Supporting Information

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SI Materials and Methods

Patients and Samples. The sample cohort comprised only patients with hypercalcemia, elevated PTH, and no evidence of secondary hyperparathyroidism. Deidentified, linked clinical data were maintained in a password-protected Health Insurance Portability and Accountability Act-compliant database.

Preparation of Dispersed Parathyroid Cells. Representative research samples were collected from pathologic samples in the operating room immediately after resection and were transported in cold (4 °C) RPMI (Invitrogen/GIBCO) with 100 U/mL penicillin and 100 μg/mL streptomycin to the laboratory for immediate processing. Parathyroid tissue was digested and dispersed parathyroid cells were isolated by a modification of the method of Brown et al. (1).

Parathyroid tissue was minced into 1- to 2-mm fragments and washed with the transport buffer at room temperature three to four times until the supernatant was visually clear of blood, fat, or other material. Dispersed cells were released from the washed tissue fragments by digestion with 10–15 mL of 1 mg/mL collagenase (Sigma) in RPMI. The tissue suspension was agitated gently on a magnetic stirring plate at 37 °C for 40–45 min with aspiration into a 10-mL plastic pipette every 15 min to mechanically disrupt the tissue. The turbid cell suspension was passed through a 100-μm mesh cell strainer (BD Biosciences). The released cells were then recovered by centrifugation at $400 \times g$ for 4 min. The cell pellet was resuspended and washed with RBC lysis buffer (155 mM NH_4Cl, 0.1 mM Sodium EDTA, 10 mM $KHCO_3$) until a clear supernatant was observed. The dispersed parathyroid cells were resuspended and maintained in Keratinocyte-SFM (Invitrogen/GIBCO) supplemented with the manufacturer's provided media supplements and with $CaCl_2$ added to a final concentration of 1.25 mM.

Dispersed parathyroid cells were suspended in PBS supplemented with 2% FBS and 2 μg/mL propidium iodide. Nonviable and fragmented cells were excluded by gating for the absence of propidium iodide uptake and for forward scatter threshold size using standard parameters at 30 psi (air pressure) and with a 70-μm nozzle. The cells pass the laser at a speed between 2,000 and 7,000 cells per second. Viable (P1 fraction) and single cells (P2 fraction) were selectively captured, and subpopulations were preparatively isolated on the basis of forward and side light scatter parameters (P3, P4, and P5 fractions) using a BD FACS Vantage flow cytometer with DIVA software (Becton Dickinson).

Electron Microscopy and Light Microscopy. After viable sorting, freshly isolated P3, P4, and P5 cells were rinsed in PBS, fixed in 4% glutaraldehyde, and then pelleted. The cell pellets were subsequently encased in molten 1% agar. Encased cell pellets were washed and postfixed in 1% OsO_4, all in cacodylate buffer. They were then washed in veronal acetate buffer (VA) and further stained in 0.5% uranyl acetate in VA. Dehydration was achieved in a series of ascending ethanol concentrations, followed by propylene oxide. Cell pellets were infiltrated with a 50/50 mixture of propylene oxide and epoxy resin (Poly/Bed; Polysciences, Inc.), followed by 100% resin. Cell samples were then baked overnight at 60 °C, and sections were cut on a Reichert-Jung Ultracut S (Leica Microsystems, Inc.), poststained with uranyl acetate and lead citrate, and viewed in a Philips CM12 (FEI Co.) electron microscope at 80 KV. Digital micrographs were taken on a 2 Vu digital 2k × 2k camera (Advanced Microscopy Techniques, Corp.).

For light microscopy, isolated P4 and P5 cells were deposited onto standard charged adhesion microscope slides at a density of 3×10^4 cells per slide using a Shandon cytospin centrifuge. The cells on the slides were fixed with 4% paraformaldehyde-PBS (CalBioChem/EMD Biosciences) for 15 min and permeabilized with 0.1% Triton X-100 (G Bioscience) for 15 min at room temperature. After blocking with 3% BSA/PBS, the cells were incubated with an anti-calcium-sensing receptor (anti-CASR) monoclonal antibody (Clone 5C10; Thermo-Fisher) at a 1:1,000 dilution, followed by an Alexa Fluor 488 goat anti-mouse IgG secondary antibody (Invitrogen). Slides were counterstained with DAPI in the mounting media (Prolong Gold; Invitrogen) to facilitate nuclear visualization. Samples were examined with an Axiovert 25 CFL inverted fluorescence microscope (Carl Zeiss) using 20× and 63× oil objective lenses. Images were acquired with an AxioCam MRm camera using AxioVision Rel. 4.8 software (Carl Zeiss). Parathyroid adenoma tissue was rinsed in PBS to remove blood, fixed in 3.7% formaldehyde, and embedded in paraffin. The paraffin-embedded tissue sections were deparaffinized through sequential immersion in xylene and ethanol following standard methods. After blocking with 5% normal goat serum in PBS, the sections were incubated with an anti-CD3 rabbit polyclonal antibody (Cat. No. IS503; Dako) at a 1:50 dilution, and reactivity was visualized by diaminobenzidine (DAB) staining and with hematoxylin/eosin counterstaining. Samples were examined with a Leica DM5500B microscope (Leica). Images were acquired with a DFC290 camera using Leica Application Suite version 2.8.1 software (Leica).

Immunoblotting and Flow Cytometry. Peripheral blood lymphocytes (PBLs) were isolated from patient-matched whole blood by Ficoll-Paque Plus (Amersham Biosciences) density gradient centrifugation. Dispersed parathyroid cells and PBLs were incubated with FITC, allophycocyanin (APC), or PE-Cy7 conjugated antibodies (anti-CD45 clone 30-F11, anti-CD3 clone HIT3a, anti-CD19 clone HIB19, anti-CD24 clone SN3, and anti-CD44 clone IM7; eBioscience) at 4 °C for 1 h, washed, and analyzed by flow cytometry using a BD FACS Canto II instrument with DIVA software. Dispersed parathyroid cells were incubated with PerCP/Cy5.5 anti-human CD4 antibody (Clone OKT4; BioLegend) and anti-CD8, PE antibody (Clone HIT8a; BD Biosciences) at room temperature for 25 min, washed, and analyzed by flow cytometry using a BD FACSAria II instrument with DIVA software. For immunotyping analysis of P3 cells in dispersed parathyroid cell preparations, the P3 population was gated according to forward and side light scatter parameters. Data were analyzed using FlowJo version 9.3.3 software.

Measurement of Intact PTH Secretion. Flow-sorted parathyroid cells were stimulated immediately with 0 mM, 0.5 mM, 1.25 mM, and 3.0 mM $CaCl_2$ for 2 h at 2×10^4 cells per well in 48-well plates, and the media were collected. After a brief centrifugation step, secreted intact PTH levels were measured in the clarified media using the intact PTH Turbo assay (Roche).

Intracellular Calcium Flux. Dispersed parathyroid cells were cultured in Keratinocyte-SFM (GIBCO) overnight before performing the calcium-flux assays. Calcium-stimulated intracellular calcium flux was determined using the Fluo-4 NW Calcium Assay (Molecular Probes/Invitrogen). Briefly, 45 min before testing, growth media were removed from the culture plates and replaced with the Fluo-4

AM agent dissolved in calcium-free Hanks' Balanced Salt Solution containing 20 mM Hepes and 2.5 mM probenecid as recommended by the manufacturer (Molecular Probes/Invitrogen). After fluorochrome loading, the cells were harvested and stimulated. Data acquisition was performed on a BD FACS Canto II (Becton Dickinson). Fluorescent output in the FL-1 channel was collected for 30 s before calcium addition and then for an additional 4 min after calcium addition. Calcium flux response was visualized from these data using the kinetic analysis module in FlowJo version 9.3.3 software (Tree Star, Inc.).

Quantitative Reverse Transcription-PCR. Total RNA was isolated using the RNeasy Micro Kit (Qiagen) and was reverse transcribed with oligo-dT primers and Quantiscript reverse transcriptase (Invitrogen) in a final volume of 20 μL for 50 min at 42 °C. CASR primers used for PCR amplification were 5′-GTGTGGAGTG TCCTGATGGG-3′ (forward) and 5′-CACGACAGAAACTC-GATCTC-3′ (reverse). For quantitative analysis of messenger RNA (mRNA) abundance, the resulting complementary DNAs were PCR amplified in a Rotor-Gene Q (Qiagen) with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Quantification of target-gene expression was obtained using Rotor-Gene Q Series Software. Relative units estimated from the quantification represent the ratio between specific mRNA molecules and GAPDH mRNA molecules in each sample.

Clonality Assays. Parathyroid adenoma samples and matched ipsilateral normal gland biopsies were embedded in O.C.T. Compound (Sakura) and snap frozen in liquid nitrogen. Samples were kept at −80 °C until use. Six serial 10-μm sections were cut with a cryostat and stained with hematoxylin and eosin for histological examination. Parathyroid cells were captured from the sections using a PixCell II Laser Capture Microdissection System (Arcturus). Peripheral blood lymphocytes (PBLs) were isolated from patient-matched whole blood using Ficoll-Paque Plus (Amersham Biosciences). Genomic DNA extraction from isolated flow-sorted parathyroid cell populations, peripheral blood lymphocytes, and parathyroid adenoma tissue was performed using Puregene Core Kit B (Qiagen). Clonality at the HUMARA locus was determined via restriction enzyme digestion and PCR amplification following the procedure described by Allen et al. (2). The PGK clonality assay was performed as described by Gilliland et al. (3).

For the human androgen receptor (HUMARA) clonality assay, five genomic DNA sources [from (*i*) PBL, (*ii*) whole tumor tissue, (*iii*) microdissected frozen sections, (*iv*) preparatively sorted P4, and (*v*) preparatively sorted P5 cells] from each patient were analyzed. For each DNA sample, two reactions were prepared: (*i*) 200 ng of DNA digested with 10 U of HhaI (NEB) and (*ii*) a no-enzyme control. All restriction-enzyme reactions were in a 100-μL total volume in the manufacturer's optimized buffer, and all incubations were overnight at 37 °C. After restriction-enzyme digestion, the DNA was purified by phenol:chloroform: isoamyl alcohol (25:24:1; Sigma) organic phase extraction followed by ethanol precipitation. The DNA was resuspended in water and added to a 30-μL total volume PCR containing oligonucleotide primers, KOD Hot Start DNA Polymerase (Novagen/EMD), and dNTPs. The primer sequences were as follows: primer 1, 5′-GCTGTGAAGGTTGCTGTTCCTCAT-3′; and primer 2, 5′-TCCAGAATCTGTTCCAGAGCGTGC-3′, as described by Allen et al. (2). Samples were amplified using an MJ Mini Personal Thermal Cycler (Bio-Rad) for 36 cycles (each comprising 20 s at 95 °C, 10 s at 60 °C, and 15 s at 70 °C), with an initial denaturation at 95 °C for 2 min. The PCR products were loaded onto a 20% polyacrylamide/TBE gel (Invitrogen), and electrophoresis was performed at 200 W for 3.5 h. The gel was stained with EtBr in TBE buffer, and bands were visualized under UV light in an AlphaInnotech Alpha Imager EP gel documentation system.

1. Brown EM, et al. (1978) Dispersed cells prepared from human parathyroid glands: Distinct calcium sensitivity of adenomas vs. primary hyperplasia. *J Clin Endocrinol Metab* 46(2):267–275.
2. Allen RC, Zoghbi HY, Moseley AB, Rosenblatt HM, Belmont JW (1992) Methylation of HpaII and HhaI sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation. *Am J Hum Genet* 51(6): 1229–1239.
3. Gilliland DG, Blanchard KL, Levy J, Perrin S, Bunn HF (1991) Clonality in myeloproliferative disorders: Analysis by means of the polymerase chain reaction. *Proc Natl Acad Sci USA* 88(15): 6848–6852.