THE INDUCTION OF C/EBPβ CONTRIBUTES TO VITAMIN D INHIBITION OF ADAM17 EXPRESSION AND PARATHYROID HYPERPLASIA IN KIDNEY DISEASE

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ABSTRACT

BACKGROUND: In secondary hyperparathyroidism (SHPT), enhanced parathyroid levels of transforming growth factor-α (TGFα) increase EGF receptor (EGFR) activation causing parathyroid hyperplasia, high PTH and also reductions in vitaminD receptor (VDR) that limit vitaminD suppression of SHPT. Since anti-EGFR therapy is not an option in human SHPT, we evaluated ADAM17 as a therapeutic target to suppress parathyroid hyperplasia because ADAM17 is required to release mature TGFα, the most potent EGFR-activating ligand.

METHODS: Computer analysis of the ADAM17 promoter identified TGFα and C/EBPβ as potential regulators of the ADAM17 gene. Their regulation of ADAM17 expression, TGFα/EGFR-driven growth and parathyroid gland (PTG) enlargement was assessed in promoter-reporter assays in A431 cells and corroborated in rat and human SHPT, using erlotinib as anti-EGFR therapy to suppress TGFα signals, active vitaminD to induce C/EBPβ, or the combination.

RESULTS: While TGFα induced ADAM17-promoter activity by 2.2-fold exacerbating TGFα/EGFR-driven growth, ectopic C/EBPβ expression completely prevented this vicious synergy. Accordingly, in advanced human SHPT, parathyroid ADAM17 levels correlated directly with TGFα and inversely with C/EBPβ. Furthermore, combined erlotinib+calcitriol treatment suppressed TGFα/EGFR-cell growth and PTG enlargement more potently than erlotinib in part through calcitriol induction of C/EBPβ to inhibit ADAM17 promoter activity, mRNA and protein. Importantly, in rat SHPT, the correction of vitaminD deficiency effectively reversed the resistance to paricalcitol induction of C/EBPβ to suppress ADAM17 expression and PTG enlargement, reducing PTH by 50%.

CONCLUSION: In SHPT, correction of vitaminD and calcitriol deficiency induces parathyroid C/EBPβ to efficaciously attenuate the severe ADAM17/TGFα synergy, which drives PTG enlargement and high PTH.

Key words: EGF receptor tyrosine kinase inhibitor, TGFα, transcriptional regulation, Vitamin D receptor.

Short summary: This work identifies an ADAM17/TGFα synergy that exacerbates EGFR-driven parathyroid hyperplasia, and a safe anti-ADAM17 strategy to improve treatment of SHPT: TGFα induces ADAM17 gene expression aggravating the vicious cycle of ADAM17 release of mature TGFα that enhances TGFα/EGFR-driven hyperplasia. Instead, correction of vitaminD and calcitriol deficiency induceC/EBPβ to down-regulate the ADAM17 gene thus attenuatingparathyroid gland enlargement and PTH elevations..

INTRODUCTION

Secondary hyperparathyroidism (SHPT) is a common and serious complication of chronic kidney disease-mineral bone disorders (CKD-MBD)[1, 2]. The degree of parathyroid hyperplasia determines both the severity of SHPT and the response to therapy[3, 4]: The former by increasing the gland capacity for parathyroid hormone (PTH) synthesis and secretion[2], and the latter by reducing parathyroid levels of the vitaminD receptor (VDR), the calcium sensing receptor (CaSR), and the anti-aging protein α-klotho, a co-receptor for FGF23[5]. All three receptors mediater the suppression of cell growth and serum PTH by vitaminD, calcium, or elevations of FGF23 (Refs). Importantly, parathyroid hyperplasia precedes the reductions in parathyroid CaSR[6]. Instead, hyperplastic growth and VDR reductions occur simultaneously and are caused by EGF receptor (EGFR) activation[7]. Since the calcitriol-VDR complex induces CaSR and klotho gene expression[8, 9], the reductions in both proteins with parathyroid hyperplasia could be secondary to VDR loss. Thus, inhibition of EGFR activation should effectively attenuate the progression of SHPT and improve the response to treatment. Fig.1A summarizes the molecular link between EGFR activation, hyperplastic growth and VDR loss.

In rat and human SHPT, the severity of parathyroid EGFR activation is determined by CKD-induced increases in parathyroid levels of transforming growth factor-α (TGFα, the most potent EGFR-activating ligand)[7, 10, 11], which are aggravated by high serum phosphate (P), low calcium (Ca) or vitaminD deficiency[11, 12]. Enhanced TGFα/EGFR signals induce the synthesis of Activator protein-2 (AP2), a transactivator of the TGFα gene[13] and of LIP (for Liver Inhibitory Protein), an oncogene and a transrepressor of the VDR gene[7, 14] TGFα/EGFR-induction of CUG-BP1 phosphorylation and its binding to the third in-frame AUG codon of the single C/EBPβ mRNA[15] (Fig. 1B) makes LIP translation prevail over that of the two other CAAT-enhancer binding protein-β (C/EBPβ* and C/EBPβ), both potent growth suppressors[16] . LIP lacks the transactivation domain but binds C/EBP sequences on

DNA with higher affinity than either C/EBPβ molecule[14] LIP acts as an oncogene and a VDR gene suppressor by competing with C/EBPβ homodimers for DNA-binding, or by forming LIP/C/EBPβ heterodimers, transcriptionally inactive to induce growth arrest[14] and VDR gene expression[7]. In nodular hyperplasia, the worst form of human SHPT, the highest TGFα levels concur with the highest LIP content and proliferation rates, and with the lowest VDR mRNA and protein[7]. Accordingly, inhibition of EGFR activation using erlotinib, a highly specific EGFR tyrosine-kinase inhibitor, effectively prevented TGFα induction of: its own gene, LIP levels, proliferation rates and reductions of VDR gene transcription, in A431 cells[7], in which growth is driven by TGFα and EGFR over-expression[17, 18]and also in established rat SHPT, in which only TGFα is enhanced[7]. In these uremic rats, a low calcitriol dose (4 ng thrice weekly) which failed to suppress PTH if given during week 4 after 5/6nephrectomy (NX) due to VDR loss, became effective if preceded by erlotinib treatment during week 3[7]. This strategy, however, was insufficient for calcitriol suppression of parathyroid hyperplasia[7].

Since erlotinib treatment is not an option in human SHPT, we searched for a target upstream from TGFα activation of the EGFR to prevent PTG growth. We focused on ADAM17, the enzyme that cleaves the membrane TGFα precursor releasing mature TGFα to the circulation, thus enhancing autocrine, paracrine and endocrine TGFα/EGFR-growth[19]. In mouse CKD, systemic inhibition of ADAM17 activity attenuated angiotensin II-induced TGFα/EGFR-driven proteinuria and damage to the renal parenchyma as effectively as global TGFα gene deletion or renal-specific EGFR inactivation[20]. However, current ADAM17 inhibitors are highly toxic to be used therapeutically in SHPT[21, 22]. To overcome this limitation, a computer analysis of the ADAM17 promoter searched for targetable regulators of ADAM17 gene expression. The identification of 13 putative C/EBP and 4 AP2 binding sites suggested that enhanced parathyroid TGFα/EGFR induction of LIP[7]and AP2[13] could increase ADAM17 gene expression further aggravating growth rates and VDR loss. Importantly,

it also revealed a potentially safe anti-ADAM17 strategy: calcitriol (1,25D) induction of parathyroid C/EBPβ, as demonstrated in other cell types[23-26]. Therefore, herein, in vivo, ex vivo and in vitro protocols tested the hypothesis that simultaneous treatment with erlotinib and calcitriol should enhance erlotinib potency to suppress parathyroid growth through an effective induction of C/EBPβ to inhibit ADAM17 gene expression.

In addition, because 25-hydroxyvitaminD (25D) enhances calcitriol/VDR-antiproliferative actions in various cancer cell types[27], we examined whether this 25D/calcitriol synergy could compensate for parathyroid VDR loss and induce C/EBPβ repression of the ADAM17-gene and PTG enlargement without erlotinib. To evaluate the contribution of 25D conversion to calcitriol to the 25D+active vitaminD synergy,calcitriol treatment had to be substituted by its analog, paricalcitol[28].

METHODS

Cell Culture and Proliferation Assays: The human epidermoid carcinoma cell line A431 (ATCC) was grown in 10%FBS DMEM (Invitrogen) at 37°C in 5%CO_2. Either 10^6 (10-cm plate) or 10^4 cells (96-well plates) were synchronized at G_0 using serum-free DMEM for 8h, and treated with erlotinib (in DMSO), 1,25-dihydroxyvitaminD (in ethanol) or the combination in 2%FBS DMEM for 60h followed by 1%BSA DMEM up to 84h.

The colorimetric 3-(4,5 dimethylthiazol-2-yl)2-5-diphenyl tetrasodium bromide assay kit (Chemicon International) measured A431 proliferation.

RT-PCR for human ADAM17: Total RNA was extracted using RNA-Bee (TEL-TEST) and quantified by UV-VIS spectrophotometer (Nanodrop Technologies). First-strand cDNA was obtained from 2μg RNA using Omniscript Reverse Transcription reagents (Qiagen). Cycling conditions for RT-PCR for ADAM17 and cyclophilinB were: 5' at 94°C, 40 or 24 cycles, respectively, of 30" at 94°C, 30" at 57°C or 54°C respectively, 45" at 72°C, with final 5'extension at 72°C. RT-PCR products were

electrophoresed in 1% agarose gels, visualized using a transilluminator (Sigma Chemical T1202) and quantified with ImageJ. Primer sequences were: ADAM17: forward:5-TCATTGACCACGTGAGCATC-3; reverse:5-TCGTCCATATGTGAGTCTGTGC-3; CycB: forward:5-GTGATCTTTGGTCTCTTCGG-3; reverse:5-CGATGATCACATCCTTCAGG-3.

Plasmids:

ADAM17-luciferase reporter: The human ADAM17 promoter fragment [-2305 to -1 before ATG] containing 13 C/EBP and 4 AP2 putative binding sites, was PCR amplified with forward primer:5-GATAAACTAATTAATCTATCC-3 and reverse primer:5-GAGTCGGTAGCGGGGCCGGGAAC-3, subcloned into T-vector (pBluescript II), sequenced, and inserted into pGL2-Basic vector (between Kpn I and Hind III).

Expression vector exclusive for human C/EBPβ: The LIP's ATG (Met) was replaced by TCC (Ser) to impede the initiation of LIP translation (Fig. 1). The 5'-fragment of the human mutant C/EBPβ was PCR amplified, forward primer:5-TATGGAAGTGGCCAACTTCTAC-3 and reverse primer:5-AGGATCCTGCGCCGCCGCCCGGCGC-3; and the human mutant C/EBPβ 3'-fragment with forward primer:5-AGGATCCGCGGCGGGCTTCCCGTACGCG-3 and reverse primer:5-ATCTAGACTAGCAGTGGCCGGAGGAGG-3. PCR fragments were cloned into T-vector (pBluescript II) and sequenced. 5'- and 3'-fragments were assembled with BamHI, and subcloned into expression vector pcDNA3.

Promoter-reporter assays: 1μg of the human ADAM17 luciferase reporter and 0.1μg of the β-galactosidase expression plasmid[7] with or without 0.1μg of the C/EBPβ expression vector were transiently transfected (Myrus Transfection reagents, using 4μl/1μg of DNA following manufacturer's protocols) into A431 cells, plated at a concentration of 3×10^5 cells/ml media/well. pGEM DNA was added when required to maintain the total amount of transfected DNA constant. Upon an overnight incubation, cells were treated with vehicle or TGFα 8nM for 24h, then lysed, and luciferase and β-

galactosidase activities measured using Luciferase reporter system (Promega) and Galacto-Light (Applied Biosystems).

Protocols for rat SHPT: Female Sprague-Dawley rats (200-225 g) underwent 5/6NX as in[7] and were fed a high P diet for 4 weeks (0.9% P, 0.6% Ca; Dyets). Protocol 1: Rats received from week 2 to 4, either vehicle (200µl of DMSO), erlotinib (6mg/kgbw, daily, in 100µl propylene glycol as in[7]; Genentech), 1,25-dihydroxyvitaminD (4ng thrice weekly in 200µl of propylene-glycol:PBS 1:1), or the combination.

Protocol 2; One week after 5/6NX, rats received either vehicle, 25-hydroxyvitaminD (800ng weekly) to correct vitamin D deficiency; paricalcitol (16ng, equivalent to 4 ng calcitriol in PTH suppression[28], thrice weekly) or the combination, for 3 weeks. A group of 5/6NX rats were fed a low P diet (0.2%P; 0.5%Ca) throughout the study.

At sacrifice, blood was drawn for analytical determinations. PTG were removed and weighed (Cahn Instruments). For immunohistochemistry, glands were fixed in formalin and paraffin included. Plasma P, creatinine, ionized Ca, and intact PTH levels were measured as described[7]. All animal protocols were approved by the Animal Study Committee at Washington University.

Human PTG: Cryopreserved 1-2 mm^3 sections of 32 human PTG were obtained at parathyroidectomy from advanced CKD patients (Vall d'Hebron Hospital, Barcelona, Spain, kindly provided by Dr. Galicia)

Immunohistochemistry: Antigen retrieval, quenching and blocking in rat PTG was performed as described[7]. Primary antibodies against ADAM17 (Genetex, 1:50), C/EBPβ (Biolegend, 1:50, directed to an epitope in the N-terminal domain absent in LIP), and PCNA (Biolegend 1:500) were added overnight at 4ºC, followed by biotinylated secondary antibody and streptavidin–horseradish peroxidase conjugate. Immune complexes were visualized with aminoethyl carbazole substrate-chromagen

(Histostain-plus, Invitrogen). For negative controls, one section was left without primary antibody. ADAM17, C/EBPβ and PCNA were quantified in at least three sections per gland, as in[12]. A semi-quantification scoring:1+(low intensity);4+(high intensity) evaluated PTGs from the 25D/paricalcitol protocol.

For human PTG, upon an overnight exposure with the primary antibody: ADAM17 (Abcam 1:200); TGFα (GF10, Calbiochem 1:20); VDR (Millipore 1:200); C/EBPβ (Biolegend 1:100), sections were washed, the appropriate Alexa-fluor secondary antibodies and Hoechst added for 1h, and quantified with ImageJ.

Western Blots: A431 cells: whole cell and nuclear extracts were obtained using 1%RIPA Buffer/protease inhibitor cocktail (Roche), or Nuclear Extract Kit (Active Motif), respectively. Protein concentration was quantified by Bio-Rad Protein Assay (Bio-Rad Laboratories). Protein was resolved by either 10% or 12%SDS-PAGE, and electroblotted onto PVDF (Immobilon-P transfer membrane; Millipore) membranes. After probing overnight at 4°C with primary antibodies (Total C/EBPβ [Santa Cruz 1:1000, directed to an epitope in the C-terminal domain common to the 3 isoforms], ADAM17 [Genetex 1:1000]) blots were visualized by enhanced chemiluminescence (SuperSignal West Pico; Pierce), and quantified with ImageJ.

Statistical Analyses: ANOVA assessed statistical differences among all experimental groups, with Bonferroni tests (or unpaired *t* test analysis when indicated) to compare selected pair of groups.

RESULTS

Calcitriol/erlotinib synergy to suppress PTG growth involves induction of C/EBPβ

Figure 2A and table 1 show that, in rat established SHPT, treatment from week 2 to 4 after 5/6NX with erlotinib and calcitriol (at a selected dose, ineffective to suppress PTG enlargement to mimic calcitriol resistance due to VDR loss), had a higher potency than erlotinib alone in inhibiting proliferation, as measured by parathyroid PCNA and PTG weight/bw, and in enhancing PTH suppression by calcitriol from 16 to 34.5%. This higher potency was unrelated to differences in serum creatinine, Ca and P levels, and associated directly with the doubling of parathyroid C/EBPβ protein ($p<0.05$) and with marked reductions in parathyroid ADAM17 immunostaining (Fig. 2B). Neither monotherapy enhanced parathyroid C/EBPβ above the levels in untreated controls. To overcome the limitation in estimating parathyroid C/EBPβ/LIP ratio immunohistochemically, as LIP antibodies recognize C/EBPβ (See Fig. 1B), the contribution of increases in C/EBPβ/LIP ratio to suppress ADAM17 gene expression was examined in A431 cells, upon reproducing the down-regulation of TGFα/EGFR-growth by erlotinib and calcitriol observed in rat SHPT.

Calcitriol/erlotinib synergy to inhibit TGFα/EGFR-growth involves the induction of C/EBPβ:LIP ratio to suppress ADAM17 gene expression

The combination of erlotinib (0.25 μM)+calcitriol (10^{-7}M, a dose ineffective to suppress A431 growth), inhibited proliferation more potently than erlotinib alone (Fig. 3A), in part through a higher suppression of ADAM17-promoter activity (Fig. 3B). Only erlotinib+calcitriol treatment, which increased nuclear C/EBPβ:LIP ratio by 9-fold (Fig. 3C), reduced ADAM17 mRNA and protein expression (Fig. 3D and 3E).

ADAM17 gene expression is induced by TGFα and suppressed by C/EBPβ

Treatment of A431 cells with 8nM TGFα for 48h caused a 2.2-fold increase in ADAM17-promoter activity (Fig. 4A), which was reduced by 43% through ectopic expression of C/EBPβ.

Accordingly, immunofluorescent analysis of human hyperplastic glands (Fig. 4B,Top) demonstrated that the highest ADAM17 levels co-localized with the highest cell membrane TGFα expression, both whole and ADAM17-cleaved TGFα precursors, (Left). Instead, the lowest ADAM17 and TGFα expression concurred with the highest nuclear C/EBPβ and VDR content (Right). Furthermore, Fig. 4B, (bottom panels) shows that parathyroid ADAM17 strongly correlated directly with TGFα (r^2=0.571, p<0.007) and inversely with C/EBPβ (r^2=0.572, p<0.03).

Next, we examined whether, in rat SHPT, 25D enhancement of calcitriol/VDR antiproliferative actions sufficed to induce parathyroid C/EBPβ to suppress ADAM17 and PTG enlargement despite VDR loss. To measure whether 25D conversion to calcitriol contributes to the 25D/calcitriol synergy, paricalcitol replaced calcitriol treatment.[28]

25-hydroxyvitaminD+paricalcitol suppresses parathyroid ADAM17 and gland enlargement in rat SHPT as effectively as erlotinib+calcitriol

Fig. 5 shows that despite similar serum creatinine, P, total and ionized Ca among all experimental groups (Table 2), and also despite the normalization of serum 25D in rats receiving 25D, only rats receiving the 25D+paricalcitol combination from week 1 after 5/6NX could prevent further PTG enlargement reducing PTH by 50%. This reduction in PTG enlargement associated to a marked reduction (7.5-fold) of parathyroid ADAM17 protein and a 2-fold increase in parathyroid C/EBPβ over untreated controls, a potency similar to that of dietary P restriction (1.95-fold p<0.01). Serum calcitriol was similar in rats receiving combined 25D+paricalcitol compared to those receiving paricalcitol alone. Thus, normalization of serum 25D sufficed to reverse the resistance to a low dose of paricalcitol in doubling C/EBPβ and in suppressing ADAM17 protein and PTG enlargement.

DISCUSSION

This work presents ADAM17 as a critical target upstream from EGFR activation to improve current vitaminD strategies to treat SHPT (Fig. 6), as it identified: 1) TGFα induction of ADAM17 promoter activity as the initiator of a powerful ADAM17-dependent autocrine loop that agravates the already enhanced TGFα/EGFR-growth signals; 2) C/EBPβ trans-repression of the ADAM17 gene as a mechanism by which active vitaminD therapy enhances the efficacy of anti-EGFR therapy; 3) A 25D/paricalcitol synergy that induces parathyroid C/EBPβ and attenuates the increases in parathyroid ADAM17 causing PTG enlargement, as efficacious as combined erlotinib-calcitriol treatment.

TGFα upregulation of ADAM17 gene expression provides a previously unrecognized pathogenic mechanism that aggravates the severe growth patterns associated with ADAM17/TGFα co-expression. Specifically, ADAM17 activity is essential to release mature TGFα, the stronger EGFR activator. In fact the ADAM17 null mouse dies perinatally due to severe developmental defects in the morphogenesis of epithelial organs including the PTG[29]. Undoubtedly, in human SHPT, ADAM17 activity is mandatory for the strong association between TGFα content and proliferation rates[7]. Furthermore, TGFα activation of the EGFR also increases ADAM17 stability[30], the phosphorylation required for ADAM17 translocation to the plasma membrane for activity[31, 32] and causes a 2.2-fold increase in ADAM17 gene expression. Clearly, TGFα induction of ADAM17 promoter activity and post-transcriptional modifications generate an ADAM17/TGFα synergy that may contribute to nodule formation, as over-expression of LIP in normal mouse mammary glands is sufficient to induce hyperproliferation and tumorigenesis[33]. The presence of several AP2 and C/EBP putative binding sites in the human ADAM17 promoter suggested that anti-EGFR therapy should prevent both TGFα induction of parathyroid AP2[13] and LIP[7] and consequently, ADAM17 gene upregulation. Since LIP antagonizes C/EBPβ actions, we examined whether part of C/EBPβ antiproliferative actions involved

ADAM17 gene suppression. We found that, in A431 cells, in which not only TGFα but also EGFR is overexpressed[17], ectopic C/EBPβ expression prevented the doubling of ADAM17 promoter activity induced by TGFα and reduced ADAM17 gene expression by 43%. This finding suggested that calcitriol induction of C/EBPβ[23-26] could provide asafe anti-ADAM17 strategy to break the deleterious ADAM17/TGFα synergy upstream from EGFR activation. The demonstration in hyperplastic PTG from patients with advanced CKD, that parathyroid ADAM17 content correlated directly with TGFα and inversely with C/EBPβ content led us to evaluate, in rat CKD, the efficacy of simultaneous anti-EGFR and anti-ADAM17 treatment in attenuating PTG hyperplasia. In established rat SHPT, in which only TGFα, but not EGFR, is overexpressed[7], simultaneous treatment with erlotinib, a suppressor of LIP synthesis, and calcitriol, an inducer of C/EBPβ expression, from week 2 to week 4 after 5/6NX, is more effective than erlotinib alone in attenuating further increases in PTG enlargement and serum PTH, despite similar serum levels of creatinine, Ca and P. The higher suppression of parathyroid cell growth by the erlotinib/calcitriol combination associated directly with the doubling of parathyroid C/EBPβ content, and with marked reductions in ADAM17 protein, supporting but not proving, the transcriptional control of the ADAM17 gene by increases in parathyroid C/EBPβ.

Next, studies in A431 cells conclusively demonstrated that inhibition of the ADAM17 gene mediated, at least in part, the higher potency of the erlotinib/calcitriol combination over that of erlotinib alone to inhibit TGFα/EGFR-driven growth. This improved outcome associated with a 9-fold elevation in C/EBPβ:LIP ratio, which resulted in significantly reduced ADAM17 mRNA and protein expression.

Since anti-EGFR is not a choice in human SHPT, and current ADAM17 inhibitors are highly toxic [21, 22], we also examined in rat SHPT whether 25D enhancement of calcitriol/VDR antiproliferative actions[27] could safely substitute for erlotinib reversal of the resistance to active vitaminD suppression of PTG growth caused by VDR loss. Intraperitoneal administration of 25D to normalize serum vitaminD

levels starting one week after 5/6NX, , effectively reversed the resistance to 16ng of paricalcitol in inducing C/EBPβ and suppressing ADAM17 expression and PTG enlargement, with a potency similar to either combined erlotinib+calcitriol treatment or P restriction, resulting in a 50% PTH reduction.

The mild elevations in serum 1,25D in rats treated with combined 25D+paricalcitol compared to those receiving paricalcitol alone suggested that local conversion of 25D to calcitriol could partially account for the synergy. However, direct activation of VDR antiproliferative actions by 25D should not be ruled out, as 25D can directly activate the VDR and also synergize with calcitriol in cells from the 1α-hydroxylase null mouse[34] or upon specific inhibition of 25D conversion to calcitriol[35].

This 25D/active vitaminD synergy is translationally relevant: Fist, it unraveled a novel mechanism supporting the recommendation by the KDIGO guidelines to correct vitaminD deficiency to improve outcomes in SHPT at all CKD stages. Secondly, it provided a potentially safe alternative for patients whose high serum calcium and phosphate levels preclude escalating the dose of paricalcitol to suppress PTH[28, 36, 37]. Thirdly, it demonstrated that the correction of vitaminD deficiency in CKD patients that are not receiving active vitaminD therapy may not suffice to reach sufficient 25D levels within parathyroid cells to compensate for VDR reductions and effectively attenuate ADAM17/TGFα-driven hyperplasia. Indeed, in CKD stage 3-4, vitaminD supplementation that normalized serum 25D and 1,25D levels, was ineffective in suppressing PTH[38-41]. Mechanistically, in hemodialysis patients, CKD-induced reductions in 25D uptake by circulating monocytes is corrected by calcitriol[42]. Similar defective 25D uptake may occur in the PTG due to CKD-induced decreases in the endocytic receptor megalin[43], highly expressed in the parathyroids[44]. Since active vitaminD induces megalin[45], combined 25D+active vitaminD could enhance both parathyroid 25D uptake and 25D/active vitaminD synergy for ADAM17suppression. A

similar synergy for 25D uptake by proximal tubular cells from the glomerularultrafiltrate should help maintain serum 25D levels[46].

An important consideration before the clinical application of this 25D+active vitaminD synergy to treat human SHPT is that in these pre-clinical studies, nutritional and active vitaminD were administered intraperitoneally. The oral formulations more commonly used in CKD patients could increase the risk for hypercalcemic and hyperphosphatemic epidoses as a result of synergic 25D/active vitaminD interactions enhancing intestinal calcium and phosphate absorption. Therefore, the safe transfer of this synergy to the bed site requires the identification of non-invasive markers of parathyroid ADAM17 inhibition.. Measurements of reductions in circulating mature TGFα levels in response to vitaminD therapy may l not be accurate, as they reflect both parathyroid and renal ADAM17 activity in CKD[20, 47]. Importantly, the results from the 25D+active vitaminD protocols also suggested that simple measurements of the ability of vitaminD supplementation alone or in combination with low doses of active vitaminD (either calcitriol or paricalcitol), to effectively normalize serum 25D above 30 ng/ml and reduce PTH by 50%, could help personalize nutritional and active vitamin D interventions to reach the appropriate parathyroid 25D/active vitamin D synergy for ADAM17 inhibition. This is a mandatory first step prior to the much needed prospective, well powered clinical studies.

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Table 1: Serum chemistry, body weight and changes in parathyroid features with treatment.

	Vehicle (n=7)	**erlotinib** (n=8)	**1,25D** (n=7)	**erlotinib+1,25D** (n=6)
ICa (mg/dl)	4.56± 0.08	4.68± 0.12	4.71± 0.07	4.76± 0.14
Creatinine (mg/dl)	1.38± 0.12	1.20± 0.12	1.20± 0.08	1.14± 0.10
Total Ca (mg/dl)	9.95± 0.16	10.32± 0.20	10.6± 0.10	10.43± 0.23
P (mg/dl)	7.74± 0.63	8.06± 0.64	7.16± 0.48	7.66± 0.63
PTH (pg/ml)	427.6± 120.0	414.9± 116.0	359.6± 62.5	280.0± 82.2
Body weight (g)	271.0± 4.8	272.4± 3.7	277.1± 4.7	272.0± 5.4
PCNA (+Nuclei/Area)	0.42± 0.08 (N=12)	0.21± 0.03 (N=14) *	0.28± 0.07 (N=9)	0.14± 0.05 (N=8) ***
CEBPβ (IOD/Area)	36.6± 5.3	37.7± 6.3	39.4± 5.5	56.6± 4.7*

Values indicate Mean±SEM in 5/6NX rats fed a high P (HP) diet and treated with vehicle, erlotinib (6 mg/kg bw, daily, ip in 100µl DMSO), calcitriol (1,25D, 4 ng thrice weekly in 200 µl of propyleneglycol:PBS 1:1)**,** or the combination, from week 2 to week 4 after 5/6NX. Abreviations: ICa=ionized calcium; PTH=parathyroid hormone; PCNA=Proliferating Cell Nuclear Antigen where n=number of rats and N=number of parathyroid glands examined. *, ** and *** indicate $p<0.05$; $p<0.01$ and $p<0.001$ vs. HP.

Table 2: Serum chemistries and parathyroid C/EBPβ content with treatment.

	Sham	LP	HP	HP+25D	HP+Pari	HP+25D+ Pari
Creatinine (mg/dl)	0.73±0.05	1.65±0.26	1.38±0.11	1.53±0.14	1.52±0.10	1.46±0.17
Total Ca (mg/dl)	9.86±0.11	10.56±0.22	9.82±0.15	9.68±0.40	9.70±0.26	9.63±0.26
ICa (mg/dl)	4.85±0.05	4.84±0.07	4.47±0.13	4.39±0.17	4.35±0.14	4.42±0.17
P (mg/dl)	5.14±0.27	5.13±0.39	8.12±0.92	7.78±1.25	9.03±0.68	8.08±1.00
PTH (pg/ml)	91.5±34.3	272.3±58.0	3,555.7±776.4	3,562±151	3098.2±437	1,550±819
25D (ng/ml)	32.2±2.2	22.1±6.8	16.6±4.0	27.4±7.4	17.9±3.3	33.1±9.8
1,25D (pg/ml)	125.3±17.8	83.6±29.7	94.3±27.9	89.7±16.7	29.7±7.6	37.8±3.3
C/EBPβ (Score)	---	3.25±0.25 (4)	1.67±0.33 ** (3)	2.0±1.0 (3)	2.0±0.58 (4)	3.0±0.41 ^ (4)

Values indicate Mean±SEM in at least 5 5/6NX rats fed either a low P (LP) or a high P (HP) diet and treated with vehicle (HP), 25D (800ng once weekly, ip in 200 µl of propyleneglycol:PBS 1:1); paricalcitol (Pari, 16 ng thrice weekly in 200 µl of propyleneglycol:PBS 1:1)**,** or the combination, from week 1 to week 4 after 5/6NX. Abreviations: ICa=ionized calcium; PTH=parathyroid hormone; 25D=25-hydroxyvitamin D; 1,25D= 1,25-dihydroxyvitamin D; C/EBPβ= parathyroid levels of C/EBPβ protein, where (number) indicates the number of parathyroid glands examined. ** indicates $p<0.01$ vs. LP and ^ indicates $p<0.06$ vs. HP.

LEGENDS TO FIGURES

Figure 1: ***Pathogenesis of severe EGFR-driven growth and VDR loss.*** A. TGFα/EGFR induction of AP2 and LIP synthesis exacerbate growth and VDR loss through TGFα gene upregulation and VDR gene downregulation. B. *Structure of the C/EBPβ mRNA and protein isoforms:* A single C/EBPβ mRNA encodes (darker lane) for 3 distinct proteins C/EBPβ* (LAP1), C/EBPβ (LAP2) and dominant negative C/EBPβ (LIP) through their translation initiation (AUG) from 3 distinct in-frame translation sites. TGFα induces LIP translation through activation and binding of CUG-BP1 to the 3rd in frame AUG. The protein diagram indicates transactivation, DNA binding and leucine zipper regions.

Figure 2: ***The higher potency of the erlotinib/calcitriol combination compared to erlotinib alone to suppress PTG enlargement in rat SHPT associates with marked reductions in parathyroid ADAM17.***

A. Scheme of the experimental protocol. B. Top: Representative immunostaining for parathyroid ADAM17 expression. Magnification X200; Bottom: Bars and error bars represent Mean±SEM of parathyroid gland (PTG) weight/body weight from 5/6NX rats fed a high P diet (HP) and receiving vehicle (HP), or calcitriol (HP+1,25D); erlotinib (HP+ertotinib), or the combination(HP+erlotinib+1,25D), at the concentrations specified in Methods. * and ** indicate $p<0.05$ or $p<0.01$ vs. HP.

Figure 3: TGFα and vitamin D regulation of ADAM17 expression.

A. Growth rates (MTT assay) of A431 cells treated for 84 h with vehicle (Control), erlotinib 0.25μM, calcitriol (1,25D) 100nM or the combination (erlotinib+1,25D). Bars and error bars represent the Mean±SEM from 3 independent experiments. ** and *** indicate $p<0.01$ and $p<0.001$ vs. controls; ^ indicates $p< 0.05$ vs. erlotinib.

B. Promoter/reporter assay of luciferase activity driven by the human ADAM17 promoter, corrected by β-galactosidase (βgal), in A431 cells treated as in A. Bars and

error bars represent the Mean±SEM of at least triplicate determinations per condition from 2 independent experiments. ** and ^ indicate p<0.01 vs. Control and p<0.05 vs. erlotinib

C: Top: Representative western blot analysis of nuclear LAP1/2 and LIP expression in A431 cells treated as in A; Bottom: Nuclear C/EBPβ (LAP1/2): LIP ratios, in A431 cells treated as in A. Bars and error bars represent the Mean±SEM from at least 4 independent experiments; ** indicates p<0.01 vs. controls.

D and E: Top panels: Representative RT-PCR for ADAM17 (D) and the loading control Cyclophillin B (CypB) and Western blot analysis (E) of pro-ADAM17 and active ADAM17 protein expression and the loading control GAPDH in A431 cells treated as in A. Bottom panels: Densitometric analysis of ADAM17 mRNA expression corrected for the housekeeping gene CypB(D) and of ADAM17 protein expression corrected for GAPDH as the loading control (E) in A431 cells treated as in A. Bars and error bars represent the Mean±SEM from at least 2 independent experiments. * indicates p<0.05 vs. controls

Figure 4: ***Increased parathyroid TGFα and ADAM17 concur with low VDR and C/EBPβ in human hyperplastic glands from SHPT.*** *A.* Promoter/reporter assay of human ADAM17 promoter activity, corrected by βgal, in A431 cells treated with vehicle (Control) or TGFα 8 nM (TGFα), and transfected with empty vector (Control+Empty vector) or with a C/EBPβ expression vector. Bars and error bars represent the Mean±SEM from 6 and 2 independent experiments with triplicate determinations each, for TGFα treatment or upon ectopic C/EBPβ expression, respectively. Results are expressed as % of respective controls.

B. Top Panels: Representative immunofluorescence of ADAM17, TGFα, VDR and C/EBPβ expression and co-localization in two distinct areas from a single patient. Merge 1: Hoechst (Blue), ADAM17 (green); TGFα (red); Merge 2: Hoechst (Blue); VDR (gray); C/EBPβ (red); ADAM17 (green). Magnification 40X.

Bottom Panel: (left): Correlation between parathyroid ADAM17 and TGFα in 11 nodules from the parathyroid glands from 4 patients: r^2=0.571, p=0.007. Bottom panel (right): Correlation between parathyroid ADAM17 and C/EBPβ in 8 nodules from the parathyroid glands from 6 patients: r^2=0.572, p=0.03.

Figure 5: 25-hydroxxyvitaminD enhances paricalcitol efficacy to suppress parathyroid ADAM17 and gland enlargement in rat SHPT

A. Scheme of the experimental protocol in rats fed either a high P (HP) or a low P diet (LP) throughout the 4 weeks after 5/6NX or sham operation, and treated with vehicle, 25-hydroxyvitaminD (HP+25D); Paricalcitol (HP+Pari) or the combination (HP+Pari+25D) at the concentrations specified in Methods, starting 1 week after NX;

B. Bars and error bars represent Mean±SEM of parathyroid gland (PTG) weight/body weight in rats treated as in A;

C. Top: Representative immunostaing for parathyroid ADAM17 expression; Bottom: Bars and error bars represent Mean±SEM of parathyroid ADAM17 expression estimated by the quantification of ADAM17 immunostaining per area (IOD/Area), respectively, from sham operated (Sham) or 5/6NX rats treated as indicated. * and *** indicate p<0.05 or p<0.001, respectively vs. LP; # and ## indicate p<0.05 or p<0.01, respectively vs. HP.

Figure 6: ***Molecular bases for ADAM17/TGFα induction of severe parathyroid hyperplasia in SHPT and its suppression by vitamin D.*** TGFα induction of ADAM17 gene transcription reinforces the vicious autocrine loop of enhanced ADAM17 release of TGFα and TGFα activation of the EGFR to induce LIP-driven growth exacerbation and ADAM17 gene transcription in A431 cells and in rat and human SH. 1,25-dihydroxyvitaminD efficacy to enhance C/EBPβ synthesis contributes to inhibit ADAM17 gene expression. 25-hydroxyvitaminD enhances 1,25-dihydroxyvitaminD actions induction of C/EBPβ to suppress ADAM17 gene expression.