ISSN 2472-1972

In Vitro Control of Genes Critical for Parathyroid Embryogenesis by Extracellular Calcium

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Background: The expression of the parathyroid transcription factors, encoded by the genes *GATA3*, *GCM2*, and *MAFB*, persists after parathyroid morphogenesis. This suggests a role of these genes in the regulatory program that governs parathyroid function in the adult. Indeed, these 3 genes form a transcriptional cascade able to activate *PTH* gene expression.

Materials and Methods: Adult adenoma parathyroid tissues were put in primary cell culture to evaluate the messenger ribonucleic acid (mRNA) expression of the *PTH* gene, of the genes involved in the calcium regulatory signaling pathway (*CaSR*, *GNA11*, and *AP2S1*), and of the 3 genes (*GATA3*, *GCM2*, and *MAFB)* involved in the parathyroid morphogenesis in the presence of different extracellular calcium concentrations from 0.1 mM to 3.0 mM.

Aim: The aim of the study was to investigate whether different extracellular calcium conditions could control the expression of transcription factors critical for parathyroid embryogenesis.

Results: The results of the experiments showed that the mRNA expression of *GATA3*, *GCM2*, and *MAFB* genes follows the same response as the *PTH* gene to extracellular calcium concentrations, with the highest expression at low calcium (0.1 mM) and the lowest at high calcium (3.0 mM). Conversely, the genes involved in the calcium signaling in the parathyroid cells showed a variable response to the extracellular calcium concentrations, with the *CaSR* and *GNA11* genes exhibiting a sensitivity to low calcium concentrations.

Conclusions: These findings indicate that transcription factors recognized for their role in parathyroid embryogenesis show a response to extracellular calcium later in adulthood that parallels the behavior of the *PTH* gene.

Freeform/Key Words: parathyroid cells, parathyroid development, calcium, *GCM2*, *GATA3*, *MAFB*

Parathyroid glands serve a pivotal function in the regulation of calcium and phosphate homeostasis by secreting parathyroid hormone (PTH), which controls the serum calcium concentration through the increase of calcium absorption from bone and calcium reabsorption

Received 11 February 2020
Accepted 22 May 2020
First Published Online 25 May 2020
Corrected and Typeset 30 June 2020

in the kidney. Parathyroid chief cells, the most abundant cells of these glands, are able to detect small changes in blood ionized calcium levels, modifying PTH release via a calcium-sensing receptor (CaSR) [1].

The discovery that transcription factors active in the embryogenesis of the parathyroid glands were also expressed postnatally in the parathyroid tissue prompted investigation of their role in adult parathyroid physiology [2]. The transcription factors essential for the development of the parathyroid glands—GATA binding protein 3 (GATA3), glial cells missing 2 (GCM2), and transcriptional activator v-maf muscoloaponeurotic fibrosarcoma oncogene homologue B (MAFB) appear all to be part of a genetic cascade capable of directly regulating *PTH* gene expression. Indeed, in the rodent model the complex of the three parathyroid morphogenesis genes controls *Pth* gene expression through the stimulation of its promoter in a hierarchic sequence, where *Gata3* is the gene activated more upstream and *MafB* is activated the more downstream, with *Gcm2* acting in the middle [3,4].

Gata3 knockout mouse embryos lack *Gcm2* expression and have defects in the third and fourth pharyngeal pouches, including absent parathyroid-thymus primordia [5]. *Gata3* transactivates the *Gcm2* gene by binding specifically to a double-*GATA*-motif within the *Gcm2* promoter. In addition, *Gata3* cooperates with *Gcm2* and *MafB* to activate the *Pth* gene expression with the ubiquitous specific protein 1 transcription factor [6].

GATA3 gene (OMIM# 146255) sits in chromosome 10p14 in humans. Its encoded product was shown to have an important role in embryogenesis, development, and cell differentiation, not only in the parathyroid glands and the thymus, but also in several other organs and tissues, including kidney, breast, nervous system, lymphocytes, and hair follicles [7-9]. Haploinsufficiency of the *GATA3* gene, caused by various types of mutations, including point mutations as well as small- and large-scale deletional mutations, cause a very rare autosomal dominant genetic hypoparathyroidism deafness renal dysplasia syndrome with the triad of affected organs variably manifested in affected patients [10]. The *GATA3* gene is located in a site on chromosome 10p13/14 where the critical DiGeorge region II is sitting and terminal deletions (10p14-10pter) are linked to the hypoparathyroidism deafness renal syndrome, whereas interstitial deletions in the 10p13-14 site are linked to the DiGeorge phenotype [11]. Moreover, the *GATA3* gene is expressed in adult parathyroid cells and can be used as a specific immunohistochemical biomarker for cells of parathyroid origin [12,13].

In *Drosophila*, the zinc-finger type transcription factor glial cell missing (GCM) acts as a developmental binary switch between glia and neurons. In mammals, there are 2 orthotics, GCM1/GCMA and GCM2/GCMB, linked respectively to placental and parathyroid development [3]. In *Gcm2*-null mice, parathyroid precursor cells die of apoptosis, and terrestrial vertebrates missing the parathyroid glands develop hypocalcemia and hyperphosphatemia, as observed in hypoparathyroidism, and die soon after birth [5,14]. In humans, the 5 exons of the *GCM2* gene (OMIM# 603716) on chromosome 6p24.2 encode a protein of 506 amino acids, and inactivating mutations in the gene were causal of hypoparathyroidism in kindreds affected by autosomal recessive or dominant disease [15,16]. The gene continues to be expressed in the adult [3], and its role in parathyroid physiology was investigated. Using rat parathyroid cells in culture [17] Kawahara et al showed that Gcm2 binds to the *PTH* gene 5' promoter to regulate its transcription [18]. A series of analyses indicate that GCM2 subsequently controls serum calcium concentration by modulating *CaSR* gene expression and promoting PTH secretion [19]. The absence or reduction of GCM2 transcription factor, both in vivo in mice and in human parathyroid cultured cells treated with *GCM2* gene siRNA, correlates with lack of or decreased expression of the *CaSR* gene, a marker of differentiation for the parathyroid cells [19].

v-MAF muscolo-aponeurotic fibrosarcoma oncogene homologous B (MAFB), is a transcription factor member of the MAF family, characterized by a basic leucine zipper region, affecting transcription positively or negatively, depending on its partner proteins [20]. MAFB plays important roles in the developmental processes of various tissues, as well as in cell-type-specific gene expression, by binding directly to MAF-recognition element–related sequences either in their promoter or enhancer regions. *MafB* gene knockout in mice has shown that its encoded protein regulates respiratory rhythmogenesis in the brain [21], monocyte and osteoclast differentiation

[22,23], and maturation of pancreatic islet α and β cells [24]. MAFB also stimulates the expression of tissue-specific genes such as F4/80 in macrophages, glucagon, and insulin in pancreatic islets [25,26]. MAFB is essential for the later steps of parathyroid development, which involve the separation from the thymus and the migration toward the thyroid gland. *MafB* expression persists after morphogenesis and in adult parathyroid glands [27]. MAFB and GCM2 interact with each other, binding directly to an evolutionally conserved region of the *PTH* gene promoter, and synergistically activate its transcription [28]. In recent studies, *Maf* heterozygote (*Maf*+/-) or knockout (*Maf*-/-) mice with induced secondary hyperparathyroidism do not develop an increased secretion of Pth, nor an enlargement of the parathyroid glands through a reduction of *Pth* and *Ccnd1* (cyclin D1) genes expression [4]. These findings suggest that *MAFB* may represent a new therapeutic target in secondary hyperparathyroidism. In humans, *MAFB* gene (OMIM# 608968) localizes on chromosome 20q12.2-q13.1, and mutations of the gene have been correlated with multicenter carpotarsal osteolysis [29] and Duane retraction syndrome 3, with or without deafness [30].

Besides the effect on *PTH* gene expression, neither the role of these transcription factors on parathyroid cell development and function nor the regulation of their expression in the parathyroid tissue is fully understood. The functional machinery that controls parathyroid physiology encompasses extracellular ionized calcium, CaSR, guanine nucleotide-binding protein subunit alpha-11 (GNA11), adaptor related protein complex 2 subunit sigma 1 (AP2S1), and PTH. The CaSR, a guanine nucleoside-binding protein (G-protein-coupled receptor [GPCR]), is abundant in the parathyroid glands and in the kidneys, where it is pivotal in the regulation of PTH secretion and in the renal excretion of calcium. Loss-of-function mutations in the *CaSR* gene lead to 3 hypercalcemic disorders: familial hypocalciuric hypercalcemia type 1, neonatal severe hyperparathyroidism, and primary hyperparathyroidism (PHPT) [31,32]. Conversely, the activating mutations of this gene cause a hypocalcemic disorder, known as autosomal dominant hypocalcemia syndrome [33]. The *GNA11* gene encodes for the subunit α11 in the trimeric G-protein and mediates the signaling of GPCRs, and the *AP2S1* gene encodes for the adaptor protein-2 σ subunit, which plays a role in the internalization of membrane proteins, including GPCRs [34]. Inactivating mutations of *GNA11* and *AP2S1* genes are responsible, respectively, for familial hypocalciuric hypercalcemia type 2 and type 3 [35].

In this study, for the first time, the expression of transcriptional factors *GATA3*, *GCM2*, and *MAFB* was evaluated in a human model of primary cultures of parathyroid adenomatous cells, along with the expression of the functional genes *CaSR*, *GNA11*, *AP2S1*, and *PTH*, under low, normal, and high extracellular calcium concentrations.

Materials and Methods

Sample acquisition

Fresh parathyroid adenoma tissue was obtained during parathyroidectomy for PHPT at Careggi and Santa Maria Nuova hospitals in Florence, Italy, between April 2018 and July 2019. Eight parathyroid tissues from 8 patients, were collected during this time period.

All patients with PHPT, sporadic and nonsyndromic, had a single parathyroid adenoma. PHPT was diagnosed based on serum calcium and intact PTH levels in the absence of other possible causes of hypercalcemia (Table 1). Of the 8 PHPT patients, 4 were male and 4 female, with a mean age of 66 ± 12. The mean size of a parathyroid adenoma was 2.2 ± 0.5 × 1.5 ± 0.5 cm. This study was approved by the Regional Ethics Committee for Clinical Trials of the Tuscany Region (12599_bio, April 2018).

Cell Cultures

Knife biopsies of the waste tissues obtained from surgery were immediately placed in McCoy's 5A medium (Sigma Aldrich, St Louis, MO, US) supplemented with sterile 22 mM HEPES (Sigma Aldrich), 100 IU/mL penicillin, and 100 μg/mL streptomycin, pH 7.4,

Table 1. Pre-operative calcium (normal reference serum range 8.5-10.5 mg/dL) and PTH (normal reference serum range 10–77 pg/mL) levels of the parathyroid adenomas

Parathyroids	Calcemia (mg/dL)	PTH (pg/mL)
1	14.6	813
2	12	702
3	14	754
4	10.6	222
5	11.8	180
6	14	1166
7	11.8	933
8	10.8	137

transported to the laboratory, and processed within 30 min from excision. The parathyroid tissues were trimmed of extraneous fatty tissue and its capsule of fibrous connective, sliced into ~ 0.2 to 0.5 mm thickness with a knife, and placed into collagenase type I 0.3 mg/ml (C-0130; Sigma Aldrich) in Coon's modified Ham's F-12 medium supplemented with fetal bovine serum (FBS) 20%, 100 IU/mL penicillin, 100 µg/mL streptomycin, overnight at 37°C in humidified air with 5% CO_2. The following day, once the collagenase was removed by washing 3 times with serum-free medium, the cells were mechanically dispersed by vigorous pipetting through 10 mL pipettes. The digested tissues were then incubated in 100 mm culture dishes in Coon's modified Ham's F-12 medium (1.12 mM calcium) supplemented FBS 10% (0.1 mM calcium), 100 IU/mL penicillin, 100 µg/mL streptomycin, incubated at 37°C in humidified air with 5% CO_2. After 24 h, once parathyroid cells were attached to the plate, they were detached with trypsin 0.4 mg/mL, collected, and transferred into 48-multiwell plates in Coon's modified Ham's F-12 medium, with FBS 10%, 100 IU/mL penicillin, 100 µg/mL streptomycin, incubated at 37°C in humidified air with 5% CO_2. After another 4 days, the medium was replaced with Dulbecco's modified eagle's medium (DMEM) and Ham's F-12 Nutrient Mixture (Sigma Aldrich), calcium-free medium, with 3 different calcium concentrations added for 1 h (0.1 mM, 1.2 mM, and 3.0 mM).

Immunofluorescence staining

The intracellular PTH and the CaSR proteins were evaluated with immunofluorescence staining in adenoma parathyroid cells after 5 days in culture in growth Ham's F12 Coon's modified medium with an addition of 10% FBS. The cells were then transferred into glass slides an incubated in DMEM medium containing 1.2 mM calcium and after 24 h fixed in 4% paraformaldehyde in Dulbecco's phosphate-buffered saline (DPBS) for 20 min at room temperature and permeabilized in 0.5% Triton X-100 in DPBS for 10 min at room temperature. Nonspecific binding sites were blocked with 2% bovine serum albumin (BSA) in DPBS for 30 min at room temperature. The staining for the PTH was done using an anti-PTH (monoclonal rabbit, SAB5500159, Sigma-Aldrich, MO, US) [36] primary antibody 1:100 in 2% BSA/DPBS for 4 h at room temperature in a humidified chamber and a fluorescein isothiocyanate conjugated anti-rabbit immunoglobulin G goat host (F0382, Sigma-Aldrich, MO, US) [37] secondary antibody 1:100 in 2% BSA/DPBS for 60 min in the dark at room temperature in humidified chamber. The staining for the CaSR was done using 1:100 anti-CaSR (monoclonal anti-human, MA1-934, clone 5C10, ADD, Thermo Fisher Scientific, Waltham, MA, US) [38] primary antibody in 2% BSA/DPBS overnight at 4°C. Revelation was obtained using 1:50 fluorescein isothiocyanate-rabbit anti-mouse immunoglobulin G secondary antibody (SA1-10651, Thermo Fisher Scientific, Waltham, MA, US) [39] in 2% BSA/DPBS for 60 min at room temperature. Cell nuclei were counterstained with 10^{-5}M propidium iodide (Invitrogen, Carlsbad, CA, US) for 30 min in the dark at room temperature in a humidified chamber after RNA digestion with 500 µg/ml RNase I-A (Sigma-Aldrich, St. Louis, MO, US) for 30 min at room temperature. Samples were rinsed 3 times with 2% BSA/DPBS between

each step. Cells were observed in laser scanning confocal microscopy using a LSM510META microscope equipped with Ar/ML458/477/488/514, HeNe543, HeNe633 laser lines (ZEISS, Oberkochen, Germany).

Messenger ribonucleic acid collection, reverse transcription, and gene expression

Cultured cells were then washed with phosphate-buffered saline (PBS), mixed with a lysis solution, containing reagents to inactivate endogenous RNAases, also added with DNAase I, and incubated at room temperature for 5 min. Cells were lysed during this incubation and RNA released into the lysis solution. A stop solution was then added to inactivate the lysis. Cell lysates were reverse transcribed to synthesize complementary deoxyribonucleic acid using a 20× enzyme mix and a 2× reverse transcription buffer. Finally, the complementary deoxyribonucleic acid was amplified by real time polymerase chain reaction (RT-PCR) using a Taqman® Gene Expression Master Mix (TaqMan® Gene Expression Cells-to-C_T ™ Kit, Thermo Fisher). The genes amplified were actin beta (*ACTB*) as housekeeping gene and *PTH*, *CaSR*, *GATA3*, *GCM2*, *MAFB*, *GNA11*, and *AP2S1* with Rotor-Gene System (Qiagen®).

Taqman primers and probes

Primers and probes (IDT DNA Technologies®) for comparative RT-PCR (Rotor-Gene Q Software) with calibrator conditions fixed in 1.2 mM calcium, were reported in Table 2. The RT-PCRs were conducted in triplicate according to the manufacturer's protocol (Taqman® Gene Expression Cells-to-C_T ™ Kit, Thermo Fisher).

Statistics

Descriptive statistics (mean and standard deviation) related to the expression of each gene (*PTH*, *GATA3*, *GCM2*, *MAFB*, *CASR*, *GNA11*, and *AP2S1*), in different calcium concentrations, were analyzed for each parathyroid. Statistical significance of the differences between means was evaluated through a mixed model approach, taking into account the 3 levels of calcium in the medium (0.1, 1.2, and 3.0 mM) as fixed factor. Random factors were the triplicates and the different parathyroids. For each test omnibus, Bonferroni-corrected post-hoc tests were conducted. Experiment-wise alpha-level was $\alpha_t = 0.05$. The statistical analyses were performed using R software.

Results

Phase contrast microscopy observations showed a characteristic morphology of in vitro-grown parathyroid glandular parenchyma (Fig. 1A), and immunocytochemistry confirmed the presence of the CaSR and PTH proteins in the presence of 1.2 mM calcium (Fig. 1B and C).

The 8 parathyroid cell primary cultures obtained were evaluated for in vitro calcium responsiveness.

Table 3 reports the results of descriptive statistics related to single parathyroid preparations. With regard to *PTH*, the 8 parathyroids had a higher value at 0.1 mM calcium compared to 1.2 mM and to 3.0 mM. Together with the *PTH*, all the transcription factors analyzed, *GATA3*, *GCM2*, and *MAFB*, demonstrated the same tendency in the response to extracellular calcium concentration in the majority of the parathyroids tested. With regard to the *CaSR* and *GNA11*, the majority of the parathyroid preparations showed the same tendency when comparing 0.1 mM to 1.2 mM, with a higher value in the lower calcium conditions and an increase in the expression at 3 mM calcium.

These trends were confirmed at an inferential level considering the mean values of each condition. With regard to the *PTH*, the mean values were different with respect to the

Table 2. Genes primers and probes

Genes	*Primer Forw (5'–>3')*	*Primer Rev (5'–>3')*	*Probe (5'/56-FAM/ZEN/3IABkFQ/-3')*
ACTB	TCGTCGCCCACATAGGAA	CTCCGGCATGTGCAAGG	TGATGGTGGGCATGGGTCAGAAG
PTH	ATGCATAAGCTGTATTTCACCAC	GACATGGCTAAAGTTATGATTGTCA	TCGGATGGGAAATCTGTTAAGAAGAGATCT
CaSR	GGCTGCTGTTTATCTCCTCTATG	CGGAGTCTGTGGAATGTATCAG	TGGGTTTCGCTGGTTACAGGCTAT
GATA3	CCTGGACTTGCATCCGAA	CCCATCACCACCTACCC	CCGAGTACAGCTCCGGACTCTT
GCM2	CGCTTCTTCTAGCTTCTGTCTC	TGATGGCAACGCGATCTT	TCAGGCCAAGGGAGTTCATGATCA
MAFB	GGGTTCATCTGCTGGTAGTT	GCTCAGCACTCCGTGTAG	TCGAGGATCTGTACTGGATGGCGA
AP2S1	CGTTTAAGACCTCCACGAAGT	CCTCTACTTCTGCATCTGTGTG	TGACAACAACCTGGCTTACCTGGA
GNA11	ATGGCACTGACGTACTGATG	GACGCTCAAGATCCTCTACAAG	TACGAGCAGAACAAGGCCAATGCG

different levels of calcium (0.1 mM low, 1.2 mM physiological, 3.0 mM high) ($F_{(2,179)}$ = 5.39, P = 0.0053), with the mean value observed at 0.1 mM calcium being significantly higher than the value at 1.2 mM (t = −2.84, P = 0.0051) and the value at 3.0 mM (t = −2.95, P = 0.0036). As expected, the *PTH* gene expression was modulated *i*n vitro with an inversely proportional tendency to extracellular calcium concentrations (Fig. 2A).

The *GATA3* gene expression was influenced by the different extracellular calcium concentrations with a trend similar to the *PTH* gene ($F_{(2,73)}$ = 13.97, P < 0.0001), with the mean value at 0.1 mM higher than the value at 1.2 mM calcium (t = −3.30, P = 0.0015) and 3.0 mM calcium (t = −5.23, P < 0.0001). Therefore, the regulation of the *GATA3* gene expression by the extracellular calcium concentrations paralleled the one of the *PTH* gene (Fig. 2B).

Also for the expression of the *GCM2* gene, statistically significant differences of the previous genes were observed ($F_{(2,84)}$ = 5.24, P = 0.0071). The value at 0.1 mM calcium was higher than the value at 1.2 mM (t = −2.50, P = 0.0145) and the value at 3.0 mM (t = −3.02, P = 0.0034) and this response paralleled what seen for the *PTH* and *GATA3* genes (Fig. 2C).

The mean values of the *MAFB* gene were also different with respect to the 3 levels of calcium ($F_{(2,85)}$ = 6.02, P = 0.0036), where the mean value observed at 0.1 mM calcium was higher than the value at 1.2 mM (t = −2.50, P = 0.0145) and the value at 3.0 mM (t = −3.33, P = 0.0013). Therefore, the expression of the *MAFB* gene followed the same trend as the *PTH*, *GATA3*, and *GCM2* genes with an inverse correlation with the extracellular calcium concentrations (Fig. 2D).

The results of the analysis of the effect on the parathyroid calcium response cascade showed that the *CaSR* gene expression was modulated by the 3 different extracellular calcium concentrations ($F_{(2,101)}$ = 4.58, P = 0.0126), with the mean value of the *CaSR* gene

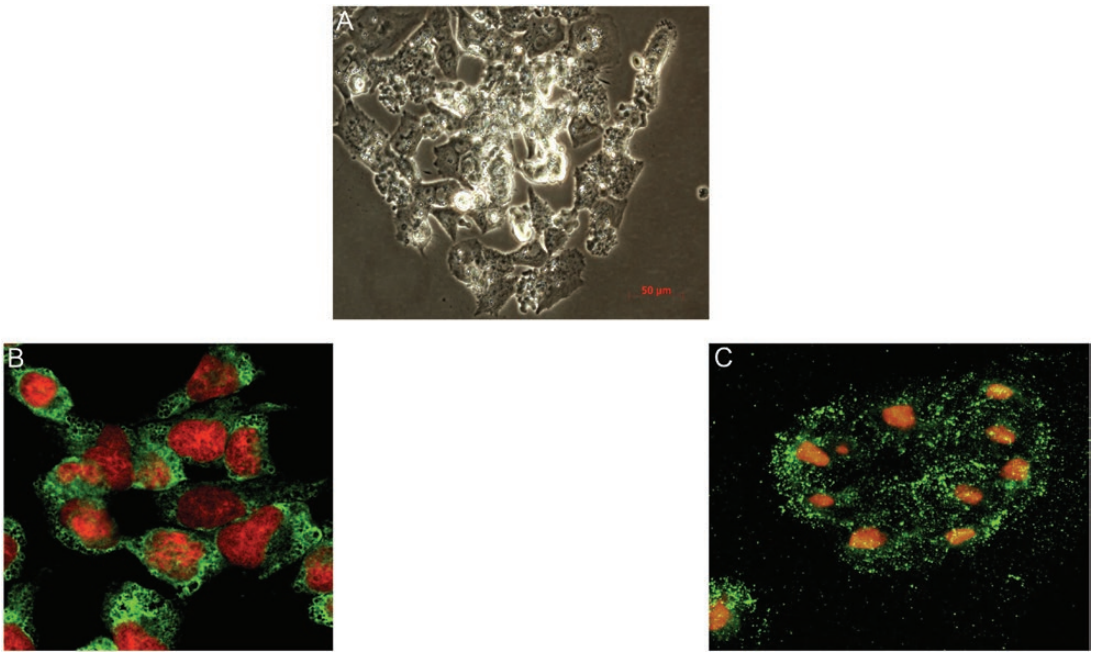

Figure 1. The intracellular PTH and CaSR proteins were evaluated with immunofluorescence staining in adenoma parathyroid cells after 5 days in culture with growth medium and then transferred into glass slides and incubated for 24 h in DMEM medium containing 1.2 mM calcium before the phase contrast analysis and the immunofluorescence staining. (A) Phase contrast image of an adenoma parathyroid primary culture (40× original magnification). (B) Parathyroid cells stained for CaSR (in green) and counterstained for nuclei (in red). Observations with laser scanning confocal microscopy, objective 40× original magnification. (C) Parathyroid cells stained for PTH (in green) and counterstained for nuclei (in red). Observations with laser scanning confocal microscopy, 40× original magnification.

Table 3. Descriptive statistics (mean and standard deviation) of single parathyroid preparations

Gene	Parathyroids	0.1 mM calcium	1.2 mM calcium	3.0 mM calcium
PTH	1	1.16 ± 0.33	0.79 ± 0.34	0.71 ± 0.16
	2	1.93 ± 0.35	1.06 ± 0.38	0.99 ± 0.53
	3	1.08 ± 0.23	0.71 ± 0.16	0.78 ± 0.16
	4	1.97 ± 1.13	0.78 ± 0.26	1.12 ± 0.24
	5	1.80 ± 0.97	1.03 ± 0.23	0.83 ± 0.25
	6	20.51 ± 19.77	1.01 ± 0.23	0.22 ± 0.28
	7	1.03 ± 0.28	0.93 ± 0.43	0.82 ± 0.09
	8	1.26 ± 0.43	1.01 ± 0.13	0.81 ± 0.15
GATA3	1	1.49 ± 0.33	1.02 ± 0.21	0.73 ± 0.10
	2	1.51 ± 0.11	1.01 ± 0.16	1.18 ± 0.21
	3	1.31 ± 0.40	1.02 ± 0.19	0.95 ± 0.18
	4	1.46 ± 0.64	1.02 ± 0.25	0.72 ± 0.12
	5	2.27 ± 0.45	1.23 ± 0.70	0.49 ± 0.11
	6	0.29 ± 0.05	0.24 ± 0.07	0.24 ± 0.03
	7	1.20 ± 0.35	1.07 ± 0.52	0.91 ± 0.32
	8	1.71 ± 1.13	1.01 ± 0.17	0.32 ± 0.14
GCM2	1	1.19 ± 0.41	1.01 ± 0.21	0.85 ± 0.12
	2	1.56 ± 0.34	1.43 ± 0.14	1.36 ± 0.30
	3	1.21 ± 0.40	1.03 ± 0.27	1.03 ± 0.25
	4	10.56 ± 2.25	1.36 ± 0.86	0.05 ± 0.02
	5	1.32 ± 0.52	1.04 ± 0.31	1.02 ± 0.29
	6	0.87 ± 0.09	0.77 ± 0.09	0.73 ± 0.14
	7	1.15 ± 0.35	0.97 ± 0.41	0.72 ± 0.18
	8	1.70 ± 1.11	1.01 ± 0.17	0.83 ± 0.25
MAFB	1	2.14 ± 1.15	1.03 ± 0.27	0.97 ± 0.50
	2	1.32 ± 0.54	1.01 ± 0.14	0.98 ± 0.16
	3	1.62 ± 0.30	1.07 ± 0.40	0.34 ± 0.11
	4	0.94 ± 0.17	1.05 ± 0.34	0.94 ± 0.16
	5	0.53 ± 0.12	0.55 ± 0.10	0.54 ± 0.19
	6	0.79 ± 0.12	0.77 ± 0.03	0.76 ± 0.04
	7	0.88 ± 0.25	0.80 ± 0.33	0.78 ± 0.17
	8	2.05 ± 1.38	1.01 ± 0.16	0.98 ± 0.47
CaSR	1	0.44 ± 0.12	0.33 ± 0.19	0.65 ± 0.37
	2	1.16 ± 0.20	1.01 ± 0.15	0.95 ± 0.15
	3	1.37 ± 0.40	1.01 ± 0.13	1.02 ± 0.22
	4	3.24 ± 1.63	1.06 ± 0.38	2.44 ± 0.57
	5	2.85 ± 0.36	1.15 ± 0.77	3.65 ± 0.54
	6	1.22 ± 0.21	1.05 ± 0.30	0.90 ± 0.16
	7	1.09 ± 0.26	0.95 ± 0.15	0.89 ± 0.08
	8	1.46 ± 0.96	1.01 ± 0.11	0.65 ± 0.17
GNA11	1	3.45 ± 5.80	1.71 ± 1.81	3.01 ± 4.56
	2	0.95 ± 0.15	1.01 ± 0.15	1.01 ± 0.22
	3	1.47 ± 0.19	1.01 ± 0.13	1.16 ± 0.18
	4	2.63 ± 0.60	1.02 ± 0.23	2.77 ± 0.48
	5	3.21 ± 1.88	1.19 ± 0.83	5.00 ± 0.99
	6	0.99 ± 0.21	1.07 ± 0.28	0.83 ± 0.26
	7	1.03 ± 0.09	0.88 ± 0.07	0.57 ± 0.16
	8	2.03 ± 1.47	1.31 ± 0.15	0.91 ± 0.36
AP2S1	1	1.40 ± 0.44	1.02 ± 0.75	1.22 ± 0.43
	2	1.33 ± 0.96	1.39 ± 0.71	1.06 ± 0.59
	3	1.02 ± 0.49	1.34 ± 0.64	1.56 ± 0.75
	4	1.09 ± 0.71	0.99 ± 0.30	1.25 ± 0.46
	5	1.08 ± 0.31	1.33 ± 0.59	1.34 ± 0.43
	6	1.23 ± 0.39	1.07 ± 0.22	1.16 ± 0.38
	7	1.18 ± 0.12	0.91 ± 0.19	1.53 ± 0.48
	8	1.35 ± 0.53	1.13 ± 0.33	1.34 ± 0.61

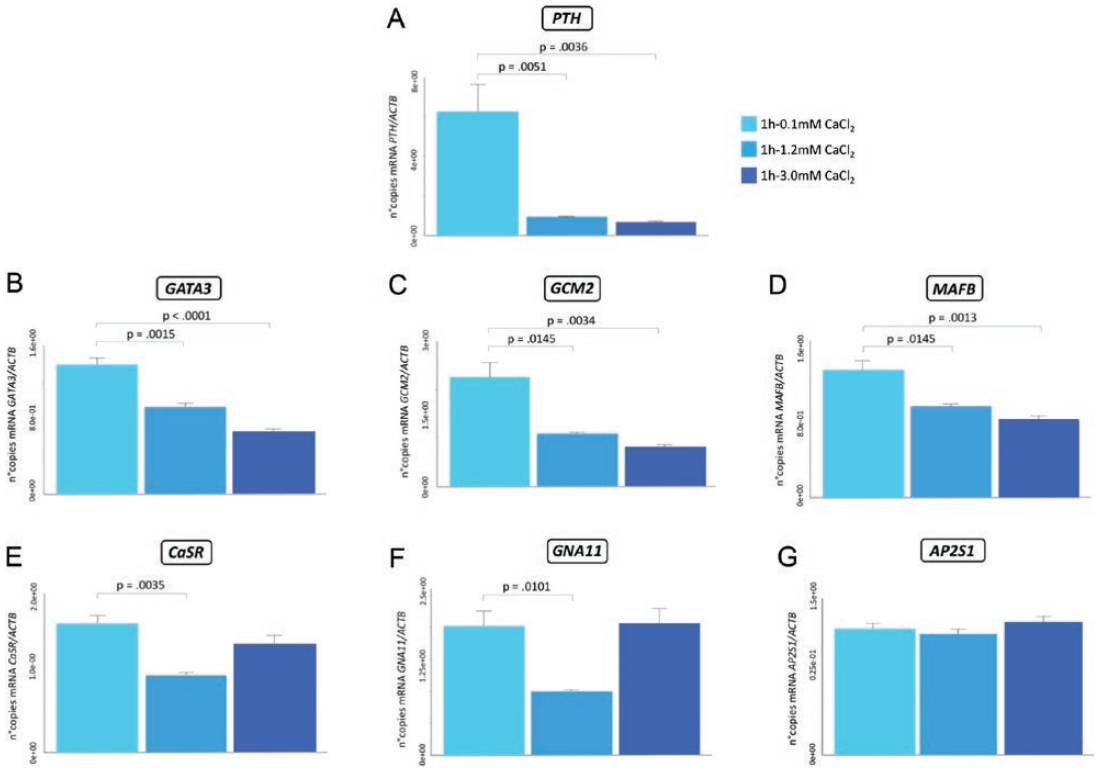

Figure 2. Genes expression: *PTH* (A), *GATA3* (B), *GCM2* (C), *MAFB* (D), *CaSR* (E), *GNA11* (F), and *AP2S1* (G) evaluated by RT-PCR in 8 different parathyroid cell cultures. messenger ribonucleic acid expression was evaluated in 3 calcium concentrations (0.1 mM, 1.2 mM, and 3.0 mM calcium) for 1 h. Data are normalized against *ACTB* messenger ribonucleic acid.

observed at 0.1 mM calcium being higher than the one at physiological concentration of 1.2 mM (t = 2.99, $P = 0.0035$). However, no differences were observed at 3.0 mM extracellular calcium concentrations when compared to 1.2 mM calcium. The expression of the *CaSR* gene was modulated between 0.1 mM and 1.2 mM, with the same trend as the previously mentioned genes, but with a different behavior at high extracellular calcium concentrations (Fig. 2E).

Similarly, the *GNA11* gene expression showed a response that paralleled the profile of the *CaSR* gene with a statistically significant difference at the 3 calcium levels ($F_{(2,97)} = 3.44$, $P = 0.036$) and a mean value at 1.2 mM calcium, lower when compared to the mean value at 0.1 mM (t = 2.62, $P = 0.0101$) (Fig. 2F).

Finally, for *AP2S1* gene expression, no statistically significant differences were seen between the 3 tested calcium concentrations ($F_{(2,101)} = 0.33$, $P = 0.721$). Unlike the expression of other genes analyzed, the *AP2S1* gene was not modulated with the increase of the extracellular calcium concentrations (Fig. 2G).

The expression of all the genes described in this paper was assessed in the cultured cells exposed to growth medium just before the challenge with various calcium concentrations in DMEM medium, and the results obtained were superimposable to what observed for each sample challenged with 1.2mM calcium (data not shown).

Discussion

Normal parathyroid cells have a very slow rate of proliferation, and in vitro primary cell cultures derived from human parathyroid cells are very difficult to obtain. Therefore, primary cultures of human parathyroid glands have mainly been derived from parathyroid

adenomas and hyperplastic glands coming from patients with hyperparathyroidism, which were shown to have a higher proliferative activity compared to normal glands [26,31]. However, the culture of human parathyroid cells presents several difficulties. There is an overgrowth of fibroblasts, which becomes evident after 8 to 10 days of culture, together with a slow in vitro proliferation exhibited by these cells, and by a decreased expression of the *CaSR* at messenger ribonucleic and protein levels. This behavior causes a decreased sensitivity of the CaSR receptor to the extracellular calcium. Moreover, a reduced calcium sensing function and calcium receptor expression, seen in adenomatous and hyperplastic cells, have been attributed to various degrees of functional dedifferentiation [32].

Besides all the limitations that pathologic human parathyroid tissues present, cells cultured from parathyroid adenomatous and hyperplastic tissues represent an accepted model for in vitro parathyroid physiopathology investigations.

Mouse embryology has made it possible to characterize the genetic control of parathyroid development. The embryonic formation of the parathyroid glands, together with the thymus, includes a complex transcriptional network that involves *HOXA3*, *PAX1/PAX9*, *EYA1*, *TBX1*, *SOX3*, and *SIX1/SIX4* genes, able to control the formation of the parathyroid/thymus common primordium [40,41]. After the generation of the third pouch, the parathyroid domain can be distinguished by the expression of the *GCM2* gene, fundamental for parathyroid survival and differentiation. In mice that lack *HOXA3*, *PAX1/PAX9*, *EYA1*, and *SIX1/SIX4* genes, parathyroid glands are either absent or hypoplastic. These genes are mediated by the effect of the *GCM2* gene, whose absent expression causes apoptosis of the parathyroid chief cells [42]. The parathyroid developing cascade includes also *GATA3* and *MAFB* genes [5,27]. Interestingly, the 3 genes, *GCM2*, *GATA3*, and *MAFB*, maintain an expression postnatally when they control the expression of the *PTH* gene [3,4].

In humans, congenital disorders were essential for understanding the role of the genes important in mouse parathyroid development, as mutations of the genes involved in mouse parathyroid morphogenesis can translate into hypoparathyroidism or hyperparathyroidism syndromes [10,11,15,16,43]. However, the role of developmental transcription factors in adult parathyroid physiology and pathophysiology is still unclear.

To understand the interactions of *GATA3*, *GCM2*, and *MAFB* genes with the calcium response machinery, primary cultures obtained from human parathyroid adenomas were utilized to evaluate the effect of different extracellular calcium concentrations on the expression of both morphogenesis and functional genes. The results obtained show for the first time an in vitro role of extracellular calcium in controlling the expression of *GATA3*, *GCM2*, and *MAFB* genes in a parallel fashion to the response of the *PTH* gene. What would be the significance of the increased *GATA3*, *GCM2*, and *MAFB* genes expression in the parathyroid cells at low extracellular calcium concentrations? A potential explanation for this finding can be read in the cooperation of the three transcription factors, known to activate the *PTH* gene [3,4], with low calcium concentrations. The mirror condition would be one of high calcium concentrations where the 3 transcription factors are inhibited by calcium with a potentiation of calcium inhibitory effect on PTH secretion. This can certainly lead to future pharmacological interventions of great interest for both hyper- and hypo-functional parathyroid conditions.

An interesting finding of the experiments carried out in this study is the increase in the expression of the *CaSR* and *GNA11* genes at low extracellular calcium concentrations, without a response of the *AP2S1* gene, the other known component of the parathyroid calcium response cascade. Regarding the potential transactivation of the *CaSR* gene by extracellular calcium, the results are controversial. Theoretically, a control exerted by calcium on *CASR* gene expression could interfere with the function of the calciostat for the receptor and, therefore, disturb the calcium homeostasis. The original studies, which excluded an effect of calcium in controlling the *CaSR* gene, were conducted in rats infused with calcium [44]. However, in those pioneering studies, the lowest calcium concentration was 0.7 mM. This concentration cannot exclude the possibility that a rescue function of increased *CaSR* gene expression could exist at very low extracellular calcium concentrations, in order to

counteract an excessive secretion of PTH. This interpretation could easily encompass the stimulating effect that the higher *GCM2* gene expression, observed at low extracellular calcium concentrations, could exert on the expression of the *CaSR* gene through P1 and P2 promoters [19]. The different response to extracellular calcium observed for the other 2 genes of the calcium response cascade, *GNA11* and *AP2S1* genes, are not easily interpretable. The different physical positions of the encoded proteins—one, *GNA11*, close to the cellular membrane and the other, *AP2S1*, intracellular—could explain the lack of response of the *AP2S1* gene to extracellular calcium concentrations.

In conclusion, the present in vitro study has shown for the first time that genes involved either in the development or in the function of the parathyroid glands are themselves controlled by extracellular calcium concentrations. These findings prompt new avenues of investigation that could unravel still unrecognized molecular mechanisms that control the parathyroid chief cells in physiological and pathological conditions.

Acknowledgments

This paper was supported by Fondazione Italiana Ricerca sulle Malattie dell'Osso (F.I.R.M.O.).

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Disclosure Summary: The authors have nothing to disclose

Data Availability: All data generated or analyzed during this study are included in this published article or in the data repositories listed in References.

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