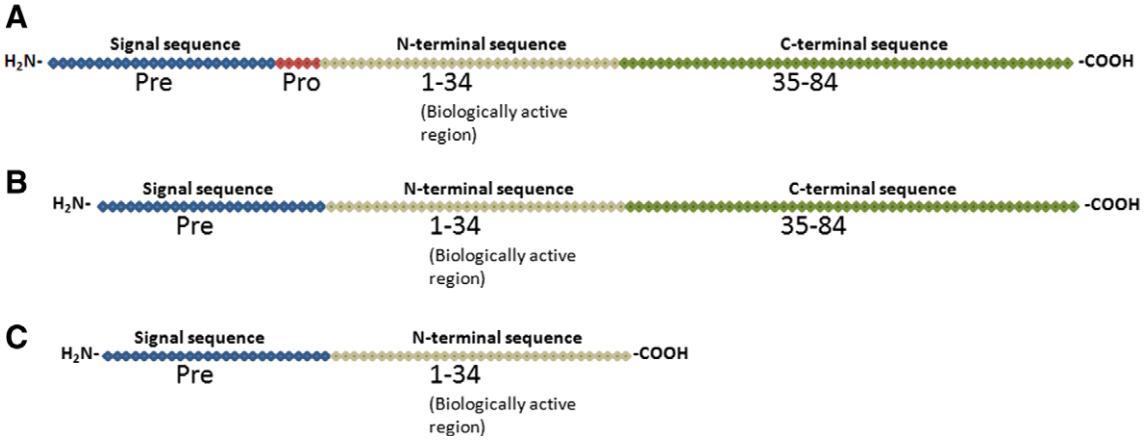

Fig. 1. Schematic depiction of the different hPTH constructs encoded by and expressed from adenoviral vectors herein. (A) The full-length native pre-pro-hPTH1–84 amino acid sequence; (B) pre-hPTH1–84 with the pro-sequence deleted; and (C) pre-hPTH1–34, with both the pro-sequence and the sequence encoding amino acids 35–84 deleted.

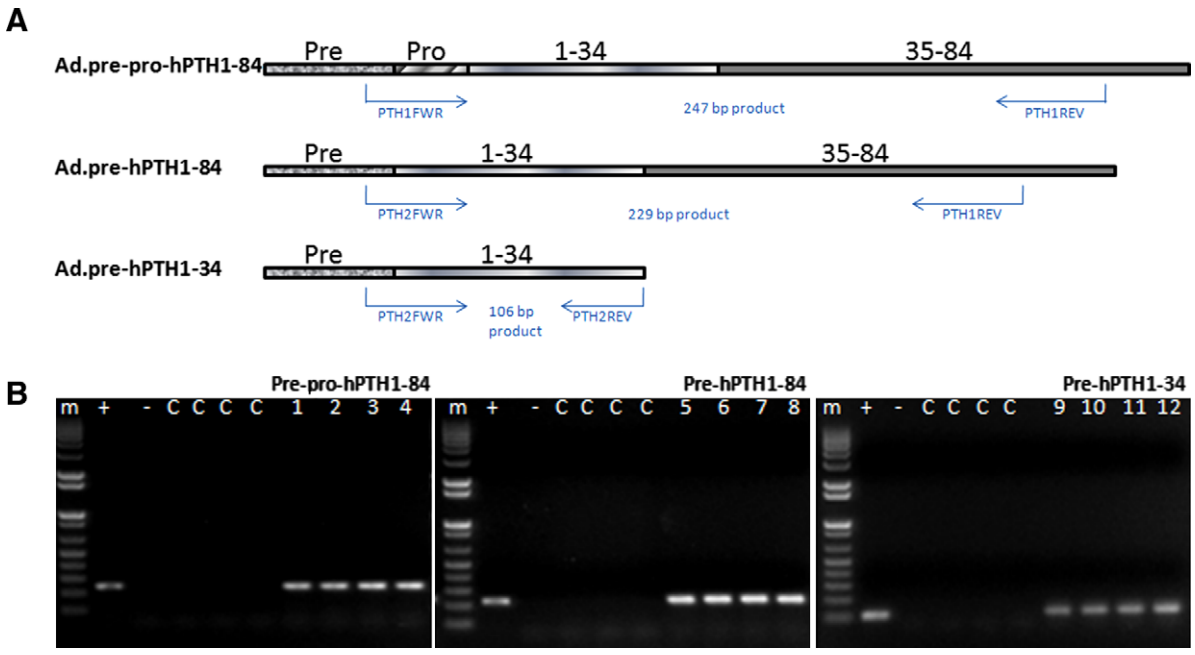

Fig. 2. Transcription of three hPTH transgenes in submandibular glands in vivo. Glands were removed two days after gene transfer, homogenized and mRNA was isolated. After cDNA synthesis, reverse transcriptase (RT)-PCR was performed with primers (A) designed to amplify regions specific to each sequence. (B) RT-PCR products visualized on 1% agarose gels, comparing results from control animals (marked 'C' in figure) with those from rats transduced with either Ad.pre-pro-hPTH1–84 (labeled 1, 2, 3 and 4, left panel), Ad.pre-hPTH1–84 (labeled 5, 6, 7 and 8, middle panel) or Ad.pre-hPTH1–34 (labeled 9, 10, 11 and 12, right panel). As a positive control (+) for each gel, the same adenoviral vector as infused in the respective animals was used directly as the PCR template. Water was used as a no template control (−). No control animal showed a positive signal, whereas all transduced animals displayed a clear and specific band of the expected size.

leading to a different amplicon unique for the specific construct used with that group of animals.

In saline infused animals ('C') and the no template control ('−') samples, no signal was present using all the three primer sets. In the animals transduced with Ad.pre-pro-hPTH1–84, a band of the expected size was visible in all animals (four representative rats are shown, labeled 1, 2, 3 and 4, left panel of Fig. 2B). In rats infused with Ad.pre-hPTH1–84, the specific amplicon band was also visible in all animals (four representative rats shown, labeled 5, 6, 7 and 8, middle panel; Fig. 2B). Finally, samples from all rats transduced with Ad.pre-hPTH1–34 also gave a specific band at the expected size (four representative rats shown, labeled 9, 10, 11 and 12, right panel; Fig. 2B). Thus, all three Ad5 constructs led to successful gland transduction in vivo.

Secretion of hPTH from transduced salivary glands

Two days after vector delivery, serum and saliva were obtained and assayed for secreted hPTH (Fig. 3; total secreted hPTH amounts shown). hPTH was undetectable in samples from saline infused animals. As we reported previously [4], hPTH secreted from submandibular glands transduced with Ad.pre-pro-hPTH1–84 exclusively sorts towards the saliva, with no hPTH found in serum. In these animals, the saliva contained extremely high levels of hPTH (>7000 pg/ml) and the average total secretion of hPTH into saliva was ~2250 pg/animal over ~15 min. Deleting the pro-sequence had a significant effect on the amount of hPTH protein secreted, i.e., animals that received Ad.pre-hPTH1–84 secreted an average total of ~580 pg hPTH into their saliva (concentration ~2400 pg/ml), with again no hPTH detected in serum. Interestingly, in rats whose glands were transduced with Ad.pre-hPTH1–34, there was no secretion of hPTH detected into either saliva or serum (Fig. 3). Thus, while there was apparently little difference in transcription levels following administration of the three constructs (Fig. 2B), there was a substantial difference in secreted hPTH protein levels following deletion of pro-sequence ± the sequence encoding amino acids 35–84.

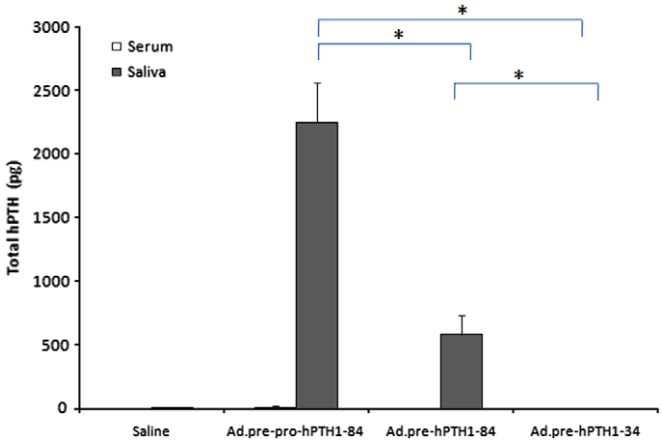

Fig. 3. Secretion of transgenic hPTH from transduced submandibular glands. Two days after the vector infusion, serum and saliva were obtained, assayed for transgenic hPTH by ELISA, and the total secreted hPTH calculated as described in Methods. Total transgenic hPTH secreted into each compartment is displayed as mean ± SEM values. Vector (5×10^9 vp/gland) delivery to both submandibular glands led to abundant secretion of hPTH into saliva, but essentially no hPTH was detected in serum. Animals infused with Ad.pre-pro-hPTH1–84 had the highest levels of hPTH in their saliva, followed by rats that received Ad.pre-hPTH1–84. Ad.pre-hPTH1–34 gene transfer led to no detectable levels of secreted hPTH. All saline infused animals had no detectable hPTH present in their saliva. $^*p < 0.05$.

Detection of hPTH in salivary gland extracts

The differences observed in secreted hPTH levels could be due to translational and post-translational processing, or due to impaired secretory abilities. To evaluate this, transduced glands were excised, homogenized, and soluble protein assayed for hPTH.

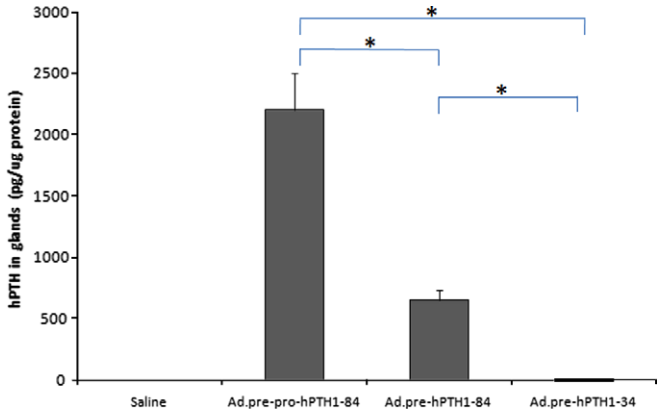

Fig. 4. Detection of transgenic hPTH protein in aqueous extracts of submandibular glands. Submandibular glands were excised and homogenized in lysis buffer two days after vector delivery. Total gland protein extracted was assayed for hPTH by ELISA as described in Methods. Ad.pre-pro-hPTH1–84 mediated gene transfer led to the highest levels of hPTH measured in gland extracts. Ad.pre-hPTH1–84 delivery resulted in much lower hPTH levels, while almost no hPTH was detectable after use of the Ad.pre-hPTH1–34 vector. All data are expressed as mean ± SEM values of hPTH, in pg per μg extracted protein. $^{*}p < 0.05$.

Ad.pre-pro-hPTH1–84 mediated gene transfer led to high levels (2202 pg/μg protein; Fig. 4) of hPTH in gland extracts, even after pilocarpine stimulation. As above, extracts of glands transduced with Ad.pre-hTPH1–84 showed significantly lower levels of hPTH (653 pg/μg protein). For extracts of glands transduced with Ad.pre-hPTH1–34, the hPTH levels measured were barely over the assay's detection limit (2.6 pg/μg protein).

Discussion

We have previously shown that following gene transfer to rat submandibular glands, transgenic hPTH overwhelmingly sorts into the saliva. The reason for this specificity is unknown and, herein, we have examined if the hPTH pro-sequence ± the C-terminal sequence (amino acids 35–84) influences this highly specific hPTH sorting behavior. Theoretically, the deletion of the pro- and C-terminal sequences could affect hPTH sorting if a sorting signal were localized to either segment. Importantly, deletion of either sequence should not alter hPTH's classical biological role in calcium homeostasis [5,7,13]. Of note, the pro-sequence of hPTH affects signal peptide processing, since its deletion can lead to ambiguity in the sites selected by signal peptidase for cleavage in rat pituitary cells in vitro [12]. As a result, (post-) translational processes could be less efficient, but the produced mature hPTH still should be functionally intact. The C-terminal region, hPTH amino acids 35–84, [18], as well as fragments of that region [19,20], have recently been shown to have a physiological role, e.g., in inhibiting 1,25-dihydroxyvitamin D synthesis. However, the first 34 amino acids alone are sufficient for the calcium elevating function of hPTH [5,7,13].

Our present results show that deletion of the pro-sequence ± the C-terminal sequence had relatively little effect on hPTH transcription. However, both deletions significantly affected hPTH protein production and secretion. This effect was particularly profound for the pro + C-terminal sequence deletions. The results seem not due to an impaired secretory pathway, as the relative amounts of hPTH found in extracts of glands following transduction with the two experimental vectors were generally similar to hPTH levels secreted. Further, the intracellular kinetics of hPTH in the secretory pathway seems generally unchanged for the pro-sequence deletion alone. Importantly, for the latter deletion, we found that no hPTH was found in serum, clearly indicating that the pro-sequence per se does not influence the sorting characteristics of transgenic hPTH in rat submandibular glands in vivo.

It was unanticipated that transduction with the Ad.pre-hPTH1–34 construct would result in essentially the complete absence of production of hPTH protein in the submandibular glands. Protein extracts of transduced glands contained very low amounts of hPTH indicating severely impaired translational or post-translational handling of the transgenic hormone. The cause of this dramatic effect is at present speculative, but could be due to impaired translocation across the ER membrane in combination with an incorrect cleavage of the signal peptide. It has been shown in COS cells that a minimum of 65 amino acid residues is needed for hPTH to cross the ER membrane and that 83 residues are needed for effective pro-sequence cleavage [21]. Such circumstances may, for example, mark any translated transgenic hPTH peptides for ubiquitination and subsequent proteasomal degradation.

In conclusion, this is the first study to examine the in vivo expression and sorting behavior of modified hPTH constructs following direct gene transfer to a polarized epithelial tissue. No changes in hPTH sorting were found following pro-sequence deletion, indicating that the key sorting determinants directing hPTH into an exocrine pathway in rat submandibular glands are not located in this region. The general approach utilized herein, involving short-term transfer of modified genes mediated by an Ad5 vector, should prove useful for investigators interested in understanding the sorting of soluble secretory proteins in polarized glandular tissues [22].

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References

[1] B.J. Baum, M.E. Berkman, Y. Marmary, C.M. Goldsmith, L. Baccaglini, S.L. Wang, R.B. Wellner, A.T.M.S. Hoque, J.C. Atkinson, H. Yamagishi, H. Kagami, A.F. Parlow, J.L. Chao, Polarized secretion of transgene products from salivary glands in vivo, Hum. Gene Ther. 10 (1999) 2789–2797.
[2] D. Castle, A. Castle, Intracellular transport and secretion of salivary proteins, Crit. Rev. Oral Biol. Med. 9 (1998) 4–22.
[3] S.U. Gorr, S.G. Venkatesh, D.S. Darling, Parotid secretory granules: crossroads of secretory pathways and protein storage, J. Dent. Res. 84 (2005) 500–509.
[4] J. Adriaansen, P. Perez, C.M. Goldsmith, C. Zheng, B.J. Baum, Differential sorting of human parathyroid hormone after transduction of mouse and rat salivary glands, Hum. Gene Ther. 19 (2008) 1021–1028.
[5] K.E.S. Poole, J. Reeve, Parathyroid hormone – a bone anabolic and catabolic agent, Curr. Opin. Pharmacol. 5 (2005) 612–617.
[6] H.C. Blair, M. Zaidi, Osteoclastic differentiation and function regulated by old and new pathways, Rev. Endocr. Metab. Disord. 7 (2006) 23–32.
[7] J.T. Potts, Parathyroid hormone: past and present, J. Endocrinol. 187 (2005) 311–325.
[8] M.W. Freeman, K.M. Wiren, A. Rapoport, M. Lazar, J.T. Potts Jr., H.M. Kronenberg, Consequences of amino-terminal deletions of preproparathyroid hormone signal sequence, Mol. Endocrinol. 1 (1987) 628–638.
[9] A.C. Karaplis, S.K. Lim, H. Baba, A. Arnold, H.M. Kronenberg, Inefficient membrane targeting, translocation, and proteolytic processing by signal peptidase of a mutant preproparathyroid hormone protein, J. Biol. Chem. 270 (1995) 1629–1635.
[10] J.F. Habener, M. Rosenblatt, P.C. Dee, J.T. Potts Jr., Cellular processing of preproparathyroid hormone involves rapid hydrolysis of the leader sequence, J. Biol. Chem. 254 (1979) 10596–10599.
[11] D.V. Cohn, J. Elting, Biosynthesis, processing, and secretion of parathormone and secretory protein-I, Recent Prog. Horm. Res. 39 (1983) 181–209.
[12] K.M. Wiren, J.T. Potts, H.M. Kronenberg, Importance of the propeptide sequence of human preproparathyroid hormone for signal sequence function, J. Biol. Chem. 263 (1988) 19771–19777.
[13] J.T. Potts, T.J. Gardella, Progress, paradox, and potential: parathyroid hormone research over five decades, Ann. NY Acad. Sci. 1117 (2007) 196–208.
[14] X. He, C.M. Goldsmith, Y. Marmary, R.B. Wellner, A.F. Parlow, L.K. Nieman, B.J. Baum, Systemic action of human growth hormone following adenovirus-mediated gene transfer to rat submandibular glands, Gene Ther. 5 (1998) 537–541.
[15] C. Delporte, B.C. OConnell, X.J. He, I.S. Ambudkar, P. Agre, B.J. Baum, Adenovirus-mediated expression of aquaporin-5 in epithelial cells, J. Biol. Chem. 271 (1996) 22070–22075.

[16] C.Y. Zheng, J.M. Vitolo, W.T. Zhang, F. Mineshiba, J.A. Chiorini, B.J. Baum, Extended transgene expression from a nonintegrating adenoviral vector containing retroviral elements, Mol. Ther. 16 (2008) 1089–1097.
[17] A.T.M.S. Hoque, L. Baccaglini, B.J. Baum, Hydroxychloroquine enhances the endocrine secretion of adenovirus-directed growth hormone from rat submandibular glands in vivo, Hum. Gene Ther. 12 (2001) 1333–1341.
[18] T.M. Murray, L.G. Rao, P. Divieti, F.R. Bringhurst, Parathyroid hormone secretion and action: evidence for discrete receptors for the carboxyl-terminal region and related biological actions of carboxyl-terminal ligands, Endocr. Rev. 26 (2005) 78–113.
[19] M. Usatii, L. Rousseau, C. Demers, J.L. Petit, J.H. Brossard, M. Gascon-Barre, J.R. Lavigne, R.J. Zahradnik, P. D'Amour, Carboxyl-terminal fragments of PTH inhibit rPTH(1–34) induced 1,25(OH)(2) vitamin D synthesis and renal CYP28B1 mRNA expression in parathyroidectornized rats, J. Bone Miner. Res. 21 (2006) S442 (abstract).
[20] M. Usatii, L. Rousseau, C. Demers, J.L. Petit, J.H. Brossard, M. Gascon-Barre, J.R. Lavigne, R.J. Zahradnik, E.F. Nemeth, P. D'Amour, Parathyroid hormone fragments inhibit active hormone and hypocalcemia-induced 1,25(OH) 2D synthesis, Kidney Int. 72 (2007) 1330–1335.
[21] S.K. Lim, T.J. Gardella, H. Baba, S.R. Nussbaum, H.M. Kronenberg, The carboxy-terminus of parathyroid-hormone is essential for hormone processing and secretion, Endocrinology 131 (1992) 2325–2330.
[22] B.J. Baum, In vivo veritas: the power of in situ manipulation of cells in a living animal, Am. J. Physiol. Cell Physiol. 297 (2009) 1333–1335.