Role of glutaminyl cyclases in thyroid carcinomas

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Abstract

CCL2 is a chemokine known to recruit monocytes/macrophages to sites of inflammation. CCL2 is also associated with tumor progression in several cancer types. Recently, we showed that the N-terminus of CCL2 is modified to a pyroglutamate (pE)-residue by both glutaminyl cyclases (QC (QPCT)) and its isoenzyme (isoQC (QPCTL)). The pE-residue increases stability against N-terminal degradation by aminopeptidases. Here, we report an upregulation of QPCT expression in tissues of patients with thyroid carcinomas compared with goiter tissues, whereas QPCTL was not regulated. In thyroid carcinoma cell lines, QPCT gene expression correlates with the mRNA levels of its substrate CCL2. Both QPCT and CCL2 are regulated in a NF-κB-dependent pathway shown by stimulation with TNFa and IL1b as well as by inhibition with the IKK2 inhibitor and RNAi of p50. In the culture supernatant of thyroid carcinoma cells, equal amounts of pECCL2 and total CCL2 were detected by two ELISAs discriminating between total CCL2 and pECCL2, concluding that all CCL2 is secreted as pECCL2. Activation of the CCL2/CCR2 pathway by recombinant CCL2 increased tumor cell migration of FTC238 cells in scratch assays as well as thyroid carcinoma cell-derived CCL2-induced migration of monocytic THP1 cells. Suppression of CCL2 signaling by CCR2 antagonist, IKK2 inhibitor, and QPCT RNAi reduced FTC238 cell growth measured by WST8 proliferation assays. Our results reveal new evidence for a novel role of QC in thyroid carcinomas and provide an intriguing rationale for the use of QC inhibitors as a means of blocking pECCL2 formation and preventing thyroid cancer metastasis.

Introduction

Chemokines represent a group of chemotactic cytokines responsible for orchestrating cell recruitment under both homeostatic and inflammatory conditions. CCL2 (monocyte chemoattractant protein (MCP1)) is a member of the CC chemokine family and has been associated with cancer progression in several types of cancers including breast, prostate, lung, and melanoma (Löberg et al. 2006, Lu et al. 2006, Koga et al. 2008, Lu & Kang 2009, Zhang et al. 2010, Fridlender et al. 2011). In papillary thyroid carcinoma (PTC), CCL2 is correlated with lymph node metastasis and tumor recurrence (Tanaka et al. 2009). Interestingly, the oncogenic fusion protein RET/PTC expressed in PTC activates the transcription factor NF-κB and induces CCL2 gene expression (Russell et al. 2003, Neely et al. 2011). Recently, others and we have shown that the chemotactic activity of CCL2 depends on a modified N-terminus of the polypeptide, particularly the formation of a pyroglutamate (pE)-residue protecting...
QPCCT between the gene expression of affected. In addition, we identified a positive correlation QPCTL carcinomas, whereas the 15-fold higher QPCT transcript levels were downregulated in malignant pheochromocytomas (Thouennon et al. 2008, Cynis et al. 2006, Cynis et al. 2011). Two distinct genes termed QPCT (QC; NM_012413) and QPCTL (isoQC; NM_017659) are coding for the QC activity. In contrast to the secreted QC, the isoQC is exclusively localized within the Golgi complex, shows 46% sequence identity to the QC, and exhibits nearly identical substrate specificity in vitro (Cynis et al. 2008, Stephan et al. 2009).

Limited data are available about the expression of QCs in cancer. Analysis of microarray datasets identified QPCT as highly expressed in melanoma (Gillis 2006), whereas the QPCT transcript levels were downregulated in malignant pheochromocytomas (Thouennon et al. 2007). Genome-wide methylation analysis identified QPCT as an epigenetically inactivated candidate tumor suppressor gene in renal cell carcinoma (Morris et al. 2011). In thyroid carcinomas, QPCT was identified as a markedly overexpressed gene using gene expression profiling studies (Jarzab et al. 2005, Fluge et al. 2006, Griffith et al. 2006). Quantitative PCR analysis of microarray data confirmed a 15-fold higher QPCT gene expression in PTC compared with normal thyroid tissues (Jarzab et al. 2005).

Here, we show higher QPCT transcript levels in thyroid carcinomas, whereas the QPCTL gene expression was not affected. In addition, we identified a positive correlation between the gene expression of QPCT and its substrate CCL2 in thyroid carcinoma cell lines. This co-regulation of QPCT and CCL2 involved the NF-kB-dependent pathway and the CCL2/CCR2 pathway promoted the migration and proliferation of thyroid carcinoma cells.

Materials and methods

Tissues, cell lines, and materials

A total of 40 thyroid tissues, including ten follicular thyroid carcinomas (FTCs), ten PTCs, ten undifferentiated thyroid carcinomas (UTCs), and ten goiter, were obtained from patients of the Department of Surgery of the University of Halle-Wittenberg by surgical resection and were stored at −80 °C until use. The Local Committee of Medical Ethics approved the use of human tissues, and all patients gave their written informed consent. Each tumor was scored based on the TNM classification (Kehlen et al. 2003). Human thyroid cancer cell lines (ML-1, TT2609-CO2, 8305C, 8508C, B-CPAP, and BHT101 (all provided by DSMZ, Braunschweig, Germany); FTC133, FTC236, and FTC238 (kindly provided by P Goretzki, Düsseldorf, Germany); and SW1736, C643, and Hhh74 (kindly provided by N E Heldin, Uppsala, Sweden) were cultured in the recommended growth medium with 10% FCS. Cell line authentication was done by DNA multiplex STR typing with 15 genetic loci and one sex determination marker (PowerPlex 16, Promega, Mannheim, Germany) and data analysis as recommended (Schwepppe et al. 2008). For stimulation experiments, cells were treated with the cytokines TNFa and IL1b (Peprotech, Hamburg, Germany) for 24 h. The compounds IKK2 inhibitor IV and U0126 MEK inhibitor (both Calbiochem, Darmstadt, Germany) were added 30 min before cytokine treatment.

Immunohistochemistry

To detect QC, immunohistochemistry was performed using the goat anti-QC antiserum #10269 (Probiodrug, Halle, Germany) at a dilution of 1:100 as described (Hartlage-Rubsamen et al. 2009). After inactivation of endogenous peroxidase with 3% H2O2 in 20% cold methanol for 20 min, sections were incubated with the primary antibody at 4 °C in a humid chamber on a shaker. After 24 h, sections were incubated with secondary biotinylated donkey anti-goat antibody (1:500, Dianova, Hamburg, Germany) for 60 min followed by the ABC method, which comprised incubation with complexed streptavidin–biotinylated HRP's (Life Technologies, Darmstadt, Germany). QC immunoreaction was visualized by incubation with DAB substrate (DAKO, Hamburg, Germany) for 10 min. After washing steps with bidest, sections were counterstained with hematoxylin and mounted in aqueous mounting medium.

RT-PCR and RNAi

RNA was isolated using the NucleoSpin RNA II kit (Macherey Nagel, Düren, Germany) according to the manufacturer’s instructions. RNA of normal thyroid was obtained from the FirstChoice Human Total RNA Survey Panel (Ambion, Life Technologies, Erlangen, Germany). RNA concentration was measured using a NanoDrop 2000 spectrophotometer (Peqlab, Erlangen, Germany). RNA (0.1–1 μg) was reverse transcribed into cDNA using
random primers (Roche, Penzberg, Germany) and SuperScript III (Life Technologies). Quantitative PCR was performed in a Rotorgene 3000 (Corbett Research, Sydney, Australia) using the Rotor-Gene SYBR Green PCR kit and the Quantitect primer assays HsQPCT and HsQPCTL (both from Qiagen, Hilden, Germany) or specific primers for CCL2 (NM_002982.3; 5'-GCCTCCAGCATGAAAGTCTC and 3'-CAGATCTCCTTGGCCACAAT) synthesized by Metabion, Martinsried, Germany. Relative amounts of gene expression were determined using the Rotorgene Software version 6.1 in comparative quantitation mode. GAPDH (NM_002046.3) and YWHAZ (NM_003406.2) were used as reference genes for expression analysis. The PCR was verified by product melting curves and single amplicons were confirmed by agarose gel electrophoresis.

For RNAi experiments, cells were transfected with siQPCT and siQPCTL (flexi tube siRNA; both Qiagen) as well as with si-p50 and si-p65 (both Santa Cruz) using Dharmafect 1 (Dharmacon; Thermo Scientific, St. Leon-Roth, Germany) in a final concentration of 25 nM. After 48 h, medium was changed to FCS free and cells were cultured or stimulated for further 24 h.

FACS analysis

Cells were detached using Accutase (PAA, Cölbe, Germany) and stained with either the anti-human CCR2-Alexa Fluor 647 nm or the IgG2b-Alexa Fluor 647 nm isotype control antibody (all Becton Dickinson, Heidelberg, Germany) at 4 °C for 45 min. After two washings with staining buffer (PBS, pH 7.2 supplemented with 2 mM EDTA and 0.5% BSA), cells were analyzed in a FACS Calibur using the CellQuest Software (Becton Dickinson).

Total and pECCL2-specific ELISA

QC inhibitor (Buchholz et al. 2009) was applied 30 min before treatment of cells with TNFa/IL1b (each 10 ng/ml). After 24 h, culture supernatant of stimulated cells was harvested and analyzed for total CCL2 and pECCL2 using specific ELISAs developed by Probiodrug as described (Cynis et al. 2011).

Scratch assay

FTC238 cells were seeded in dishes with culture inserts (ibidi, Martinsried, Germany) according to the manufacturer’s instructions. After cell attachment in RPMI 1640 with 10% FCS, culture inserts were removed creating a 500 μm cell-free gap. Experiments were done in medium without FCS containing 20 and 50 ng/ml pECCL2. IKK2 (10 μM) and anti-CCR2 (10 μg/ml, Biozol, Eching, Germany) were added 30 min before CCL2 stimulation. After 12 h of incubation, images were taken and analysis was done using the web-based image analysis platform of Wimasis (www.wimasis.com).

Transwell chemotaxis assay

Potency of monocytic THP1 cells (CLS, Eppelheim, Germany) for migration into the lower compartment containing conditioned culture media of different thyroid cell lines was quantified in a transwell assay as described (Cynis et al. 2011). After 90 min, migrated THP1 cells were counted using FACS Calibur based on 5000 beads per sample in BD Trucount tubes (BD, Heidelberg, Germany) as reference standard. Thyroid carcinoma cells (5 × 10⁵ cells/well) were cultured in six-well plates in medium with 10% FCS for 1 day. The day after, the medium was changed to medium with 0.1% BSA. Cells were treated with IKK2 (10 μM) or QC inhibitor (10 μM) and conditioned cell culture supernatant was collected after 24 h. Neutralizing antibodies polyclonal anti-MCP1 (10 μg/ml; Peprotech) or monoclonal anti-MCP1/CCL2 (10 μg/ml; R&D, Wiesbaden, Germany) were added to the conditioned medium 30 min before starting the transmigration assay. THP1 cells were treated with anti-CCR2 antibody (10 μg/ml; Biozol) 30 min before performing the assay.

Proliferation assay

FTC238 or SW1736 cells (1000 cells/well) were seeded in a 96-well plate in RPMI/10% FCS or were treated with siRNA as described. Next day, the medium was changed to RPMI 1640 without FCS containing IKK2 inhibitor, CCR2 antagonist solved in DMSO (sc-2025252; Santa Cruz, Heidelberg, Germany), and the medium, DMSO control, and compounds were replenished every 24 h. After 72-h treatment, WST-8 (Promokine, Heidelberg, Germany) was added and absorption was determined at 450 nm (Spectrafluor Plus Microplate Reader, Tecan, Crailsheim, Germany). The reference wavelength of 600 nm was subtracted from the sample absorption at 450 nm.

Statistical analysis

Values are expressed as mean ± S.D. Statistical analyses comprised Student’s t-test, one-way ANOVA followed by Tukey’s post hoc analysis and correlation analysis.
GraphPad Prism 4 (GraphPad Software, Inc., La Jolla, CA, USA) was used.

**Results**

**High QPCT transcript levels correlate with high CCL2 mRNA expression in thyroid carcinomas**

Using qPCR analysis, we measured the QPCT gene expression in tissues of patients with FTC, PTC, and undifferentiated anaplastic thyroid carcinoma in comparison to control tissues of patients with goiter (mean 1.0; median 0.83; range 0.5–1.8) and tissue of normal thyroid (mean 1.09). As shown in Fig. 1A, the QPCT gene expression was up 1.2-fold in UTC (range 0.2–7.5), 3.0-fold in FTC (range 0.6–8.5), and 3.2-fold in PTC (range 0.7–10.0) respectively. In contrast, the QPCTL gene expression was not increased (Fig. 1B) with comparable QPCTL levels in FTC (0.6-fold; range 0.6–1.8), PTC (0.48-fold; range 0.2–1.2), and UTC (0.35-fold; range 0.2–1.9) compared with the goiter tissue (range 0.46–1.8) and normal thyroid tissue (1.4-fold).

We also quantified the QPCT transcript levels by qPCR in 12 cultured thyroid carcinoma cell lines: FTC133, FTC236, FTC238, ML-1, TT2609-CO2 (all follicular), B-CPAP (papillary), BHT101, 8305, 8508C, SW1736, C643, and Hth74 (all undifferentiated). Among all cell lines studied, QPCT was expressed with the highest amounts in SW1736 and FTC238 cells (Fig. 2A). FTC133

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**Figure 1**

Comparison of the relative amounts of gene transcripts for QPCT (A) and QPCTL (B) in tissues of patients with thyroid carcinoma, goiter tissues (each group, n = 10), and normal thyroid tissues (n = 3) respectively. Results of qPCR are given as comparison to the mean expression level in goiter tissues as described in the Materials and methods section. (C) QC expression in FTC and PTC as well as in FTC238 cells shown by immunohistochemistry.
cells express similar amounts of QPCT mRNA as measured in normal thyroid tissues. Expression of QC protein was detected by immunohistochemistry. Strong QC immunoreactivity was observed in the cytoplasm of FTC238 cells and in thyroid carcinoma cells of patients with FTC and PTC (Fig. 1C). Furthermore, we determined the gene expression of the QC substrate CCL2. SW1736 and FTC238 cells with strong QPCT expression also showed the highest CCL2 mRNA levels (Fig. 2B). Correlation analysis verified a significant relationship between the gene expression of QPCT and CCL2 with $P=0.0015$ and $r^2=0.65$ (Fig. 2C).

Next, we measured the ratio between pECCL2 and total CCL2 using two ELISA systems, which permitted discrimination between total CCL2 and pECCL2 fraction. In the culture supernatant, similar levels of total CCL2 and pECCL2 were detected, demonstrating that all CCL2 is secreted in its pE variant (Fig. 2D). However, SW1736 cells expressed low levels of CCL2 protein. Therefore, we see a discrepancy between high amounts of QPCT and CCL2 mRNA vs low amounts of CCL2 protein, suggesting a block at translational level or a transcriptional feedback regulation mechanism.

NF-κB-dependent regulation of QPCT and CCL2

NF-κB signaling is induced by oncogenic proteins such as Ret/PTC, Ras, and BRAF, making this signaling pathway an interesting therapeutic target in thyroid cancer (Bauerle et al. 2010). We stimulated FTC238 cells with TNFa and IL1b, cytokines known to activate the NF-κB signaling pathway. After 6 h of treatment, transcript levels of QPCT and CCL2 were quantified. The combination of TNFa and IL1b (each 100 ng/ml) moderately induced QPCT gene expression (1.85-fold) and increased the CCL2 mRNA levels in FTC238 cells (up to 15-fold compared with the unstimulated control; Fig. 3A).

In addition, we treated FTC238 and SW1736 cells with the inhibitor of the NF-κB pathway, IKK2 compound IV, and with the ERK/MAPK inhibitor U0126. Only the IKK2 inhibitor was able to abolish the TNFa/IL1b-induced gene expression of QPCT and its substrate CCL2 (Fig. 3B). siRNA-mediated knock-down of p65 and p50 decreased both CCL2 mRNA and protein amounts, whereas QPCT transcript levels were only reduced by p50 RNAi in FTC236 and FTC238 cells (Fig. 3C and D). In conclusion, we demonstrated for the first time that the NF-κB pathway is involved in the regulation of both QPCT and CCL2 in thyroid carcinomas.
ratio of pECCL2 and total CCL2 was not influenced, regulating enzyme and substrate (Fig. 4A left and B). The level, whereas mock and non-template control did not co-silencing reduced CCL2 at mRNA and protein for 6 h. (B) After treatment with the appropriate inhibitor IKK2 or or 100 ng/ml) of TNFa and IL1b or with combinations of both cytokines (A) FTC238 cells were stimulated with different concentrations (1, 10, or 100 ng/ml) for further 24 h. Results of qPCR (C) and total CCL2 ELISA (D). *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Physiological relevance of CCL2 in thyroid carcinoma cells

In order to study the relevance of CCL2, we used cell lines derived from a single patient’s tumor tissue: FTC133 derived from the primary tumor, FTC236 obtained from a neck lymph node metastasis, and FTC238 from a lung metastasis. Of the three cell lines, FTC236 showed a moderate invasive behavior and FTC238 a high invasive potential, whereas FTC133 did not (Demeure et al. 1992, Hoelting et al. 2001). The invasive behavior of these human thyroid cancer cell lines positively correlated with their CCL2 expression levels (Fig. 2D). Stimulation with TNFa/IL1b increased the CCL2 protein up to eightfold in FTC236 and FTC238 cells. The basal CCL2 amounts in FTC133 were below the limit of quantitation (LOQ) but increased with cytokine stimulation (data not shown). In line with the basal levels, in the culture supernatant of stimulated cells, equal amounts of pECCL2 and total CCL2 were detected, suggesting that all CCL2 was present in the mature pECCL2 form (Fig. 4B and C). Both QPCT and QPCTL silencing reduced CCL2 at mRNA and protein level, whereas mock and non-template control did not co-regulate enzyme and substrate (Fig. 4A left and B). The ratio of pECCL2 and total CCL2 was not influenced, suggesting that the remaining enzyme activity is sufficient to modify the N-terminus of the reduced CCL2 level. In contrast, addition of an inhibitor of the QC activity to TNFa/IL1b-treated FTC236 and FTC238 cells resulted in a strong reduction of pECCL2 amounts (Fig. 4C). The QC inhibitor did not affect the TNFa/IL1b-mediated increase in QPCT and CCL2 transcript levels (data not shown). Therefore, combination of QPCT/QPCTL RNAi and low-dose QC inhibitor treatment demonstrated that QC activity is responsible for cyclization of CCL2 in thyroid carcinomas (Fig. 4B, right). The QC inhibitor in a concentration of 2.5 μM reduced the ratio of pECCL2/total CCL2 to 0.84 (Fig. 4C) or in the NTC control to 0.69 (Fig. 4B), whereas the combination of QPCT/QPCTL RNAi and 2.5 μM QC inhibitor to 0.37. Our results indicated a role of QCs in the formation of the pE-residue and stabilizing modification of CCL2 in thyroid carcinoma cells, likely increasing the half-life of the receptor acting form of this protein.

In addition, we addressed the potential effects of CCL2 on motility and the CCL2/CCR2 pathway in proliferation of FTC238 cells. Employing flow cytometry, we first confirmed cell surface expression of the CCL2 receptor CCR2 in FTC238 cells (data not shown). Next,
we tested the effect of recombinant CCL2 on motility of FTC238 cells in scratch assays. FTC238 cells cultured in the presence of CCL2 (50 ng/ml) displayed significantly enhanced motility after 12 h and were able to close the 500 \( \mu \text{m} \) scratch faster than FTC238 cells without chemokine treatment. Treatment of cells with NF-\( \kappa \)B inhibitor IKK2 or anti-CCR2 antibody reduced the FTC238 motility (Fig. 5A).

Recently, we reported a higher potency of pECCL2 compared with immature CCL2 in attracting of monocytes at concentrations up to 25 ng/ml (Cynis et al. 2011). Conditioned medium of cultured thyroid carcinoma cells was used as chemoattractant for testing the migration activity of monocytic THP1 cells in a transwell chemotaxis assay. CCL2-neutralizing antibodies significantly suppressed the cell migration, confirming the relevance of thyroid carcinoma cell-derived CCL2 in attraction of monocytes. The migration index was reduced more than half with anti-CCL2-treated conditioned medium of FTC238 and BHT101 cells and to 0.61 and 0.82 with medium of FTC236 and SW1736 cells respectively.

Furthermore, inhibition of QC activity by the QC inhibitor, the NF-\( \kappa \)B pathway by IKK2, or incubation of THP1 cells with anti-CCR2 significantly diminished THP1 migration (Fig. 5A).

To study the influence of an activated CCL2/CCR2 pathway on cell proliferation, cells were cultured for 72 h and cell proliferation was measured using WST-8 assays. While the addition of recombinant CCL2 did not influence the cell proliferation (data not shown), treatment FTC238 cells with the CCR2 antagonist reduced cell proliferation in a concentration-dependent way whereas the CCR2-negative SW1736 cell line was not affected (Fig. 6A and B). QPCT silencing and inhibition of NF-\( \kappa \)B signaling by IKK2 inhibited FTC238 proliferation significantly (Fig. 6C).

**Discussion**

In recent years, growing evidence suggests an involvement of the CCL2/CCR2 pathway in cancer progression. In PTC, CCL2 is correlated with lymph node metastasis and tumor...
recurrence and is discussed as a marker for aggressive behavior of this tumor type (Tanaka et al. 2009). Recently, others and we described that the pE protein modification is an important posttranslational step in CCL2 maturation (Cynis et al. 2011, Chen et al. 2012). Owing to the cyclization of the N-terminal CCL2 glutaminyl precursor into pE, the positive charge of the N-terminus is lost resulting in resistance against cleavage by aminopeptidases such as dipeptidyl peptidase 4/CD26, which is highly expressed in FTC and PTC (Kehlen et al. 2003). However, the immature CCL2 can also be cleaved by aminopeptidase P and matrix metalloproteinase-1 and the resulting truncated CCL2 variants can only bind with lower affinity or act as antagonist to the CCL2 receptor CCR2. In contrast to DP4 and aminopeptidase P, the mature pE-modified N-terminus of CCL2 is not protected against MMP1 cleavage (McQuibban et al. 2002, Cynis et al. 2011). Furthermore, inhibition of QC activity by a competitive inhibitor or by RNAi suppressed monocyctic cell migration (Cynis et al. 2011, Chen et al. 2012). Hence, the prevention of pE-formation has the potential to enhance chemokine degradation by suppression of N-terminal maturation and, thereby, opening alternative degradation pathway and this impairs the bioactivity of CCL2. Here, we show that both the enzymes QC (gene name QPCT) and isoQC (gene name QPCTL) responsible for the pE-formation of CCL2 are expressed in human thyroid carcinomas. While the amount of QPCTL transcripts was unchanged, the QPCT transcript levels were increased in thyroid cancer tissues, particularly in patients with PTC and FTC, compared with goiter tissues. Intriguingly, there was a positive correlation between the gene expression of QPCT and its substrate CCL2 in thyroid carcinoma cell lines. CCL2 is known to be regulated by the

**Figure 5**

Autocrine and paracrine effects of CCL2 on cell motility. (A) Effect of CCL2 on the invasive capacity of FTC238 cells. Results of scratch assays using the Wimasis image analysis platform. Results are given as difference of cell-covered area in percent (%) at time point 12 and 0 h (n = 4). (B) Dependence of THP1 monocyte migration on CCL2 secreted in conditioned medium of different thyroid carcinoma cell lines assessed by transwell migration assay. Neutralizing antibodies anti-CCR2 and anti-CCL2 (polyclonal; Peprotech, and monoclonal; R&D) were added 30 min before performing the migration assay. FTC238 and FTC236 cells were treated with IKK2 (10 μM) and QC inhibitor (10 μM), and conditioned medium was collected after 24 h for assessing the potency to attract THP1 cells. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
transcription factor NF-κB (Ueda et al. 1994, 1997). For the QPCT promoter, a potential NF-κB binding site was detected at position −2677 bp of the ATG transcriptional start codon. NF-κB is constitutively activated in thyroid carcinomas where it controls the expression and function of genes involved in different cellular activities, such as proliferation, invasion, and apoptosis (Bauerle et al. 2010, Pacifico & Leonardi 2010). Proinflammatory cytokines, like TNFa and IL1b, activate the NF-κB pathway and are involved in the transcriptional co-regulation of QPCT and its substrate CCL2. Inhibition of the activation of NF-κB in thyroid carcinoma cells by an inhibitor of IKK2 prevented the co-regulated expression of both QPCT and CCL2, whereas an inhibitor of the ERK/MARK pathway (U0126) did not. Silencing of NF-κB protein p50 reduced both QPCT expression and CCL2 RNA and protein levels but did not reduce the pECCL2 amounts (data not shown). In contrast, p65 RNAi did not affect QPCT gene expression, suggesting that another NF-κB subunit heterodimerize with p50. Thus, the NF-κB activating transformation through oncoproteins Ret/PTC3, Ras, BRAF, or miR-146 is involved in QPCT and CCL2 upregulation in thyroid carcinomas (Bauerle et al. 2010, Pacifico et al. 2010, Neely et al. 2011). Recently, we reported that in mouse, the isoQC is an important regulator of monocyte infiltration under inflammatory conditions through pE-formation of CCL2 (Cynis et al. 2011). In the human, we did not observe a regulation of QPCTL, neither in thyroid tissues nor under stimulatory conditions in vitro. The co-regulatory effect of QPCT and QPCTL RNAi in reducing CCL2 mRNA levels was also seen in other cells as lung carcinomas or endothelial cells (A Kehlen & M Haegele, unpublished observations, 2012) and is independent of the siRNA pool used. Whether the reduced CCL2 protein itself or other mechanisms are involved in this process needs further investigations to understand the regulation of QPCT/QPCTL and its substrate CCL2. Silencing of QPCT and QPCTL decreased gene expression of CCL2 but did not influence the ratio of pECCL2/total CCL2 protein, suggesting that the remaining enzyme activity is sufficient to transform the reduced CCL2 amounts. In addition to the enzymatic reaction, there is a spontaneous cyclization of Q1CCL2 to pECCL2 in the phosphate-containing culture medium. Even when applying high concentrations of QC inhibitor, there was no complete inhibition of the pECCL2 formation. Nevertheless, using the combination of QPCT/QPCTL RNAi and low-dose QC inhibitor treatment, we demonstrated that the QC activity is responsible for the maturation of Q1CCL2 to pECCL2. Further studies have to clarify the involvement of both the

Figure 6
Impact of CCL2/CCR2 pathway on proliferation of FTC238 cells. CCR2-positive FTC238 and CCR2-negative SW1736 cells were treated with the CCR2 antagonist for 72 h before performing WST-8 assay (A). FTC238 cells were treated with IKK2 inhibitor or transfected with siRNA specific for QPCT and QPCTL and the effect on cell proliferation was quantified via WST-8 (B). Measurements were taken at OD 450 nm (n=4). **, P < 0.01; ***, P < 0.001.
glutaminyl cyclases, isoQC or QC, under normal and stimulated/pathological conditions in pECCL2 formation of thyroid cancers.

CCL2 is correlated with lymph node metastasis and is discussed as an independent predictive factor for tumor recurrence in PTC (Tanaka et al. 2009). Here, we report that FTC238 cells express the CCR2 and can respond to CCL2 with increased tumor cell motility as shown in scratch assays. Intriguingly, the addition of a CCR2 antagonist to CCR2-expressing thyroid carcinoma cells reduced their proliferation, indicating autocrine functions of CCL2 in addition to its pro-migratory effect. Treatment of FTC238 cells with exogenous CCL2 did not influence the cell proliferation but it increases cell motility. The reason for this discrepancy could be the time point of measurements and therefore higher amounts of endogenous secreted CCL2 in the proliferation compared with the migration assay. Apart from promoting tumor cell growth, CCL2 has multifunctional effects on normal host cells in the tumor microenvironment (Zhang et al. 2010). CCL2 promotes angiogenesis through its interaction with vascular endothelial cells (Salcedo et al. 2000). Here, we show the paracrine effect of thyroid carcinoma cell-derived CCL2 on the chemotaxis of monocytic THP1 cells. As a chemotactic monocytes and macrophages, CCL2 recruits tumor-associated macrophages that support tumor growth and extravasation of tumor cells (Sica et al. 2006, Qian et al. 2011). In the bone microenvironment, CCL2 activates tumor-induced osteoclastogenesis as a major step in bone metastasis (Lu et al. 2007, Lu & Kang 2009). In PTC and FTC, the common sites of distant metastases are lung and bone (Shoup et al. 2003). Thus, CCL2 may be involved in the formation of distant metastasis. In line, endothelial CCR2 signaling induced by colon carcinoma cells controls metastasis by promoting tumor cell extravasation via increase in vascular permeability (Wolf et al. 2012). In addition, CCL2 attracts immunosuppressive Tregs and tumor-associated lymphocytes, contributing to the successful evasion of cancer cell from anti-tumor immune response. Blockade of CCL2 has been shown to augment cancer immunotherapy (Fridlender et al. 2010). In PTC, tumor-associated lymphocytes and increased FoxP3+ Treg frequency correlate with more aggressive cancer progression (French et al. 2010).

In conclusion, we demonstrated activated NF-κB signaling to increase QPCT expression in thyroid carcinomas. pE formation catalyzed by QCs is an important posttranslational step in CCL2 maturation protecting against proteolytic degradation, e.g. by dipeptidyl peptidase 4/CD26. The vast majority of secreted CCL2 was in

Figure 7
Role of pECCL2 in thyroid carcinoma. Constitutively activated NF-κB increased the expression of QPCT and its substrate CCL2. Pyroglutamate formation catalyzed by QC is an important posttranslational step in CCL2 maturation, resulting in pECCL2 being secreted by the thyroid cancer cells. pECCL2 can bind to CCR2 expressed on the surface of thyroid carcinoma cells and contributes to higher invasiveness and proliferation. In addition, pECCL2 attracts monocytes and other CCR2-positive cells that support tumor proliferation and metastasis.
the form of pECCL2 and contributed to higher invasiveness, presumably by binding to its receptor CCR2 expressed on the surface of thyroid carcinoma cells. In addition to this autocrine function, CCL2 increases monocytic chemotaxis (Fig. 7). CCL2 influences cancer tumorigenesis by modulating tumor cells and their microenvironment. The inhibition of QC/isoQC-mediated activity and pECCL2 formation resulting in the destabilization and proteolytic degradation of this pro-migratory chemokine may be an attractive novel target in preventing thyroid cancer metastasis.

Declaration of interest
A Kehlen, M Haegle, K Menge, and K Gans are employees of Probiodrug. H-U Demuth serves as CSO of Probiodrug and Managing Director of Ingenium Pharmaceutical GmbH, a daughter company of Probiodrug, and holds stocks of the Probiodrug group.

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