Impaired basolateral sorting of pro-EGF causes isolated recessive renal hypomagnesemia

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Primary hypomagnesemia constitutes a rare heterogeneous group of disorders characterized by renal or intestinal magnesium (Mg2+) wasting resulting in generally shared symptoms of Mg2+ depletion, such as tetany and generalized convulsions, and often including associated disturbances in calcium excretion. However, most of the genes involved in the physiology of Mg2+ handling are unknown. Through the discovery of a mutation in the EGF gene in isolated autosomal recessive renal hypomagnesemia, we have, for what we believe is the first time, identified a magnesiotropic hormone crucial for total body Mg2+ balance. The mutation leads to impaired basolateral sorting of pro-EGF. As a consequence, the renal EGFR is inadequately stimulated, resulting in insufficient activation of the epithelial Mg2+ channel TRPM6 (transient receptor potential cation channel, subfamily M, member 6) and thereby Mg2+ loss. Furthermore, we show that colorectal cancer patients treated with cetuximab, an antagonist of the EGFR, develop hypomagnesemia, emphasizing the significance of EGF in maintaining Mg2+ balance.

Introduction
Magnesium (Mg2+) is established as a central electrolyte in a large number of cellular metabolic reactions, including DNA synthesis, neurotransmission, and hormone receptor binding. It is a component of GTPase and a cofactor for Na,K-ATPase, adenylate cyclase, phosphoinositide kinases, and phosphofructokinase (1). Mg2+ is also important for the regulation of parathyroid hormone release (2, 3). Accordingly, Mg2+ deficiency (plasma Mg2+ concentrations below 0.70 mM) has an effect on multiple body functions. Symptoms of Mg2+ deficiency are mostly related to muscle dysfunctioning, such as tetany, prolonged QT interval, and cardiac arrhythmias (4). Children with hypomagnesemia often present with tetany and/or convulsions. Hypomagnesemia is a problem frequently observed in more than 10% of hospitalized patients and occurrences can be as high as 65% in intensive care patients (5). A long-term complication seen in many adult patients with chronic hypomagnesemia is chondrocalcinosis, which can lead to impairment of joint function (4). Mg2+ deficiency can be secondary to systemic diseases (for instance, diabetes mellitus and Crohn disease) or to the use of osmotic agents, diuretics, and drugs such as cyclosporin and cisplatin (6). In addition, primary Mg2+ deficiency is observed in several monogenetic disorders. Failure of early diagnosis or noncompliance with treatment can be fatal or result in permanent neurological damage.

The plasma Mg2+ concentration is regulated within a narrow range by changes in urinary Mg2+ excretion in response to altered uptake by the intestine. Thus, the kidney plays a key role in Mg2+ homeostasis (4, 7). Most renal reabsorption of Mg2+ occurs in the proximal tubule and the thick ascending limb of the loop of Henle via a passive paracellular transport process, but the fine-tuning of the Mg2+ excretion takes place in the distal convoluted tubule (DCT), where Mg2+ is reabsorbed via an active transcellular transport process (6, 7). Apical entry into DCT cells is mediated by the Mg2+-permeable channel TRPM6 (transient receptor potential cation channel, subfamily M, member 6) driven by a favorable transmembrane voltage (8). The mechanism of basolateral Mg2+ transport into the interstitium is unknown. Mg2+ has to be extruded against an unfavorable electrochemical gradient, most likely by a Na+/Mg2+- dependent exchange mechanism and/or a Mg2+ ATPase. Finally, 3%–5% of the filtered Mg2+ is excreted in the urine.

Despite the critical role in Mg2+ handling, the exact mechanisms of transepithelial Mg2+ transport remain obscure. Studies of disorders with primary hypomagnesemia are very important to gaining more insight into the molecular and cellular mechanisms that underlie Mg2+ (re)absorption. Genetic studies in families with hereditary renal Mg2+ wasting syndromes have identified several genes that are either directly or indirectly involved in active Mg2+ handling. In the past few years, genetic studies of inborn errors of the Mg2+ balance revealed several new proteins unexpectedly involved in transepithelial Mg2+ transport in the DCT, e.g., thiazide-sensitive sodium chloride cotransporter (NCC), the γ subunit of the Na,K-ATPase, and the previously mentioned epithelial Mg2+ channel, TRPM6 (9–12).

The aim of the present study was, therefore, to elucidate the gene defect and molecular mechanism underlying isolated reces-

Nonstandard abbreviations used: DCT, distal convoluted tubule; FE, fractional excretion; IRH, isolated recessive renal hypomagnesemia; MDCK, Madin-Darby canine kidney; NCC, thiazide-sensitive sodium chloride cotransporter; pSAD, plasmid SAD; TRPM6, transient receptor potential cation channel, subfamily M, member 6.

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sive renal hypomagnesemia (IRH), which is characterized by renal Mg\textsuperscript{2+} loss. To this end, a homozygosity-based mapping strategy and mutation detection was performed. In addition, the molecular mechanism of IRH was studied in vitro using patch clamp analysis and in vivo using clinical studies in humans.

**Results and Discussion**

IRH is characterized by low serum Mg\textsuperscript{2+} levels and mental retardation. Two affected sisters, V3 and V4 (Figure 1A), displayed low serum Mg\textsuperscript{2+} levels (0.53–0.66 mM) in combination with urinary fractional excretion (FE) values of Mg\textsuperscript{2+} of 4.8% and 3.6%, respectively. These values are well above an FE of 2%, indicating renal Mg\textsuperscript{2+} wasting as previously described (5, 13). Thus, the fact that the urinary excretion of Mg\textsuperscript{2+} was in the normal range while serum Mg\textsuperscript{2+} values were hypomagnesemic points to a diminished tubular Mg\textsuperscript{2+} reabsorption. Apart from hypomagnesemia, other biochemical abnormalities were not identified; in particular, there was no disturbance in urinary Ca\textsuperscript{2+} excretion (14). Both sisters showed psychomotor retardation during childhood, and presently they are moderately mentally retarded women who suffer from epileptic seizures. The previously identified genes involved in renal Mg\textsuperscript{2+} handling encoding the thiazide-sensitive sodium chloride cotransporter NCC (SLC12A3), paracellin-1 (CLDN16), the γ subunit of the NaK-ATPase (FXYD2), and the epithelial Mg\textsuperscript{2+} channel (TRPM6) have been excluded for these 2 affected sisters (Figure 1A) (8, 11, 12, 15). To determine the disease locus of this consanguineous family, a homozygosity-based mapping strategy with a set of highly polymorphic microsatellite markers spread over the genome was performed and followed by fine mapping. This resulted in the identification of a critical linkage interval with LOD score 2.66.

**Figure 1**

Molecular analysis of EGF. (A) Pedigree of the family in which specific members suffer from IRH. Filled symbols represent affected individuals, half-filled symbols are heterozygous for the mutation C3209T, and open symbols represent clinically unaffected family members not screened for the mutation. Slash marks indicate deceased individuals, and double lines show consanguinity. (B) Schematic representation of the critical interval between polymorphic markers D4S2623 and D4S1575 on chromosome 4q and the intron-exon structure of the EGF gene, depicting the identified mutation. The position of the polymorphic markers is indicated by vertical bars. The horizontal arrow below the schematic boxed representation of EGF depicted between the polymorphic markers indicates the localization and orientation of the EGF gene. Cen, centromeric; tel, telomeric. (C) Genomic mutation sequence analysis of EGF in wild-type, heterozygous, and affected individuals. The mutated nucleotide and resulting amino-acid change is shown under the affected individual’s sequence. The black bar under that sequence indicates the mutated codon. The black bars under the sequences of the WT and heterozygous specify codon 1070 of EGF. The affected individuals both have a homozygous mutation C3209T in exon 22, resulting in the amino acid substitution P1070L. (D) Schematic model of pro-EGF, which resides as a type I membrane protein at the plasma membrane. The position of the P1070L mutation is depicted. (E) Sequence homology analysis of juxtamembrane domain and flanking residues. P1070 is strongly conserved among different species and forms the second proline of a basolateral PXXP sorting motif. The indicated colors represent functional conservation of the amino acids: red, small plus hydrophobic; blue, acidic; magenta, basic; green, hydroxyl plus amine plus basic; gray, others. TM, transmembrane domain.
The expression of EGF and EGFR mRNA was determined by RT-PCR on various human tissues. Signals for EGF were detected in, e.g., kidney, salivary gland, prostate, and cerebrum whereas no signals were detected in the adrenal gland, cerebellum, liver, lung, and placenta. The EGFR showed a ubiquitous expression pattern since PCR amplification products were obtained in all tissues tested. hEGF, human EGF; hEGFR, human EGFR.

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(B) Immunohistochemical analysis of EGF (green) and thiazide-sensitive sodium chloride cotransporter (NCC, red) in rat kidney sections (upper panel, overview of a cortical region; lower panel, magnified image of an immunopositive tubule). EGF colocalized with NCC, a marker for the DCT. Original magnification, ×180 (B, top panels); ×360 (B, bottom panels).

The EGF gene consists of 24 exons encoding a large type I membrane-anchored precursor protein of 1,207 amino acid residues. Its secondary structure is well conserved among species and includes a large extracellular domain, a transmembrane domain, and a short cytoplasmic domain (17). EGF has a profound effect on cell differentiation in vivo and is a potent mitogenic factor for a variety of cultured cells of both ectodermal and mesodermal origin (18). EGF belongs to the EGF-like family of growth factors, which bind with high affinity to the EGFR. Other members of this family are TGF-α, amphiregulin, heparin-binding EGF-like growth factor, betacellulin, and epiregulin (19–21). These membrane-anchored growth factor precursors are characterized by the fact that they are biologically active at the cell surface although they can be proteolytically cleaved to release soluble, diffusible factors (19–21).

To study the tissue distribution of pro-EGF and EGFR, we performed RT-PCR on various human tissues (Figure 2A). For EGF, PCR amplification products were detected in, e.g., kidney, salivary gland, cerebrum, and prostate, whereas no expression was detected in the adrenal gland, liver, cerebellum, and placenta. The EGFR showed a ubiquitous expression pattern since all tissues tested were positive by RT-PCR analysis (Figure 2A). Immunohistochemistry on rat kidney sections showed that EGF is consistently coexpressed with NCC, a marker of the DCT (22) (Figure 2B). Interestingly, the immunopositive staining is mainly localized along the apical domain in DCT. Coffey and coworkers demonstrated that human pro-EGF overexpressed in Madin-Darby canine kidney (MDCK) cells is delivered equally to the apical and basolateral membrane but is found predominantly at the apical membrane domain (23). They proposed that preferential ectodomain cleavage at the basolateral surface explains the apparent apical localization of pro-EGF. Sack and Talor provided evidence for the existence of specific binding sites in tubular basolateral membranes, suggesting a physiologic role of EGF in the kidney (24). In the kidney, the EGFR was detected in glomerular endothelial cells, peritubular capillaries, and arteriolar walls as well as along the thick ascending limb of Henle loop and DCT (25, 26). Immunohistochemical analysis revealed that the EGFR was located particularly along the basolateral membrane of the tubular cells. The DCT reabsorbs approximately 10% of the filtered Mg²⁺ load, and the reabsorption rate in this segment defines the final urinary Mg²⁺ excretion, as virtually no reabsorption takes place beyond this section (1). In kidney, the epithelial Mg²⁺ channel TRPM6 is specifically expressed in DCT, where it forms the rate-limiting step of epithelial Mg²⁺ transport (8). Thus, pro-EGF and TRPM6 are both predominantly

(P < 0.004) on chromosome 4 of 18.4 cM between markers D4S2623 and D4S1575 (Figure 1B). A search for candidate genes within this region revealed the EGF gene, which was considered a highly relevant positional candidate since it had previously been linked to electrolyte homeostasis (16). We sequenced EGF in the affected individuals (Figure 1A) and identified the homozygous mutation C3209T in exon 22, which cosegregated with the disorder and was absent in 126 ethnically matched control chromosomes (Figure 1C). The mutation caused the substitution of the highly conserved proline 1070 within the cytoplasmic tail of pro-EGF by leucine (P1070L) (Figure 1, D and E). We found that both parents and the 2 unaffected sisters (Figure 1A) exhibited the homozygous wild-type genotype (Figure 1, A and C).
expressed in DCT, the main site of active renal Mg\(^{2+}\) reabsorption (1, 16, 17, 27). To determine whether EGF can regulate the activity of TRPM6, human embryonic kidney 293 (HEK293) cells were transiently transfected with TRPM6 and treated for 30 minutes with EGF (10 nM), resulting in increased channel activity (Figure 3A). Western blot analysis demonstrated endogenous expression of EGFR in these HEK293 cells (data not shown). EGF dose dependently stimulated TRPM6 activity with half maximal effective concentration of 1.7 nM, an apparent affinity that is in the physiological range as reported previously (Figure 3B) (24).

Previous in vivo studies showed that the predominant form of EGF released from epithelial cells is the high-molecular mass 160–170 kDa EGF, as found at high concentrations in serum, urine, and milk (28, 29). To investigate the functional effect of the identified mutation, HEK293 cells were transfected with wild-type or mutant pro-EGF and after 2 days their supernatant was collected. TRPM6-expressing HEK293 cells were subsequently incubated for 30 minutes with these preconditioned media and analyzed for channel activity using the patch clamp technique. Interestingly, the supernatant obtained from mutant pro-EGF–expressing HEK293 cells failed to stimulate TRPM6 activity whereas a significant channel activation was observed with the wild-type EGF–containing supernatant (Figure 3C). Quantification of EGF in both supernatants using ELISA indicated that the secretion of mutant pro-EGF is impaired (Figure 3D).

To demonstrate that the observed stimulatory effects are due to EGFR activation, a preincubation with cetuximab was performed. This IgG human/mouse chimeric monoclonal antibody competitively inhibits the activation of the EGFR. It binds externally to the EGFR to block binding of the ligand and subsequent signal transduction mediated via the receptor-associated tyrosine kinase and prevents phosphorylation of the EGFR and other downstream proteins.
proline in the cytoplasmic cell (31). Our mutation substitutes the second highly conserved motif PXXP (with X being an arbitrary amino acid), determining basolateral sorting motifs were identified, including the sequence gate in detail the cellular metabolism of pro-EGF. Recently, several cal and basolateral membranes (23). This prompted us to investi
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sured at +80 mV and treated with mock supernatant, EGF super
natant, or EGF supernatant plus cetuximab, respectively; P < 0.05, n = 6). Thus, the stimulatory effect of the supernatant on TRPM6 activity is due to direct activation of the EGFR.

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in line with a recent report showing that a significant number of similarly treated patients developed hypomagnesemia (30). Our patients treated with cetuximab displayed an FE of Mg\textsuperscript{2+} of 5.1%, which is inappropriately high given their hypomagnesemia (Figure 4B). As previously established by Agus (5), an FE of Mg\textsuperscript{2+} above 2% in the presence of hypomagnesemia indicates renal Mg\textsuperscript{2+} loss. The 2 affected sisters with IRH exhibited a similar relatively high Mg\textsuperscript{2+} excretion profile (Figure 4B). The urinary Mg\textsuperscript{2+} patterns of the patients treated with EGFR antibodies, the patients described in our Dutch family, and patients with hypomagnesemia with sec
dondary hypocalcemia (HSH; OMIM 602014) (11, 12) were identi
cal and suggest mutual defects in renal TRPM6 activity. Indeed, preincubation with cetuximab abolished the stimulatory effect of the supernatant on TRPM6 activity as shown by patch clamp analysis of TRPM6-expressing HEK293 cells (Figure 3E). Of note, the apically and basolaterally collected mock medium did not affect TRPM6 activity. These observations suggest that the basolateral release of mutant pro-EGF is diminished, seriously hampering the EGF-dependent activation of the basolaterally localized EGFR (Figure 3F).

Clinical trials directed to the treatment of patients with colorectal cancer have demonstrated that cetuximab is synergistic with chemotherapy for these patients (32). We observed that serum Mg\textsuperscript{2+} levels gradually decreased in all studied colorectal cancer patients on cetuximab treatment (Figure 4A). This is

Figure 4
Effect of cetuximab treatment on Mg\textsuperscript{2+} balance and TRPM6 activity. (A) Changes in serum Mg\textsuperscript{2+} levels from baseline over time during cetuximab therapy are shown for 20 colorectal cancer patients. Solid lines represent individual linear regression lines of the data points for each individual patient. Open symbols denote end of treatment. (B) Cetuximab treatment leads to renal Mg\textsuperscript{2+} loss and hypomagnesemia. Serum samples and urine (over a 24-hour period) was collected from 8 patients at baseline in normomagnesemic conditions (open circles), 12 patients on cetuximab treatment in hypomagnesemic conditions (filled circles), and patients (V3, V4) with IRH (triangles), then analyzed for FE of Mg\textsuperscript{2+}. FE Mg\textsuperscript{2+} was plotted against the serum Mg\textsuperscript{2+} concentrations for the tested individuals. Hypomagnesemia in combination with an inappropriately high excretion of Mg\textsuperscript{2+} was observed in patients with the P1070L mutation as well as in individuals treated with cetuximab. Large circles represent the averaged values of patients on cetuximab treatment (filled) and patients at baseline in normomagnesemic conditions (open). Asterisk indicates a significant difference in serum (Mg\textsuperscript{2+}) compared with that in control patients. P = 0.001; n = 8–12. (C) Histogram depicting the current densities at +80 mV of TRPM6-transfected cells that were

proteins in the signal transduction cascade (30). The stimulatory effect of the supernatant collected from pro-EGF–expressing HEK293 cells on TRPM6 was significantly inhibited by preincuba
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erally, the mutation should prevent the ligand from reaching its receptor. Interestingly, He and coworkers have shown previously that a 22–amino acid sequence in the EGFR juxtamembrane domain contains auton
omous sorting information necessary for basolateral localization of the receptor in MDCK cells (31). This sorting motif contains a polyproline core comprising residues P667 and P670 (667PXXP670). Site-directed mutations at P667 and P670 were associated with impaired basolateral delivery of the EGFR.

To determine the effect of the mutation on the sorting of pro-EGF in polarized epithelial cells, MDCK cells stably expressing either wild-type or mutant pro-
EGF were grown on semipermeable filter supports until they reached confluency. After 2 days, media of the basolateral and apical compartment were collected individually. Next, HEK293 cells expressing TRPM6 were treated with the collected preconditioned media of wild-type pro-EGF, mutant pro-EGF, or mock-
expressing cells. Equal stimulation of TRPM6 channel activity was observed when the cells were incubated with the apically collected media of wild-type or mutant pro-EGF–expressing MDCK cells (Figure 3E). However, stimulation of TRPM6 channel activity was only observed with basolaterally collected wild-type pro-EGF medium, not with the basolateral mutant pro-EGF media (Figure 3E). Of note, the apically and basolaterally collected mock medium did not affect TRPM6 activity. These observations suggest that the basolateral release of mutant pro-EGF is diminished, seriously hampering the EGF-dependent activation of the basolaterally localized EGFR (Figure 3F).

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Through the discovery of an EGF mutation in IRH, we have for the first time, to our knowledge, identified a magnesiotropic hormone crucial for total body Mg²⁺ balance that directly stimulates renal tubular Mg²⁺ reabsorption via activation of the epithelial Mg²⁺ channel TRPM6. Moreover, it raises the question of whether EGF plays a role in mental development since our IRH patients (Figure 1A) are mentally retarded. EGF is present in cerebrospinal fluid, and EGF mRNA has been detected in the developing brain of various species. Furthermore, EGF is a well-known neurotrophic factor regulating the development of various neuronal cells (33). Futamura et al. reported that serum EGF levels were markedly decreased in schizophrenic patients and suggested that EGF might provide a link between impaired EGF signaling and the pathology/etiology of schizophrenia (34). An association between an EGF polymorphism and schizophrenia has been suggested although data from several studies are conflicting (35, 36).

Interestingly, in our Dutch family with IRH, 1 unaffected, EGF P1070L–carrier sister (Figure 1A) suffers from schizophrenia, and 2 brothers and 1 sister of the carrier mother (Figure 1A) are known to have cluster A (Diagnostic and statistical manual of mental disorders, fourth edition, text revision) personality disorders. In addition, EGF could be involved in Mg absorption in the intestine, a site that also expresses TRPM6. If not, please revise sentence. Future therapeutic intervention might be able to treat hypo- and hypermagnesemia by regulating EGFR activity.

**Methods**

**Statistics.** In all experiments, data are expressed as mean ± SEM. Overall statistical significance was determined by ANOVA. Where differences between the means of 2 groups were significant, they were analyzed by pairwise comparison using Scheffe’s method. For the statistical analysis in Figure 4B, an unpaired Student’s t test was performed. P < 0.05 was considered significant. Statistical analysis was performed using SPSS software 12.0 (SPSS).

**Subjects.** Informed consent was obtained from the parents and unaffected daughters of the family in this study. The parents gave proxy consent for the 2 affected daughters in this study. In this family, 2 Dutch sisters suffered from primary renal Mg²⁺ loss associated with normocalciuria. These women are the offspring of a consanguineous mating, and since both parents did not display this disorder, the inheritance pattern was likely to be autosomal recessive. The clinical data of this family have previously been described in detail by Geven et al. (14). In short, 2 patients of this family, V3 and V4, had serum Mg²⁺ levels of 0.53 mM and 0.56 mM and urinary Mg²⁺ values of 3.9 mmol/24 h and 3.7 mmol/24 h, respectively. Given the low serum Mg²⁺ levels, urinary Mg²⁺ excretion was relatively high, indicating a renal Mg²⁺ reabsorption defect. Both patients suffer from epileptic seizures that started in their first year of life and are controlled by conventional antiepileptic drugs. During childhood, they showed psychomotor retardation, and they are presently moderately mentally retarded women.

**Statistical analysis was performed using SPSS software 12.0 (SPSS).**
HEK293 cells were transiently transfected. MDCK type I cells (41) were grown in DMEM containing 5% (v/v) FCS (HyClone), 13 mM NaHCO$_3$ and 5% (v/v) fetal calf serum, 2 mM l-glutamine, and 10 μg/ml Ciproxin at 37°C in a humidity-controlled incubator with 5% (v/v) CO$_2$. For stable transfection of MDCK cells, 25 μg circular DNA was transfected using the Ca$^{2+}$-phosphate precipitation technique (42). Cells were trypsinized 24 hours after transfection, seeded on Petri dishes, and cultured in DMEM containing 75 μg/ml hygromycin B (Invitrogen). Between 10 and 14 days after transfection, individual colonies were selected by means of cloning rings and expanded. Total RNA was extracted from each colony that had been grown until confluent in a 24-well culture plate using TRIzol. Total RNA Isolation Reagent (Invitrogen) according to the manufacturer’s protocol. The obtained RNA was subjected to DNase treatment (Promega) to prevent genomic DNA contamination. Thereafter, 1.5 μg of RNA was reverse transcribed by Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen) as described previously (43). The cDNA was used to determine pro-EGF expression with the primers described in Supplemental Table 2. MDCK cells stably expressing plasmd CB7–EGF, plasmd CB7–EGF–P1070L, or empty vector (5 clones of each) were used to seed 10 24-mm Transwell filter wells of each condition (Corning Inc.). We seeded 1.5 × 10$^5$ cells/cm$^2$ on permeable filter supports; these were grown for 4 days until they reached confluence and an electrical resistance of greater than 2,400 Ω-cm$^2$. Next, the media were replaced by DMEM medium as described below but without FCS and harvested after 2 days.

**Transfection of HEK293 cells.** HEK293 cells were transiently transfected with pSAD-EGF, pSAD-EGF–P1070L, or empty-vector pCNeo-IREs-GFP cDNA using polyethyleneimine (Polysciences Inc.). Six million cells were seeded on a petri dish (57 cm$^2$) (10 plates per vector) in a total volume of 5 ml DMEM. After 3 hours, the cells were transfected. The transfection mix was concentrated 400 times using Centriprep YM-3 followed by Microcon YM-3 (Millipore). Prior to concentration of the collected HEK293 medium, 2 ml of the collected FCS-free culture medium was kept separately and used for patch clamp analysis.

**Human EGF ELISA.** EGF concentrations were determined in 400 times concentrated culture supernatant by using Centriprep columns and a human EGF ELISA kit according to the manufacturer’s protocol (RayBiotech Inc.).

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