Effect of GLP1R agonists
taspoglutide and liraglutide
on primary thyroid C-cells from
rodent and man

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Abstract
Glucagon-like peptide 1 (GLP1) analogs have been associated with an increased incidence of thyroid C-cell hyperplasia and tumors in rodents. This effect may be due to a GLP1 receptor (GLP1R)-dependent mechanism. As the expression of GLP1R is much lower in primates than in rodents, the described C-cell proliferative lesions may not be relevant to man. Here, we aimed to establish primary thyroid cell cultures of rat and human to evaluate the expression and function of GLP1R in C-cells. In our experiments, GLP1R expression was observed in primary rat C-cells (in situ hybridization) but was not detected in primary human C-cells (mRNA and protein levels). The functional response of the cultures to the stimulation with GLP1R agonists is an indirect measure of the presence of functional receptor. Liraglutide and taspoglutide elicited a modest increase in calcitonin release and in calcitonin expression in rat primary thyroid cultures. Contrarily, no functional response to GLP1R agonists was observed in human thyroid cultures, despite the presence of few calcitonin-positive C-cells. Thus, the lack of functional response of the human cultures adds to the weight of evidence indicating that healthy human C-cells have very low levels or completely lack GLP1R. In summary, our results support the hypothesis that the GLP1R agonist-induced C-cell responses in rodents may not be relevant to primates. In addition, the established cell culture method represents a useful tool to study the physiological and/or pathological roles of GLP1 and GLP1R agonists on normal, non-transformed primary C-cells from rats and man.

Key Words
- GLP1 receptor
- C-cell
- primary cultures
- taspoglutide
- liraglutide
- human
- rodent

Introduction
Glucagon-like peptide 1 (GLP1) is an incretin hormone that promotes glucose-dependent stimulation of insulin and suppression of glucagon secretion, delays gastric emptying, and reduces energy intake. GLP1 also increases β-cell mass via stimulation of β-cell proliferation (Doyle & Egan 2007). The actions of GLP1 are mediated by a GLP1

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receptor (GLP1R). Long half-life GLP1R agonists, such as liraglutide and taspoglutide, have been envisaged for the treatment of diabetes.

During non-clinical development, carcinogenicity studies with liraglutide (EMA 2009, Bjerre Knudsen et al. 2010), exenatide (EMA 2006, 2011), and taspoglutide (unpublished data) resulted in increased incidence of thyroid C-cell hyperplasia and tumors in rats and mice. The findings observed in thyroid C-cells of rodents (Parks & Rosebraugh 2010) might potentially be caused by a GLP1R-dependent mechanism. However, while direct proliferation as response to GLP1R activation has been shown for pancreatic β-cells (Brubaker & Drucker 2004, Doyle & Egan 2007), the effect on thyroid C-cells is believed to be due to sustained stimulation of the GLP1R on the C-cells, leading to sustained increased calcitonin production and release followed by the observed C-cell hypertrophy and finally proliferation (Crespel et al. 1996, Costante et al. 2007). Accordingly, increased calcitonin is a known clinical marker of C-cell tumors, as well as in rodents showing C-cell hyperplasia and tumors (Pilling et al. 2007). Moreover, it has been shown that C-cell hypertrophy in mice is GLP1R dependent and is not observed in Glp1r knockout mice (Madsen et al. 2012). It has also been postulated that the increased synthesis and secretion of calcitonin by C-cells, the subsequent proliferation, and eventually adenoma and carcinoma formation (Bjerre Knudsen et al. 2010) are GLP1R dependent and possibly species specific, as liraglutide induces acute release of calcitonin in rats and mice but not in monkeys. In addition, there is only inconclusive evidence indicating that GLP1R agonists may cause C-cell carcinomas in primates; in particular, as expression of GLP1R in healthy human thyroids is still a matter of debate. Indeed, based on a group of patients with several thyroid pathologies and applying immunohistochemistry techniques, Grier et al. showed that neoplastic and hyperplastic lesions of thyroid C-cells in human express the GLP1R. In patients with papillary thyroid carcinoma, only 33% of the subjects expressed GLP1R, which co-localized with calcitonin expression in the anatomically healthy lobe, but no data were included from fully healthy control tissue (Gier et al. 2012). Other published reports failed to detect GLP1R in human C-cells; Waser et al. (2011) could not detect GLP1R in normal human thyroids using autoradiography. Concordantly, Bjerre Knudsen et al. (2010) were not able to detect mRNA or protein of GLP1R in human C-cells using in situ hybridization or in situ ligand labeling respectively. Thus, it is still controversial whether C-cell proliferative lesions observed in rodents following chronic GLP1R agonist treatment are relevant to man (Capen 2008, Chiu et al. 2012).

C-cell lines from rats and humans have already been used to investigate some of the molecular aspects of GLP1R agonistic action and possible species differences (Bjerre Knudsen et al. 2010). However, these cell lines originate from medullary thyroid carcinoma (MTC), represent already transformed cells, and are thus not well suited to study events such as early proliferation and alterations ultimately associated with cell transformation (Parola 2009). With regard to non-transformed primary cells, however, there is no well-established or widely used primary thyroid cell or tissue culture model to assess C-cell function. Reports in the literature of primary thyroid tissue cultures describe the use of tissue pieces/slices (Mu 1976, Yamamoto et al. 1986, Zerek-Melen et al. 1990, Toda et al. 2011, Vickers et al. 2012) or isolated cells (Nishiyama & Fujii 1989, Clark et al. 1995, Lu et al. 1999). However, these thyroid cell culture systems have been mainly used for the assessment of thyrocyte function, whereas functional C-cell response has rarely been demonstrated (Endo et al. 1988). This is not surprising, as C-cells in the thyroid are much less abundant than thyrocytes and thus are difficult to isolate and evaluate.

In our work, and with the specific purpose of studying cellular and molecular events in naïve C-cells from rats and humans upon GLP1R agonist treatment, we established and optimized primary thyroid cultures containing functional thyrocytes and C-cells of both species. We present data on the characterization of primary rat and human thyroid cell cultures, on the expression of GLP1R, and on the functional response of the cultures toward the GLP1R agonists liraglutide and taspoglutide.

Materials and methods

Test compounds
Liraglutide (BIM-51282, WAA.490-63-61) was kindly provided by Dr J Dong (Ipsen, Milford, MA, USA), and taspoglutide (RO5073031-001 Batch BS0811SA03) was provided by F. Hoffmann-La Roche Ltd.

Thyroid cell isolation
Rat thyroids were harvested from male albino Wistar rats (Harlan, Füllinsdorf, Switzerland) according to a method adapted from Endo et al. (1988). The rats were anesthetized with sodium pentobarbital (120 mg/kg, i.p.), and the thyroid gland was removed (including parathyroid), washed, and temporarily stored in Hank’s balanced salt
solution (HBSS, Gibco 14175). For the digestion, tissue was cut into small pieces with a scalpel and suspended in 2 ml digestion buffer consisting of 5 mg/ml collagenase type 2 (4176 Worthington, lot S8K10703) in HBSS four times for 15 min. The incubation was performed in an Eppendorf Thermomixer at 37 °C under constant shaking (800 r.p.m.). After each incubation period, the tubes were removed and the remaining undigested tissue was let to sediment to the bottom of the tubes. The supernatant (0.5 ml) containing dissociated cells was transferred to 10 ml fresh culture medium (Ham’s F12 culture medium; Gibco 21765) containing 5% FCS (Gibco 10106), 1% penicillin (10 000 U/ml)/streptomycin (10 000 μg/ml) solution (Gibco 15140), 1.3 μM insulin (Sigma I6634), 100 nM hydrocortisone (Sigma P4153), 6.1 nM transferrin (Sigma T8158), and 25 nM H-GLY-HIS-LYS-OH (GU03-104 Ivy Fine Chemicals, Cherry Hill, NJ, USA). This cell suspension was stored at 37 °C until the end of the whole digestion procedure. Fresh digestion buffer (0.5 ml) was added to the remaining non-digested tissue and the digestion process was continued for a further 15 min. Finally, all cell suspensions were pooled, filtered through a 100 μm nylon mesh, and centrifuged at 800 g for 10 min. The pellet was resuspended in culture medium (1 ml medium per 13 mg initial thyroid tissue).

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rat C-cell line was kept in culture in DMEM (ATCC, Cat # 30-2002), supplemented with 15% horse serum at 37 °C in 5% CO2 atmosphere. The medium for both lines was exchanged twice weekly.

Transfection of TT cells

To obtain a stable transfected human C-cell line, TT cells were seeded in six-well plates at a density of 500 000 cells/well. Upon 24-h incubation in Optimem containing the GlP1R vector and lipofectamine, the medium was exchanged for F12K medium (Invitrogen, Cat # 21127) supplemented with 10% FBS and 200 μg/ml G418. The cells were further incubated for 4 weeks. Once harvested, a limiting dilution was performed in order to have one cell/well in 96-well plates. The cells were cultured for 4 months with periodic medium exchanges. Due to the long duplication times (~80 h), the cells were pooled, tested for GlP1R expression, and used as a polyclonal cell line.

Cell staining by immunohistochemistry

Primary thyroid cells were cultured on collagen-coated Lab Tek II four-well chamber slides (154526 Nalge Nunc International, Naperville, IL, USA) using 1 ml of the cell suspension per chamber. At the indicated time points, the culture medium was removed and 0.5 ml of a 10% formalin solution in PBS was added. After 30-min fixation period, the formalin solution was removed and 1 ml PBS containing 0.1% NaN3 was added for storage at 4 °C until further processing. Immunostaining was performed using a primary rabbit polyclonal IgG, anticalcitonin antibody (Zymed 18-0012, Life Technologies, Zug, Switzerland), diluted 1:200, followed by incubation with a secondary anti-rabbit IgG antibody, raised in goat and FITC-labeled (Invitrogen A31627), diluted 1:500. At the end of the immunostaining procedure, a drop of Prolong Gold antifade reagent with DAPI (Invitrogen, Cat # P36935) was added. High content imaging (HCI) was performed using an EVOTEC Opera system.

Determination of triiodothyronine production and release

Cells cultured in 24-well plates were treated with 10 or 40 mU TSH (300 μl/well) to induce triiodothyronine (T3) production and release from thyroid follicular cells. At indicated time points after the beginning of treatment, 60–100 μl of the cell culture supernatant was transferred into labeled tubes and stored at −20 °C for determination of
rat or human T3 concentrations using a commercial enzyme immunoassay (Rodent T3 EIA Test Kit; 1-800-745-0843, Endocrine Technologies, Inc., Newark, CA, USA) for rat or a RIA (T3 RIA Test Kit; 131.100, BRAHMS, Hennigsdorf, Germany) for human T3 respectively.

**Determination of calcitonin production and release**

Cells cultured in 24-well plates were treated with 300 μl/well test compound or vehicle dissolved in medium. Treatment with 3 mM CaCl2 served as the positive control to induce calcitonin production and release from thyroid C-cells. At indicated time points after treatment start, 60–100 μl of the cell culture supernatant was transferred into labeled tubes and stored at −20°C for determination of rat or human calcitonin concentrations in supernatants using a commercial enzyme immunoassay (calcitonin rat EIA kit; EK-014-06, Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) for rat or RIA (calcitonin human RIA kit; S-2097 Peninsula Laboratories, BACHEM Holding AG, Bubendorf, Switzerland) for human calcitonin respectively.

**qRT-PCR**

Total RNA was extracted with Trizol (Invitrogen, Cat # 15596-026, following the manufacturer’s instructions). One microgram (or 0.1 μg for low-yield RNA preparations) was used for synthesis of single-stranded cDNA using a commercially available cDNA synthesis kit with an RT Primer mix that ensures cDNA synthesis from all regions of RNA transcripts (QuantiTect Reverse Transcription Kit, Qiagen). The obtained cDNA was used as a template for several PCRs using appropriate specific primers and TaqMan Fast Universal PCR Master Mix (Applied Biosystems). The production of double-stranded amplification products was measured using the ABI-PRISM HT 7900 sequence detection system (Applied Biosystems). All TaqMan primers and probes were ordered from Applied Biosystems. The assay IDs were Rn00569199_m1 (Rat Glp1r), Rn00562406_m1 (Rat GAPDH), Hs01100741_m1 (Human GLP1R), and Hs99999905_m1 (Human GAPDH). Expression levels were normalized to GAPDH. The expression of genes with Ct values above 32 was considered below the limit of quantification.

**Panomics branched DNA assay**

Total RNA (extracted following manufacturer’s instructions with Trizol, Invitrogen, Cat # 15596-026) was used to detect expression of specific transcripts. Linearity of the assay was verified by analyzing a fivefold dilution series of the samples (250, 50, and 10 ng RNA) with a human or rat GLP1R probe set and a housekeeping gene probe set (PPIB and ACTB for the human- and the rat-derived RNA respectively), using the QuantiGene 2.0 assay (Panomics, Cat # QS009, Affymetrix inc., Santa Clara, CA, USA). Briefly, the RNAs and the probe sets were dispensed into 96-well capture plates and incubated for 16 h at 55°C. The signal was amplified by hybridizing with a pre-amplifier (1 h at 55°C incubation) and an amplifier (1 h at 55°C incubation), followed by hybridization with a labeled probe (1 h at 50°C incubation). The signal was then detected by adding the chemoluminogenic 2.0 substrate and reading the luminescence in a Victor spectrophotometer (Perkin Elmer 1420, Schwerzenbach, Switzerland). All samples were run in triplicates.

**In situ hybridization**

The mRNA targets GLP1R and calcitonin in primary C-cell cultures were detected using the RNAscope multiplex fluorescent assay (Advanced Cell Diagnostics, Inc., Hayward, CA, USA). The assay was performed following the manufacturer’s instructions. Briefly, rat thyroid cells were plated in culture dishes at 80–90% confluence and air-dried for 20 min. The fixed cells were then dehydrated with ethanol and air-dried for 20 min. Following 30 min of protease treatment, the samples were hybridized for 2 h at 40°C with the set of probes (Rn-GLP1R-C1; Rn-CALCA-C3; Rn-dapB-C2; Advanced Cell Diagnostics, Inc.). After washing the slides, four rounds of amplification reactions were performed with amplifier molecules and labeled probes, conjugated to multiple fluorophores. Next, a counterstain with DAPI was performed, and the cells were visualized with a fluorescent microscope (Leica DMI 4000B).

**Detection of GLP1R protein**

**Exendin-Cy5 staining** The cells were washed with PBS and incubated for 60 min with Exendin-Cy5 (1:1000 dilution in PBS containing 2% goat serum) and Phalloidin-Alexa Fluor 488 (Invitrogen, Cat # A12379). After washing with PBS, the cells were fixed (Fix/Perm solution, BD, Cat # 554714, Becton Dickinson AG, Allschwil, Switzerland) for 30 min at 4°C. After washing 3× with Perm buffer, a drop of Prolong gold antifade reagent with DAPI (Invitrogen, Cat # P36935) was added to each well. The cells were visualized in a confocal microscope (Leica DMI 4000B).
Exendin/calcitonin staining

The cells were washed with PBS and incubated for 30 min with Exendin-Cy5 (1:1000 dilution in PBS containing 5% goat serum). After washing 3× with PBS, the cells were fixed for 30 min with 4% paraformaldehyde (PFA) in PBS at 4°C. After washing 2× with PBS, the cells were blocked and permeabilized with 0.2% Triton in PBS containing 10% goat serum for 60 min. The cells were washed 3× with PBS and incubated with calcitonin mAb (1:200 in 5% goat serum 0.1% Triton in PBS; NovusBio, Cambridge, UK, Cat # NBP1-3051) for at least 1 h. The cells were washed 3× with PBS and incubated with goat anti-mouse Alexa Fluor 568-Ab (1:200 in 2% goat serum 0.1% Triton in PBS; Invitrogen, Cat # A-1101) for 60 min. After washing 3× with PBS, a drop of Prolong gold antifade reagent with DAPI was added to each well. The cells were visualized in a confocal microscope.

Results

Primary cell culture systems

We established primary thyroid tissue cell cultures from rats and humans (Fig. 1). These cultures were viable during the whole duration of the experiments based on ATP measurements (data not shown). Moreover, TSH-induced release of T₃ from thyrocytes showed that both the human and the rat cell cultures were functional after isolation and plating. Primary rat cell thyroid cell cultures were responsive to TSH stimulation, although the response was quite variable with regard to its amplitude (Table 1). T₃ release was below the detection limit under basal culture conditions but became measurable upon TSH stimulation (10 or 40 ng/ml). All rat cell preparations were responsive to TSH, while this was not the case for primary human cultures. TSH responsiveness was used as a quality measure for human cultures and observed in six out of 13 tested cell preparations (Table 2).

Calcitonin release by C-cells upon Ca²⁺ stimulation was also measured in rat and human thyroid cultures. In rat primary cultures, Ca²⁺-triggered calcitonin release was dependent on concentration and time (Fig. 2A). In addition, calcitonin release was Ca²⁺ specific, as similar concentrations of Mg²⁺ (in the form of MgCl₂) did not increase calcitonin release (Fig. 2B). Phenytoin, a known calcitonin release inhibitor in vitro and in vivo, was able to reverse the effect (Fig. 2B). In the rat cultures, stimulation with Ca²⁺ elicited an induction of calcitonin release into the medium with a mean fold increase of 28-fold. However, similar to the TSH-induced T₃ release, the amplitude of the response was variable and ranged from

<table>
<thead>
<tr>
<th>Cell preparation</th>
<th>T₃ in cell culture supernatant (ng/ml)</th>
<th>Calcitonin in cell culture supernatant (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TSH-stimulated T₃ release</td>
<td>Ca²⁺-stimulated calcitonin release</td>
</tr>
<tr>
<td>Control</td>
<td>BLQ</td>
<td>BLQ</td>
</tr>
<tr>
<td>10 ng/ml TSH</td>
<td>0.58±0.48</td>
<td>1.40±0.16</td>
</tr>
<tr>
<td>40 ng/ml TSH</td>
<td>2.31±0.34</td>
<td>3.4</td>
</tr>
<tr>
<td>Calcium in cell culture supernatant (ng/ml)</td>
<td>3 mM Ca²⁺ 88.2±51.7</td>
<td>4.83±0.48</td>
</tr>
<tr>
<td>Fold induction</td>
<td>23.6</td>
<td>19.4±2.7</td>
</tr>
<tr>
<td>Lowest</td>
<td>110</td>
<td>6.2</td>
</tr>
<tr>
<td>Highest</td>
<td></td>
<td>110 ±56.6</td>
</tr>
</tbody>
</table>

BLQ, below limit of quantification for rat T₃ (~0.4 ng/ml).
Possible reasons for the only partial calcium responsiveness of cell cultures that responded both to TSH and to calcium GLP1R agonists was therefore only performed with cell specimens originating from the central core of the thyroid lobe. A post hoc analysis was performed to assess possible reasons for the only partial calcium responsiveness of human cell cultures (Fig. 3). Neither patient age, diagnosis nor the ischemia time showed any association with the calcium responsiveness. The only parameter that showed a correlation to response was the anatomical localization of the tissue: cultures prepared from tissue samples of human cell batches (Fig. 3). Neither patient age, diagnosis nor the ischemia time showed any association with the calcium responsiveness. The only parameter that showed a correlation to response was the anatomical localization of the tissue: cultures prepared from tissue samples taken randomly.

The proportion of C-cells in primary rat thyroid cultures was in the range of 0.1 and 1% (Fig. 4A) while the proportion of C-cells in the human cultures was about ten times less than that in the rat cultures (Fig. 4B).

### Expression of calcitonin and GLP1R

Calcitonin mRNA in thyroid tissue could be detected in both rat and human primary thyroid cell cultures (Fig. 4) and calcitonin release into the medium could also be measured upon calcium stimulation (Table 2), we attribute the lack of transcript to the limits of detection coupled with the low abundance of C-cells. Gene expression levels of GLP1R were below the limit of quantification for qRT-PCR in both rat and human thyroid tissues. Consistently, no expression of GLP1R was detected in cell cultures (data not shown). Using a different assay to detect gene expression (bDNA, Panomics), we confirmed the lack of expression of GLP1R in the human thyroid tissues, whereas in rat, the expression levels detected were at or slightly above the limit of detection in thyroid tissue (Fig. 5B), suggesting that the low proportion of C-cells present in the samples might be an important limiting factor to detect a, on top, low expressed receptor. Indeed, using in situ hybridization, we were able to demonstrate that mRNA of Glp1r was expressed in primary rat thyroid cultures and co-localized with calcitonin (Fig. 6). Thus, on an mRNA level, we could qualitatively confirm the expression of GLP1R to C-cells in the rat thyroid cell cultures.

For the determination of the protein expression of GLP1R on the cell membrane, the lack of suitable, specific, commercially available anti-GLP1R antibodies led us to the use of a fluorescent-labeled synthetic peptide (Exendin-C5y) known to bind to the GLP1R with high affinity (Roche Penzberg, personal communication). For this approach, cells expressing high levels of GLP1R

### Table 2: T<sub>3</sub> and calcitonin production from human primary thyroid cell cultures. Calcitonin values (upon 3 mM Ca<sup>2+</sup> stimulation for 6 h) and T<sub>3</sub> release (upon 40 mU/ml TSH stimulation for 48 h) for all primary human thyroid cell cultures. Data are mean ± s.d. of triplicates

<table>
<thead>
<tr>
<th>Cell culture</th>
<th>Calcitonin (ng/ml)</th>
<th>T&lt;sub&gt;3&lt;/sub&gt; (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CaCl&lt;sub&gt;2&lt;/sub&gt; 3 mM</td>
</tr>
<tr>
<td>098094</td>
<td>0.085 ± 0.033</td>
<td>0.028 ± 0.017</td>
</tr>
<tr>
<td>098098</td>
<td>0.071 ± 0.022</td>
<td>0.065 ± 0.017</td>
</tr>
<tr>
<td>108004</td>
<td>0.067 ± 0.005</td>
<td>0.141 ± 0.017</td>
</tr>
<tr>
<td>108005</td>
<td>0.026 ± 0.02</td>
<td>0.039 ± 0.01</td>
</tr>
<tr>
<td>108007</td>
<td>0.035 ± 0.019</td>
<td>0.035 ± 0.017</td>
</tr>
<tr>
<td>108021</td>
<td>0.106 ± 0.009</td>
<td>1.541 ± 0.326*</td>
</tr>
<tr>
<td>108026</td>
<td>0.062 ± 0.013</td>
<td>0.06 ± 0.026</td>
</tr>
<tr>
<td>108030</td>
<td>0.048 ± 0.003</td>
<td>0.07 ± 0.068</td>
</tr>
<tr>
<td>108037</td>
<td>0.048 ± 0.017</td>
<td>0.037 ± 0.011</td>
</tr>
<tr>
<td>108052</td>
<td>1.866 ± 0.235</td>
<td>&gt; 5*</td>
</tr>
<tr>
<td>108076</td>
<td>0.025 ± 0.018</td>
<td>0.066 ± 0.011*</td>
</tr>
<tr>
<td>108100</td>
<td>0.062 ± 0.008</td>
<td>0.071 ± 0.017</td>
</tr>
<tr>
<td>108102</td>
<td>0.056 ± 0.018</td>
<td>0.111 ± 0.031*</td>
</tr>
</tbody>
</table>

*P ≤ 0.05 (t-test). BLQ, below level of quantification of human T<sub>3</sub> ~ 0.06 ng/ml.
(TT-GLP1R+) were constructed and used as a labeling control. The identification of C-cells in primary cultures was achieved by counterstaining with Calcitonin-Alexa 488. As shown in Fig. 7, GLP1R could be detected on the membrane of the transfected cells (Fig. 7A) but not on the C-cells present in rat primary cultures (Fig. 7B). Thus, the presence of the GLP1R on the surface of the C-cells could not be detected using this methodology and we have no direct evidence for GLP1R protein expression on C-cells, neither in rat nor in human primary cultures.

Effect of GLP1 receptor agonists liraglutide and taspoglutide on cell cultures

In order to assess GLP1R-mediated functional response as a surrogate for GLP1R detection, primary thyroid cell cultures from rats and man were stimulated with GLP1R agonists for 6 or 24 h. In rats, both tested GLP1R agonists, liraglutide and taspoglutide, elicited the release of...
calcitonin from primary thyroid cultures into the medium. This calcitonin release was concentration dependent, with taspoglutide being slightly more potent than liraglutide based on the concentrations needed to cause a significant effect (Fig. 8). The levels of calcitonin release elicited by GLP1R agonists were much lower than that caused by Ca\textsuperscript{2+}. Slightly increased calcitonin mRNA expression was also observed after exposure to liraglutide and taspoglutide, although the effect did not reach statistical significance. Ca\textsuperscript{2+} treatment on the other hand did not alter the expression of calcitonin mRNA, despite the much more pronounced induction of calcitonin release (Fig. 9). This is indirect evidence for the existence of GLP1R on the rat C-cell, responsible for GLP1R-mediated calcitonin release.

As already mentioned, the responsiveness of human primary thyroid cultures regarding CaCl\textsubscript{2}-induced calcitonin release varied between donors and only \(\sim 40\%\) of human primary thyroid cultures were responsive to CaCl\textsubscript{2}

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**Figure 4**

Calcitonin immunostaining and C-cell quantification in rat and human thyroid cell cultures. % Calcitonin immunopositive cells, quantified with High Content Analysis. (Top panel) Primary rat thyroid cells, (bottom panel) primary human thyroid cells. Each panel shows data from one experiment. Average \(\pm\) S.D. for four wells per condition. Control = Samples stained without primary calcitonin antibody. Inset: DAPI nuclear stain indicated in red and calcitonin immunostain in green.

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**Figure 5**

Calcitonin and Glp1r mRNA in thyroid tissue and various cell cultures. Gene expression in thyroid tissue, primary thyroid cells in culture, and the thyroid C-cell lines MTC 6–23 (rat) and thyroid tissue (human). All data are mean \(\pm\) S.D. of two to three biological replicates for tissue specimen or cultures form different donors. (A) qRT-PCR results for calcitonin expression. *Value below limit of quantification (Ct value > 32). (B) DNA results for GLP1 receptor expression. *Value below limit of quantification (581 RLU for human; 787 RLU for rat).
stimulation. Thus, the number of experiments using GLP1R agonists in human primary thyroid cultures showing responsiveness toward Ca\(^{2+}\) is limited to three preparations (\(n=3\); batches 10B021, 10B052 and 10B102 in Table 2) of which one was not responsive to TSH stimulation. Regarding the functional response upon stimulation with GLP1R agonists, none of these three batches of human thyroid primary cultures responded with a significant increase in calcitonin secretion to treatment with 1 \(\mu M\) liraglutide or taspoglutide, although this concentration significantly increased calcitonin release in rat cultures to 192\(\pm\)68 and 149\(\pm\)28% of that in control for liraglutide and taspoglutide respectively (Fig. 8).

**Discussion**

Here, we succeeded in establishing primary thyroid cell culture systems for rat and human tissue showing functional integrity of the primary thyrocytes and of the primary thyroid C-cells in culture. The rat cultures showed functional physiological responses, namely T\(_3\) secretion by thyrocytes upon stimulation with TSH and calcitonin release from C-cells upon stimulation with CaCl\(_2\). These responses were qualitatively comparable between different cell preparations but large quantitative differences were observed between batches. In human thyroid cultures, thyrocyte function (T\(_3\) release) was not seen in all preparations. Regarding C-cell functional response, calcitonin release from non-stimulated cells was often at the limit of detection but could be induced by Ca\(^{2+}\) stimulation in five out of 13 preparations. The lack of response to Ca\(^{2+}\) in some preparations was not related to general tissue deterioration, age of the patient, or tissue ischemia time. However, there was an association between the localization of the tissue specimen used to isolate the cells and the ability of the cultures to respond to Ca\(^{2+}\) stimulation in line with the fact that C-cells are not homogeneously distributed in the thyroid and also are very rare (Gmuender et al. 1983). Only cell cultures that had functionally viable C-cells were used for the assessment of GLP1R expression.

In the fresh tissues, the expression levels of calcitonin were much higher in rat than in human thyroids, probably due to the higher proportion of C-cells in rat compared...
thyroid cultures showed no or very low expression levels and man. Rat and human thyroid tissues as well as primary thyroid tissue and more specifically on C-cells from rats approaches to demonstrate the expression of GLP1R in calcitonin-positive cells than did human thyroid cultures. Thyroid cultures contained approximately ten times more. This could be confirmed by HCI analysis, showing that rat thyroid cultures would contain more C-cells than human cultures. Thus, it was expected that rat primary thyroid cell cultures would contain more C-cells than human cultures. For Ca^{2+}-induced calcitonin release, there were seven independent repeats (independent batches of cells) performed in duplicate or triplicate. *Significant difference form

with human thyroid (Wolfe et al. 1974, Feinstein et al. 1996). Thus, it was expected that rat primary thyroid cell cultures would contain more C-cells than human cultures. This could be confirmed by HCI analysis, showing that rat thyroid cultures contained approximately ten times more calcitonin-positive cells than did human thyroid cultures.

Once the cultures were established, we pursued several approaches to demonstrate the expression of GLP1R in thyroid tissue and more specifically on C-cells from rats and man. Rat and human thyroid tissues as well as primary thyroid cultures showed no or very low expression levels of GLP1R, i.e. levels below or around the limit of quantification of the qRT-PCR and bDNA techniques. In the rat cell cultures, however, using in situ hybridization, we were able to detect Glp1r mRNA in C-cells, co-localized with calcitonin expression. Although GLP1R expression was reported to be co-localized with C-cell markers also in human tissue in some publications (Giet et al. 2012), Bjerre Knudsen et al. (2010) were not able to detect GLP1R in normal human thyroid by in situ hybridization or ligand binding. In our experiments, we could not assign the GLP1R expression to C-cells in human cultures and the few weekly GLP1R-positive human tissue (determined by qRT-PCR/bDNA assay) samples may arise from other cell types, as published data suggested the presence of the GLP1R in endothelial tissue or thyrocytes (Bullock et al. 1996, Nystrom et al. 2004). In agreement with published data (Bjerre Knudsen et al. 2010), there were clear differences in the expression of GLP1R in rat (MTC 6–23) compared with human (TT) medullar carcinoma cell lines. While human TT cells showed minimal expression (values just above the limit of detection with both qRT-PCR and bDNA), the rat C-cell line and transfected TT cells had very high expression levels. At the protein level, Cy5-labeled Exendin provided a good means for the detection of GLP1R in transfected cells overexpressing the hGLP1R.
However, this method failed to detect GLP1R in the rat MTC 6–23 C-cell line, despite the fact that these cells express GLP1R, pointing toward a sensitivity issue with the Cy5-exendin labeling method. Accordingly, GLP1R protein expression could not be detected on either rat or human primary cell cultures using this method. Thus, and in agreement with others, we could not provide direct evidence that GLP1R is expressed on primary human C-cells.

In light of the technical difficulties to prove expression of GLP1R at the mRNA or protein level, we measured functional responses of the cells upon stimulation with GLP1R agonists, as an indirect measure of the presence of the functional receptor. We assessed calcitonin release following stimulation of the cell cultures with liraglutide and taspoglutide. Both liraglutide and taspoglutide elicited a modest increase in calcitonin release and slightly induced calcitonin transcript expression in rat primary thyroid cell cultures. Based on the concentrations needed to elicit this effect, taspoglutide appeared to be more potent than liraglutide. This is in line with the pharmacological potency of the two molecules on the GLP1R and their potency to stimulate cAMP in the MTC 6–23 cell line. Taspoglutide has been shown to have a similar potency as the native hGLP1 (Bjerre Knudsen et al. 2010 supplementary information, Sebokova et al. 2010) while liraglutide has been shown to have an at least tenfold lower potency than native hGLP1 (Bjerre Knudsen et al. 2010). Although we do not show reversal of calcitonin release or transcript increase using GLP1R antagonists, the potency difference of liraglutide and taspoglutide provides evidence for a GLP1R-mediated effect and thus for the presence of functional GLP1R on primary rat thyroid C-cells. Conversely, no induction of calcitonin release was observed when stimulating human primary thyroid cultures with either liraglutide or taspoglutide, irrespective of the presence of functional C-cells substantiated by the ability of these cultures to respond to calcium stimulation with increased calcitonin release into the medium.

In conclusion, the lack of functional response of the human cultures to GLP1R agonists adds to the weight of evidence indicating that human C-cells have very low levels or completely lack functional GLP1R. Conversely, using primary rat thyroid cells in culture, mRNA expression and a functional response were observed upon stimulation with GLP1R agonists. Assuming that the formation of C-cell tumors by GLP1R agonists is GLP1R dependent as shown in mice (Madsen et al. 2012), our results further support that the GLP1R agonist-induced C-cell responses in rodents may not be relevant to humans. It can, however, not be fully excluded that the lack of response toward GLP1R agonists in our human primary thyroid cultures is due to the low proportion of C-cells and a very low, non-detectable, calcitonin release. However, increasing the proportion of C-cells in human cultures is not feasible due to the low proportion of C-cells in human thyroid. Additional enrichment of C-cells before cell culture by, e.g. FACS-sorting, as reported by Moerch et al. (2007), is hindered by the lack of an established cell surface marker for C-cells. Despite some current limitations, the cell culture method and results reported here may open new avenues to study the putative physiological and/or pathological roles of GLP1 and GLP1R agonists on normal, non-transformed primary C-cells.

Declaration of interest
The authors declare that they are employed by pharmaceutical companies as stated in the affiliation declaration and were co-developing taspoglutide until development was suspended in 2011.

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