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# Chemerin inhibits IGF-I-induced progesterone and estradiol secretion in human granulosa cells

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**BACKGROUND:** Chemerin is a novel adipokine involved in the regulation of adipocyte development, inflammation and metabolic functions. To date, no role of this adipokine in reproductive functions has been described. In the present study, we identified chemerin and its receptor, CMKLR1 (chemokine-like receptor 1), in primary human granulosa cells (hGCs) and in a human ovarian granulosa-like tumour cell line (KGN). We also investigated the effects of recombinant human chemerin (rhChem) on steroid production and on various signalling pathways.

**METHODS AND RESULTS:** By RT–PCR immunoblotting and immunohistochemistry, we showed that chemerin and CMKLR1 are expressed in hGCs and KGN cells. By ELISA, we also found chemerin in human follicular fluid and we observed that in 8 of 10 women the chemerin level was at least 2-fold higher in follicular fluid than in plasma. rhChem (10 or 100 ng/ml) significantly decreased insulin-like growth factor-I (IGF-I) ( $10^{-8}$  M)-induced secretion of progesterone and estradiol (as determined by radioimmunoassay) but did not affect basal-or FSH ( $10^{-8}$  M)-induced steroid secretion in hGCs and KGN cells. In parallel, it also decreased IGF-I-induced p450 aromatase protein levels without affecting the protein levels of other factors involved in steroidogenesis (steroidogenic acute regulatory protein, 3-beta-hydroxysteroid dehydrogenase and p450 side-chain cleavage enzyme) in hGCs cells. All these changes were associated with a decrease in the IGF-I-induced tyrosine phosphorylation of IGF-I receptor beta subunit and phosphorylation of mitogen-activated protein kinase extracellular signal-regulated kinases 1/2 (MAPK ERK1/2) and Akt. In hGCs and KGN cells, rhChem also decreased IGF-I-induced thymidine incorporation. Finally, we showed that rhChem rapidly activates MAPK ERK1/2, MAPK P38 and Akt phosphorylation and more slowly AMP-activated protein kinase phosphorylation under basal conditions (no IGF-I or FSH) in primary hGC cells.

**CONCLUSIONS:** Taken together, chemerin and its receptor (CMKLR1) are present and active in hGCs. Chemerin reduces IGF-I-induced steroidogenesis and cell proliferation through a decrease in the activation of IGF-IR signalling pathways in primary hGCs.

**Key words:** adipokine / ovary / steroidogenesis / growth factors / signalling pathways

## Introduction

Adipose tissue (AT) is not only an energy storage organ but it is also able to secrete a number of hormone-like compounds. Among the main endocrine products of the AT are the proteins: leptin, adiponectin, resistin, visfatin and chemerin. Chemerin, also known as retinoic acid receptor responder protein 2 (RARRES2), is a novel adipokine that regulates adipocyte development and metabolic function (Roh *et al.*, 2007). It is a 16 kDa protein secreted in an inactive form as pro-chemerin and is activated through cleavage of the C-terminus by inflammatory and coagulation serine proteases (Zabel *et al.*, 2005).

In humans, the plasma chemerin levels (~100–200 ng/ml, Bozaoglu *et al.*, 2010; Hu and Feng, 2011) are correlated with body fat, glucose and lipid metabolism, and inflammation. For example, plasma chemerin levels in patients who had undergone bariatric surgery for the purpose of weight loss were significantly reduced after surgery, and correlated with BMI and fat mass (Ress *et al.*, 2010; Sell *et al.*, 2010). A majority of human data indicate that plasma chemerin is elevated in obesity/diabetes (Bozaoglu *et al.*, 2007) and in inflammatory states (Pasceri and Yeh, 1999; Wittamer *et al.*, 2003). Recent animal studies reported the parallel findings that obese and diabetic mice have elevated circulating levels of

chemerin (Ernst *et al.*, 2010; Parlee *et al.*, 2010). Chemerin acts through its receptor, the G protein-coupled receptor, CMKLR1 (chemokine-like receptor 1), (Gantz *et al.*, 1996). Activation of CMKLR1 with its ligands results in intracellular calcium release, inhibition of cAMP accumulation and phosphorylation of mitogen-activated protein kinase extracellular signal-regulated kinases 1/2 (MAPK ERK1/2), through the G<sub>i</sub> class of heterotrimeric G proteins (Yoshimura and Oppenheim, 2011). CMKLR1 is also able to activate the phosphatidylinositol 3-kinase/Akt signalling pathway (Yoshimura and Oppenheim, 2011).

During the last few years, new roles of adipokines have been emerging in the field of fertility and reproduction. Indeed, leptin, first described over a decade ago, has been thoroughly studied regarding reproduction and at several levels of the reproductive axis, from the pituitary to gonads (Moschos *et al.*, 2002). Adiponectin and adiponectin receptors (AdipoR1 and AdipoR2) are expressed in many reproductive tissues, including the central nervous system, ovaries, oviduct, endometrium and testes (Mitchell *et al.*, 2005). They influence gonadotrophin release in pituitary cells, steroid secretion in ovarian cells, normal pregnancy and assisted reproduction outcomes. In humans, high levels of adiponectin are associated with an improved menstrual function and better outcomes in assisted reproductive cycles (Michalakis and Segars, 2010). We previously identified resistin in rat and bovine ovary and showed that it can modulate *in vitro* granulosa cell steroidogenesis and proliferation in a species-specific manner (Maillard *et al.*, 2011). In humans, the precise reproductive role of resistin remains controversial. Indeed, Seow *et al.* (2005) showed that resistin was not a major determining factor in the growth and maturation of oocytes during ovarian stimulation, whereas Chen *et al.* (2007) demonstrated a negative correlation between serum resistin levels and the number of oocytes retrieved during IVF. Chemerin and CMKLR1 have been shown to be expressed in mouse ovary and placenta and in human placenta (Goralski *et al.*, 2007).

However, to date, no studies have described the presence of chemerin and CMKLR1 in the human ovary. In the present study, we identified chemerin and CMKLR1 in human ovarian follicles. We also investigated the effects of human recombinant chemerin on steroid production and on the activation of various signalling pathways in granulosa cells from women undergoing IVF and in KGN cells.

## Materials and Methods

### Patients

Twenty-two infertile women (36 ± 4 years old) attending the IVF unit of the department for ICSI cycles were recruited in 2011. The aetiology of infertility was mechanical, unexplained or male factor infertility without any known endocrinopathy [polycystic ovarian syndrome (PCOS), hyperprolactinemia, hypo- and hyperthyroidism]. Patients gave their written informed consent and did not receive any monetary compensation for participating in the study. From each patient, only one cycle was included in the study.

### KGN cell culture

The human ovarian granulosa-like tumour cell line, KGN, was obtained from Drs Masatoshi Nomura and Hajime Nawata, Kyushu University, Japan (Nishi *et al.*, 2001). KGN cells are undifferentiated and maintain

physiological characteristics of ovarian cells, including the expression of functional FSH receptor and the expression of aromatase. They were cultured in Dulbecco's minimal essential medium/F12 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum and antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin obtained from Sigma) in a 5% CO<sub>2</sub> atmosphere at 37°C.

### Hormones and reagents

Purified ovine FSH-20 (oFSH; lot no. AFP-7028D, 4453 IU/mg, FSH activity = 175 times the activity of oFSH-S1) used for culture treatment was a gift from NIDDK, National Hormone Pituitary Program, Bethesda, MD, USA. Recombinant human insulin-like growth factor-I (IGF-I) was from Sigma. Recombinant human chemerin (rhChem) was obtained from R&D (Lille, France). Human ovary sections embedded in paraffin were obtained from Euromedex (Souffelweyersheim, France).

### Antibodies

Mouse monoclonal antibody to human chemerin for western blot was obtained from R&D system. Rabbit polyclonal antibodies to chemerin for immunohistochemistry were purchased from Phoenix France SAS (Strasbourg, France). Rabbit polyclonal antibodies to chemerin receptor (CMKLR1), phospho-ERK1/2 (Thr202/Tyr204), phospho-p38 (Thr180/Tyr182), phospho-Akt (Ser 473) and phospho-AMP-activated protein kinase (AMPK) alpha Thr172 were purchased from New England Biolabs Inc. (Beverly, MA, USA). Rabbit polyclonal antibodies to ERK2 (C14) and p38 (C20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies to AMPKα1 were obtained from Upstate Biotechnology Inc (Lake, Placid, NY, USA). Mouse monoclonal antibodies to Vinculin and p450 aromatase were obtained from Sigma and Serotec (Varilhes, France), respectively. Rabbit polyclonal antibodies against p450 side-chain cleavage enzyme (p450scc), steroidogenic acute regulatory (StAR) and 3-beta-hydroxysteroid dehydrogenase (3-β-HSD) were generously provided by Dr Dale Buchanan Hales (University of Illinois, Chicago, IL, USA) and Dr Van Luu-The (CHUL Research Center and Laval University, Canada), respectively. All antibodies were used at 1/1000 dilution in western blotting.

### Isolation and culture of human granulosa cells

Granulosa cells were collected from pre-ovulatory follicles during oocyte retrieval for IVF. The ovarian stimulation protocol and IVF and ICSI procedures used have already been reported (Guerif *et al.*, 2004). After isolation of cumulus-oocyte complexes (used for IVF), follicular fluids were pooled and centrifuged (400g, 10 min). To remove most of the red blood cells, the pellet was centrifuged (400g, 20 min) on a two layer, discontinuous Percoll gradient (40%, 60% in Ham's medium, Gibco-BRL; Life Technologies, Cergy Pontoise, France). The 40% fraction was collected and treated with a haemolytic medium (NH<sub>4</sub>Cl 10 mmol/l in Tris-HCl, 10 mmol/l, pH 7.5; Sigma, Isles d'Abeau, France) to remove as many as possible of the remaining red blood cells. Following centrifugation, the pellet was washed with a fresh medium (Hams F12); cells were counted in a haemocytometer and cell viability was determined using Trypan Blue dye exclusion. Cells were cultured in McCoy's 5A medium supplemented with 20 mmol/l HEPES, penicillin (100 U/ml), streptomycin (100 mg/l), L-glutamine [3 mmol/l, 0.1% bovine serum albumin (BSA), 0.1 µmol/l androstenedione, 5 mg/l transferrin, 20 µg/l selenium] and 5% fetal bovine serum (FBS). The cells were initially cultured for 48 h with no other treatment and then incubated in fresh culture medium with or without test reagents for the appropriate time. All cultures were performed under a water-saturated atmosphere of 95% air/5% CO<sub>2</sub> at 37°C. We performed four cultures (one per week). Each culture was

performed by pooling cells obtained from different follicles from one patient. In each culture, each treatment (chemerin in the presence or in the absence of IGF-I or FSH) was applied in quadruplicate or duplicate, as indicated in the legend of figures.

## RNA isolation and RT-PCR

Total RNA was extracted from human granulosa cells (hGCs), human ovarian granulosa tumour-derived cell line cells (KGN) and from human visceral or subcutaneous adipose tissue (Vis AT, Sc, AT), using Trizol reagent according to the manufacturer's procedure (Invitrogen). RT-PCR was performed to assay the expression of chemerin and CMKLR1 in hGCs and KGN cells and in Vis and Sc AT. RT of total RNA (1  $\mu$ g) was carried out in a 20  $\mu$ l reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 200  $\mu$ M of each deoxynucleotide triphosphate (Amersham, Piscataway, NJ, USA), 50 pmol of oligo(dT)15, 5 U of ribonuclease inhibitor and 15 U of Moloney murine leukemia virus reverse transcriptase. RT reaction was performed at 37°C for 1 h. The sets of primers for chemerin and CMKLR1 were: forward chemerin 'agacaagctgccggaagagg', reverse chemerin 'tggagaaggcgaactgtcca', forward CMKLR1 'acttctgttcaactcttct' and reverse CMKLR1 'cagctttaga caatggtga'. PCRs were carried out using 2  $\mu$ l of the RT reaction mixture in a volume of 50  $\mu$ l containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 mM of each deoxynucleotide triphosphate, 10 pmol of each primer and 1 U of Taq polymerase. The samples were processed for 35 PCR cycles (95°C, 1 min; 58°C, 1 min; 72°C, 1 min), with a final extension step at 72°C for 10 min. PCR products were visualized in an agarose gel (1.5%) stained with ethidium bromide, and the DNAs were extracted from the agarose using the gel extraction kit QIAEX II (Qiagen, Hilden, Germany) and sequenced by the Genome Express company (Meylan, France). PCR amplifications with RNA were performed in parallel as negative controls. RT-PCR consumables were purchased from Sigma (Isles d'Abeau), except that Moloney Murine Leukemia Virus reverse transcriptase and RNase inhibitor (RNasin) were from Promega (Madison, WI, USA).

## Immunoprecipitation and immunoblotting

KGN cells, freshly collected hGCs purified on Percoll gradient and *in vitro* cultured hGCs and adipose tissues (Sc and Vis AT) were homogenized as previously described (Chabrolle et al., 2009; Pierre et al., 2009). Lysates were incubated on ice for 30 min and then centrifuged at 12 000g for 20 min at 4°C. The protein concentration in the resulting supernatants was then determined using the bicinchoninic acid protein assay.

To measure IGF-1R $\beta$  tyrosine phosphorylation, lysates containing 100  $\mu$ g of protein were incubated with IGF-1R $\beta$  antibodies (1:1000 dilution) for 16 h at 4°C. Immune complexes were precipitated by incubation with protein G-agarose for 1 h at 4°C as described previously (Dupont et al., 2000). Immunoprecipitates were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Blots were blocked by incubation for 1 h at room temperature with 5% BSA dissolved in Tris-buffered saline supplemented with 0.1% Tween-20 and then probed with PY20 antibodies (1:1000 dilution). After extensive washing, immune complexes were detected using horse-radish peroxidase (HRP)-conjugated secondary antibodies (Amersham Biosciences) and an enhanced chemiluminescence detection system. Blots were then stripped and re-probed with IGF-1R $\beta$  antibodies. The radiographs were scanned, and the optical density of each band was measured using the software Scion Image (4.0.2 version). In order to determine MAPK ERK1/2, Akt, AMPK phosphorylation levels and the amount of P450 aromatase, CMKLR1 and chemerin, the lysates (50  $\mu$ g of protein) were then directly subjected to electrophoresis on 10% (w:v) SDS-PAGE under reducing conditions and transferred onto nitrocellulose membranes (Schleicher and Schuell, Ecqueville, France).

Membranes were incubated overnight at 4°C with appropriate antibodies (final dilution 1:1000). The results are expressed as the intensity signal in arbitrary units after normalization allowed by the presence of MAPK ERK2, Akt, AMPK total (for ERK1/2, Akt and AMPK phosphorylation, respectively) and vinculin (for p450 aromatase) as an internal standard. The effect of rhChem was analysed on three or four different primary hGC cultures. Each primary culture was derived from a different patient.

## Immunohistochemistry

Human ovary sections were deparaffinized, hydrated and microwaved for 5 min in antigen unmasking solution (Vector Laboratories, Inc., AbCys, Paris, France), and then allowed to cool to room temperature. After washing in a phosphate-buffered saline (PBS) bath for 5 min, sections were immersed in peroxidase-blocking reagent for 10 min at room temperature to quench endogenous peroxidase activity (DAKO Cytomation, Dako, Ely, UK). After two washes in a PBS bath for 5 min, non-specific background was eliminated by blocking with 5% lamb serum in PBS for 20 min, followed by incubation overnight at 4°C with PBS containing rabbit primary antibody raised against either chemerin (1:100) or CMKLR1 (1:100). Sections were washed twice for 5 min each time in a PBS bath and were incubated for 30 min at room temperature with a 'ready to use' labelled polymer-HRP anti-rabbit (DakoCytomation Envision Plus HRP system, Dako, Ely, UK). The sections were then washed twice in PBS and the staining was revealed by incubation at room temperature with 3,3'-diaminobenzidine tetrahydrochloride (Liquid DAB + Substrate Chromogen System, DakoCytomation). Negative controls involved replacing primary antibodies with rabbit immunoglobulin G (IgG).

## Thymidine incorporation into granulosa cells

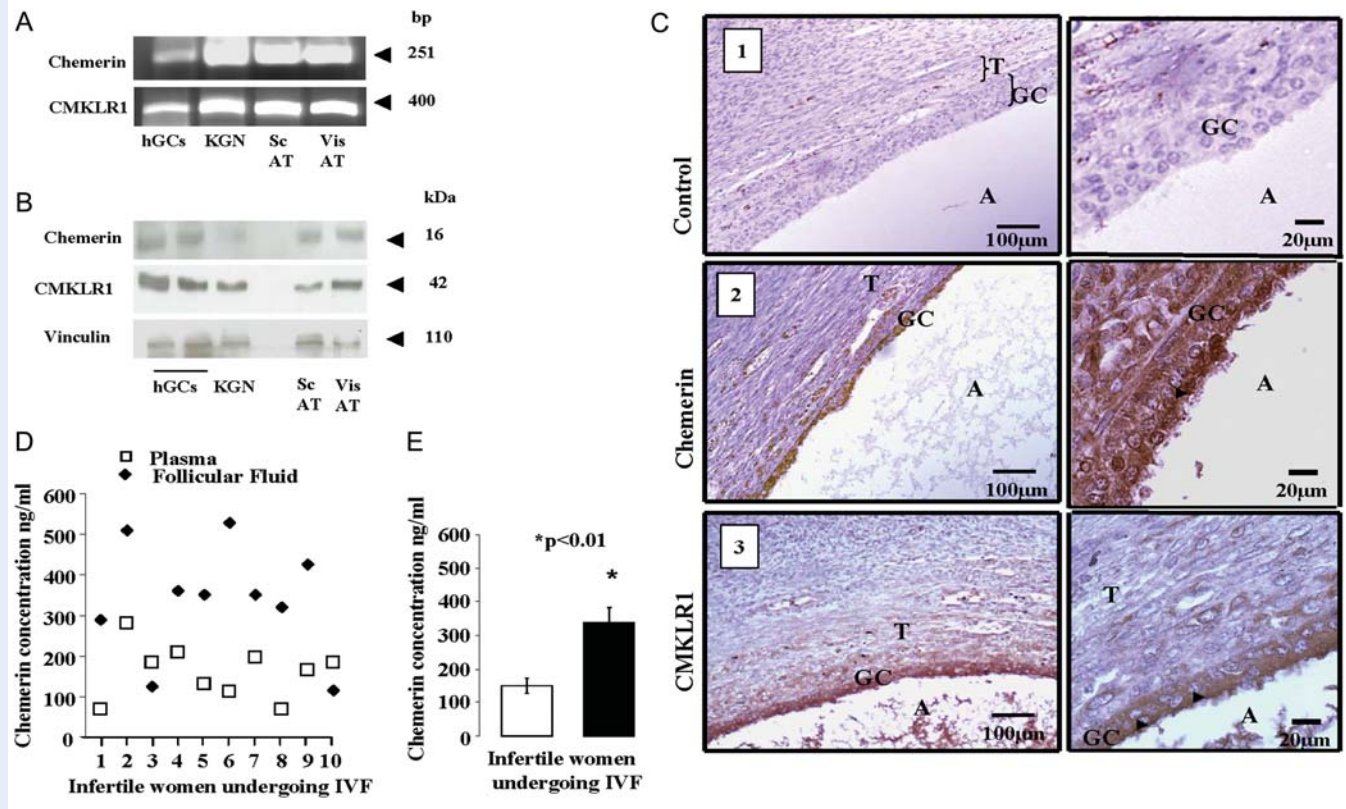
Primary hGCs (2  $\times$  10<sup>5</sup> viable cells/500  $\mu$ l) were cultured in McCoy's 5A medium and 10% FBS during 48 h and were then serum-starved for 24 h followed by the addition of 1  $\mu$ Ci/ $\mu$ l of [<sup>3</sup>H]-thymidine (Amersham Life Science, Arlington Heights, IL, USA) in the presence or absence of chemerin and/or IGF-I (10<sup>-8</sup> M). After 24 h of culture, the excess of thymidine was removed by washing the cells twice with PBS and then they were fixed with cold trichloroacetic acid 50% for 15 min and lysed by NaOH 0.5 N. The radioactivity was determined by scintillation fluid (Packard Bioscience) counting in a  $\beta$ -photomultiplier.

## Progesterone and estradiol radioimmunoassay

The concentration of progesterone and estradiol (E<sub>2</sub>) in the culture medium of KGN and hGCs cells was measured after 48 h of culture by a radioimmunoassay protocol as previously described (Chabrolle et al., 2009; Pierre et al., 2009). The limit of detection of progesterone was 12 pg/tube (60 pg/well), and the intra- and inter-assay coefficients of variation were <10 and 11%, respectively. The limit of detection of E<sub>2</sub> was 1.5 pg/tube (7.5 pg/well), and the intra- and inter-assay coefficients of variation were <7 and 9%, respectively. Results were expressed as the concentration of steroids/cell protein concentration/well. Results are means  $\pm$  SEM of data obtained from KGN cells (at four different passages) and primary hGCs (four cultures, one per week). Each primary hGCs culture was performed pooling cells obtained from different follicles from one patient. In each culture, each treatment (chemerin in the presence or in the absence of IGF-I or FSH) was applied in quadruplicate.

## Chemerin plasma and follicular fluid levels

Chemerin plasma and follicular fluid concentrations were measured using a human chemerin ELISA kit (BioVendor, Heidelberg, Germany). This ELISA



**Figure 1** Chemerin and CMKLRI expression in hGCs and measurement of the chemerin concentration in plasma and follicular fluid of women treated with IVF. **(A)** Total RNA from fresh hGCs obtained from women undergoing IVF (hGCs) and human ovarian granulosa tumour-derived cell line (KGN) was extracted as described in 'Materials and Methods' section. RT-PCR was performed by using primers designed to amplify fragments of chemerin (251 pb) and CMKLRI (400 pb). Human visceral (Vis AT) and subcutaneous (Sc AT) adipose tissues were used as a positive control for chemerin and CMKLRI expression [mRNA (A) and protein (B, see below)]. Tissues or cells from four different patients were used and two replications within patient were performed. **(B)** Protein extracts (50  $\mu$ g) were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and incubated with specific antibodies against chemerin and CMKLRI. Equal protein loading was verified by reprobing membrane with an anti-vinculin antibody. Tissues or cells from four different patients were used and two replications within patient were performed. **(C)** Localization of chemerin and CMKLRI in human ovarian follicle by immunohistochemistry. The three panels on the right-hand side are higher magnifications of part of the panels on the left. DAB-immunoperoxidase staining was performed on paraffin-embedded human ovary using antibodies against chemerin (2), CMKLRI (3) or no primary antibodies but rabbit IgG (1). Immuno-specific staining is brown. The sections were counterstained with haematoxylin. Chemerin and CMKLRI are detected in granulosa cells (GCs) and theca cells (T) of ovarian follicle. A, antrum. Bars = 100 or 20  $\mu$ m. Immunohistochemistry was performed on two different human ovary slides from each of four patients **(D and E)**. Plasma ( $\square$ ) and follicular fluid ( $\blacklozenge$ ) levels of chemerin after collecting blood and follicular fluid of 10 infertile women undergoing *in vitro* fertilization, on the day of oocyte retrieval. Each concentration was determined by ELISA method as described in 'Materials and Methods' section. The individual data (D) or the mean of these data (E) are represented. \* $P < 0.05$ .

kit does not distinguish between active chemerin and inactive prochemerin.

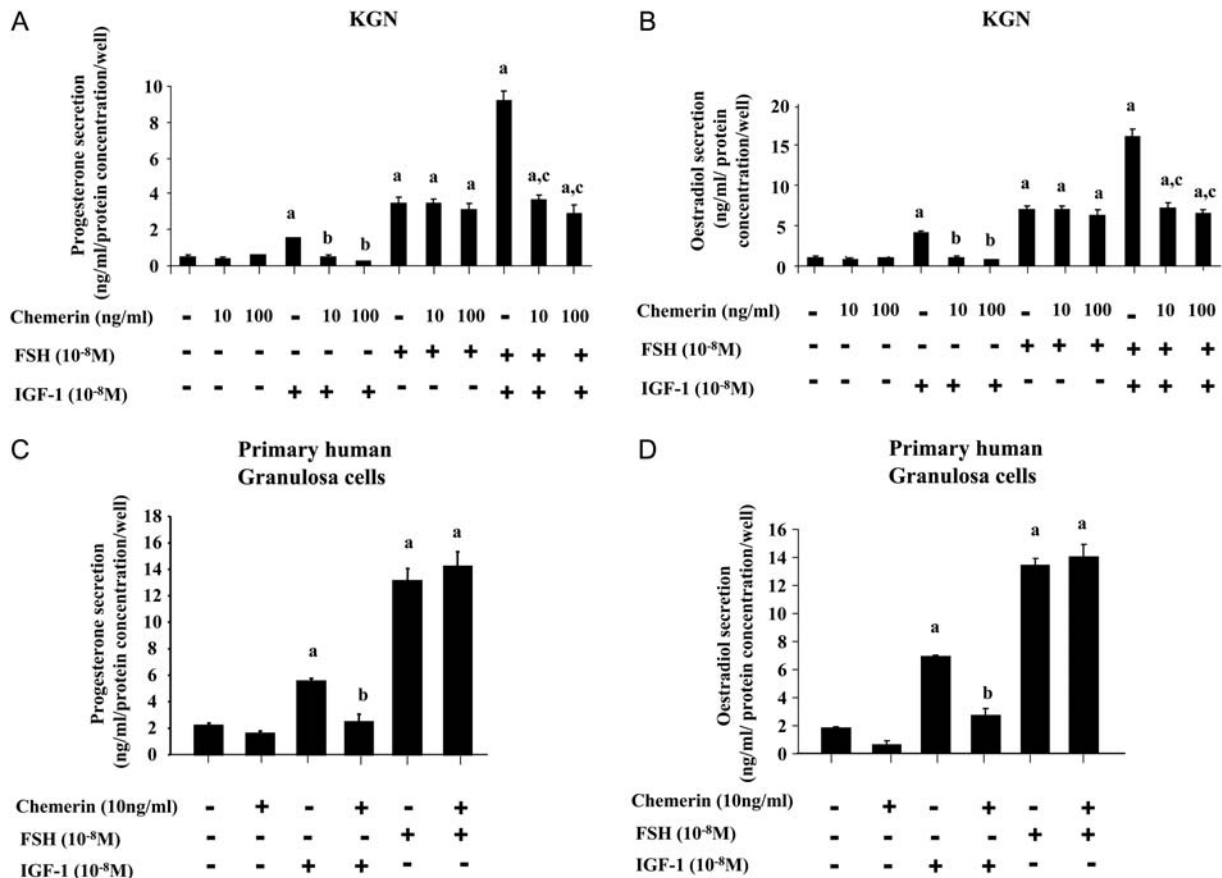
### Statistical analysis

All experimental data are presented as the mean  $\pm$  SEM. A *t*-test was used for statistical comparison of the means between two groups. A one-way analysis of variance (ANOVA) was used for multiple comparisons involving more than two treatment groups. The Student–Newman–Keuls test was used for *post hoc* comparison of the significant ANOVA. A  $P < 0.05$  was considered significant (in each experiment, 'n' is taken as the number of cultures).

## Results

### Chemerin and CMKLRI expression in hGCs

We determined the expression of chemerin and its receptor CMKLRI in fresh hGCs and in the hGC line KGN. As shown in Fig. 1A, we amplified by RT-PCR two cDNAs corresponding to the fragments of chemerin (251 pb) and CMKLRI (400 pb). Vis AT was used as a positive control for chemerin and CMKLRI expression. The transcripts of chemerin and CMKLRI were largely expressed in granulosa cells and as expected in ATs (Fig. 1A). Immunoblotting protein extracts revealed one band corresponding to chemerin (16 kDa) and another band corresponding to CMKLRI (42 kDa), showing



**Figure 2** Effect of rhChem on basal and FSH and/or IGF-I-stimulated progesterone and E<sub>2</sub> secretions by KGN (A, B) and primary hGCs (C, D) hGCs (KGN and primary hGCs) were cultured in medium with serum and then in serum-free medium in the absence or in the presence of chemerin (10 and/or 100 ng/ml) ± FSH (10<sup>-8</sup> M) and/or IGF-I (10<sup>-8</sup> M) for 48 h as described in 'Materials and Methods' section. The culture medium was collected, and progesterone (A, C) and E<sub>2</sub> (B, D) production was measured by radioimmunoassay and the data are represented as the progesterone or E<sub>2</sub> concentration (ng/ml)/cellular protein concentration/well. Results are means ± SEM of the four cultures of primary hGCs and four independent experiments (at four different passages) for KGN cells. For primary hGCs, each culture was performed by pooling cells obtained from different follicles from one patient. In each culture, each treatment (chemerin in the presence or in the absence of IGF-I or FSH) was applied in quadruplicate. <sup>a</sup>P < 0.05 compared with the untreated control, <sup>b</sup>P < 0.05 compared with the IGF-treated, <sup>c</sup>P < 0.05 compared with the IGF/FSH-treated samples.

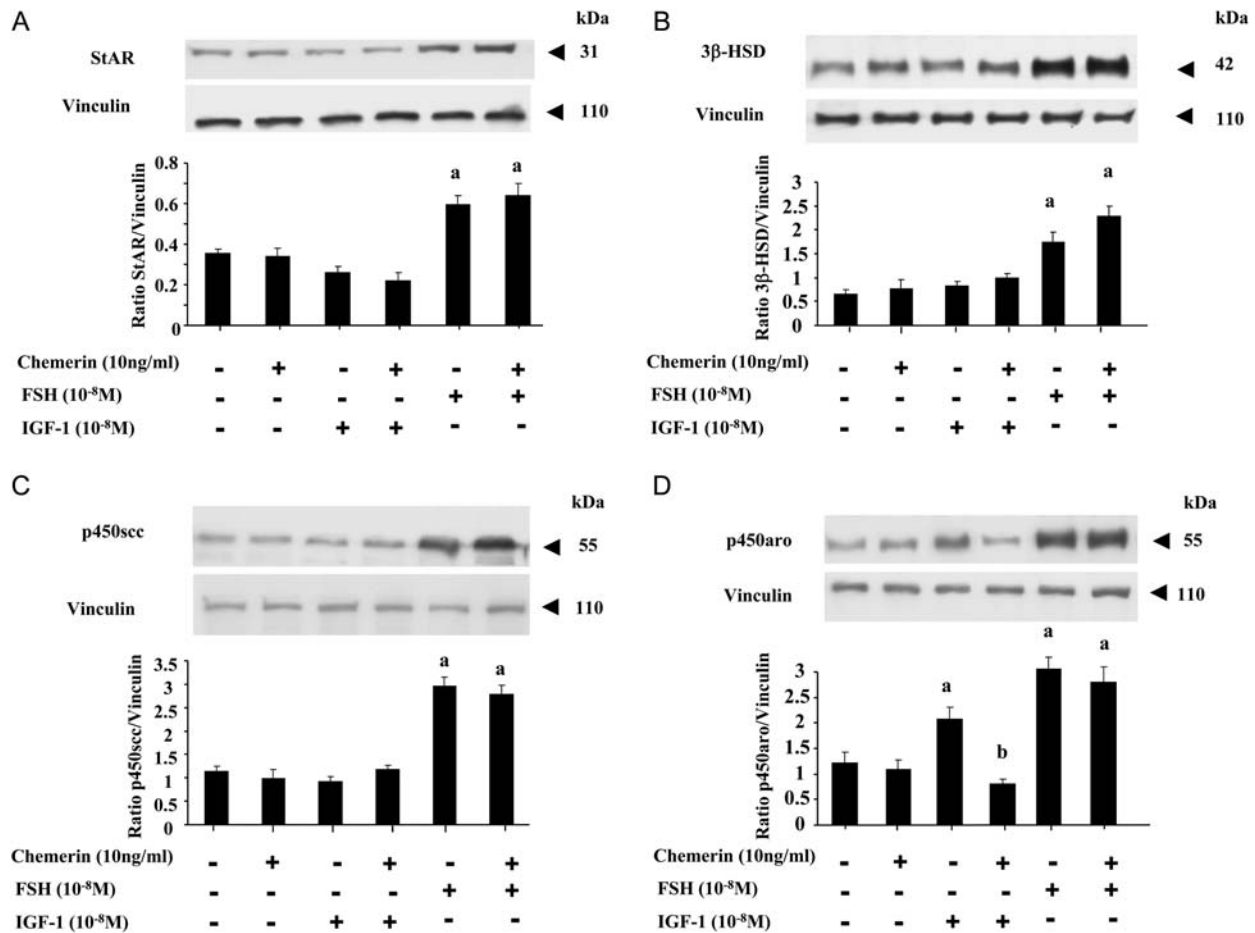
that chemerin and its receptor are produced in human ovary, and, in particular, in fresh granulosa cells (Fig. 1B). Immunohistochemistry with human ovarian sections confirmed the presence of chemerin and CMKLR1 in human follicles (Fig. 1C). CMKLR1 and its ligand were detected in granulosa cells and less abundantly in theca cells (Fig. 1C). At the cellular level, the staining for chemerin was mostly cytoplasmic (Fig. 1C2). For CMKLR1, the staining was cytoplasmic and also at the membrane level (see arrow Fig. 1C3). Thus, chemerin and its receptor (CMKLR1) are expressed in human ovary and more particularly in granulosa cells.

### Chemerin plasma and follicular fluid levels in women

We measured the concentration of chemerin in plasma and follicular fluid of 10 infertile women. As shown in Fig. 1D, chemerin level was at least 2-fold higher in follicular fluid than in plasma in 8 of 10 subjects by pooling cells (Fig. 1D, P < 0.05).

### Effect of human recombinant chemerin on basal and FSH- or IGF-I-stimulated progesterone and E<sub>2</sub> production in KGN and human primary granulosa cells

We next investigated the effects of chemerin treatment on steroidogenesis in KGN and primary hGCs. Cells were incubated in serum-free medium with human recombinant chemerin (10 or 100 ng/ml) for 48 h in the presence or absence of FSH (10<sup>-8</sup> M) and/or IGF-I (10<sup>-8</sup> M). As expected, FSH and IGF-I treatment alone increased progesterone and E<sub>2</sub> secretion in KGN cells (Fig. 2A and B, Pierre et al., 2009) and primary hGCs cells (Fig. 2C and D, Chabrolle et al., 2009). In KGN cells, this effect was enhanced when FSH and IGF-I were combined (Fig. 2A and B, P < 0.001). In these latter cells, the IGF-I-induced secretion of progesterone (Fig. 2A, P < 0.001) and E<sub>2</sub> (Fig. 2B, P < 0.001) in the absence or in the presence of FSH was decreased by 3-fold with 10 and 100 ng/ml chemerin treatments, whereas in the absence of IGF-I chemerin (10



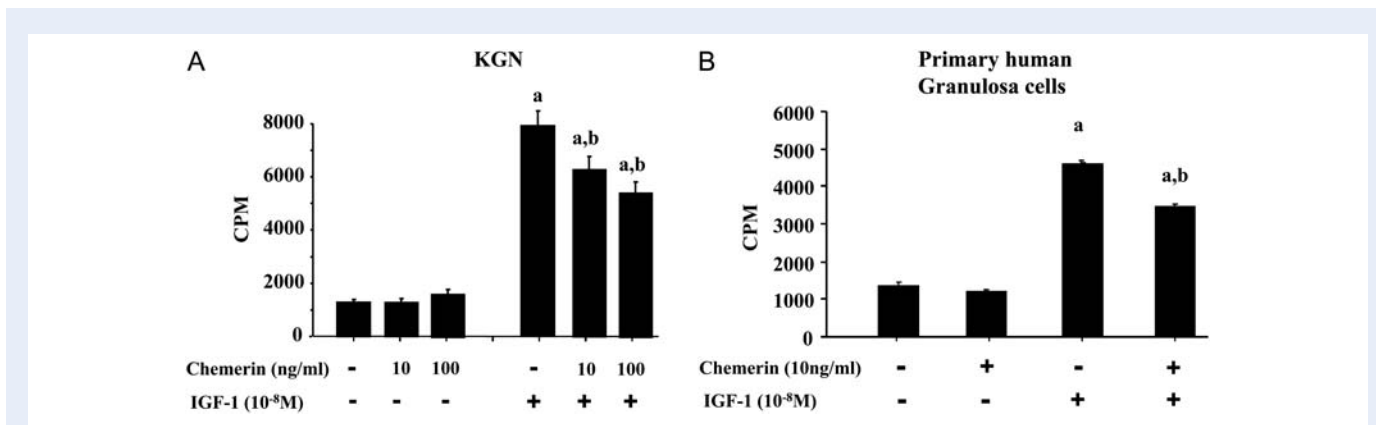
**Figure 3** The effect of human recombinant chemerin on the amount of StAR, 3-β-HSD, p450scc and p450 aromatase proteins in primary hGCs. Protein extracts from primary hGCs, cultured for 48 h in the absence or in the presence of chemerin (10 ng/ml) ± FSH (10<sup>-8</sup> M) or IGF-1 (10<sup>-8</sup> M), were submitted to SDS-PAGE as described in 'Materials and Methods' section. The membranes were probed with antibodies against StAR, 3-β-HSD, p450scc or p450 aromatase. Equal protein loading was verified by reprobing membranes with an anti-vinculin antibody. Blots were quantified and the StAR, 3-β-HSD, p450scc and p450 aromatase/vinculin ratios are shown. Results are representative of at least four cultures of granulosa cells. Each culture was performed by pooling cells obtained from different follicles from one patient. In each culture, each treatment (chemerin in the presence or in the absence of IGF-1 or FSH) was applied in duplicate. The results are expressed as mean ± SEM. <sup>a</sup>*P* < 0.05 compared with the untreated control, <sup>b</sup>*P* < 0.05 compared with the IGF-treated.

and 100 ng/ml) had no effect on steroid production (Fig. 2A and B). In primary hGCs, similar results were observed with chemerin 10 ng/ml in response to IGF-1 (Fig. 2C and D). We confirmed these data with chemerin 100 ng/ml (data not shown). Thus, chemerin decreased IGF-1-stimulated progesterone and E<sub>2</sub> production in hGCs.

Then, we investigated the effects of human recombinant chemerin on the amount of the StAR protein, an important cholesterol carrier, and on the protein amount of three key enzymes of steroidogenesis, p450scc, 3-β-HSD and p450 aromatase in the primary hGCs cells. Chemerin treatment (10 ng/ml) for 48 h did not affect the protein level of StAR, p450scc and 3-β-HSD in basal state or in response to IGF-1 or FSH (Fig. 3A–C). By contrast, chemerin decreased the amount of the p450 aromatase in the presence of IGF-1 (*P* < 0.05, Fig. 3D), but had no significant effect in its absence.

### Effects of human recombinant chemerin on hGC proliferation and viability in response to IGF-1

We also studied the effect of human recombinant chemerin on the number of KGN and primary hGCs in response to IGF-1. We determined [<sup>3</sup>H]-thymidine incorporation and cell viability of primary hGCs treated with 10 or 100 ng/ml chemerin for 24 h in the presence or in the absence of IGF-1 (10<sup>-8</sup> M). As expected, IGF-1 treatment significantly increased [<sup>3</sup>H]-thymidine incorporation in KGN (Fig. 4A) (Pierre *et al.*, 2009) and primary granulosa cells (Fig. 4B) (Chabrolle *et al.*, 2009). In KGN cells, chemerin treatment (10 and 100 ng/ml) significantly reduced IGF-1-induced thymidine incorporation (*P* < 0.05, Fig. 4A). Similar results were observed with chemerin (10 ng/ml) in human primary granulosa cells (Fig. 4B). As revealed by the staining with trypan blue, chemerin (10 or 100 ng/ml) had no effect



**Figure 4** The effect of human recombinant chemerin on hGCs thymidine incorporation. Thymidine incorporation was determined in KGN (**A**) and primary hGCs (**B**) cultured for 24 h in the presence or absence of human recombinant chemerin (10 or 100 ng/ml)  $\pm$  IGF-I ( $10^{-8}$  M) as described in 'Materials and Methods' section. Results are representative of at least four cultures of granulosa cells. For primary hGCs, each culture was performed by pooling cells obtained from different follicles from one patient. In each culture, each treatment (chemerin in the presence or in the absence of IGF-I or FSH) was applied in quadruplicate. The results are expressed as mean  $\pm$  SEM. <sup>a</sup> $P < 0.05$  compared with the untreated control, <sup>b</sup> $P < 0.05$  compared with the IGF-treated.

on the cell viability in the absence or in the presence IGF-I in both KGN and primary hGCs cells (data not shown). Thus, human recombinant chemerin decreased IGF-I-induced thymidine incorporation without affecting the viability of hGCs.

### Effect of human recombinant chemerin on signalling pathways in primary hGCs

Since chemerin treatment (10 ng/ml) significantly decreased IGF-I-induced steroidogenesis and proliferation in primary hGCs. We next investigated whether some components of IGF-1R signalling could be affected by human recombinant chemerin in these cells. Cells were incubated in serum-free medium with human recombinant chemerin (10 ng/ml) for 48 h in presence or absence of IGF-I ( $10^{-8}$  M) or FSH ( $10^{-8}$  M) (the same conditions than those used to determine the effect of chemerin on progesterone and E<sub>2</sub> production). IGF-I treatment ( $10^{-8}$  M, 48h) significantly increased IGF-I receptor beta subunit (Fig. 5A), MAPK ERK1/2 (Fig. 5B) and Akt (Fig. 5C) phosphorylation levels when compared with the basal state (no IGF-I or FSH), whereas FSH treatment ( $10^{-8}$  M, 48 h) significantly increased only MAPK ERK1/2 phosphorylation (Fig. 5A–C). As shown in Fig. 5A–C chemerin treatment (10 ng/ml, 48 h) totally abolished the IGF-I effects on these signalling components, whereas it did not affect those stimulated by FSH. Thus, the negative effect of chemerin on IGF-I-induced steroidogenesis is probably due primarily to a reduction in the amount of IGF-I receptor and consequently an inhibition of the activation of the downstream elements of IGF-1R.

Several adipokines including adiponectin, resistin and leptin have been shown to activate or inhibit AMPK, MAPK and Akt in various cell types ((Hegyi et al., 2004; Chabrolle et al., 2009; Pierre et al., 2009; Maillard et al., 2011)). Thus, we examined which signalling pathways could be activated by chemerin (10 ng/ml) in primary hGCs. As shown in Fig. 6A–C, human recombinant chemerin (10 ng/ml) rapidly (after 1 min of stimulation), but transiently significantly increased, phosphorylation of MAPK ERK1/2 (Fig. 6A), MAPK P38 (Fig. 6B) and Akt (Fig. 6C), whereas it significantly increased more slowly

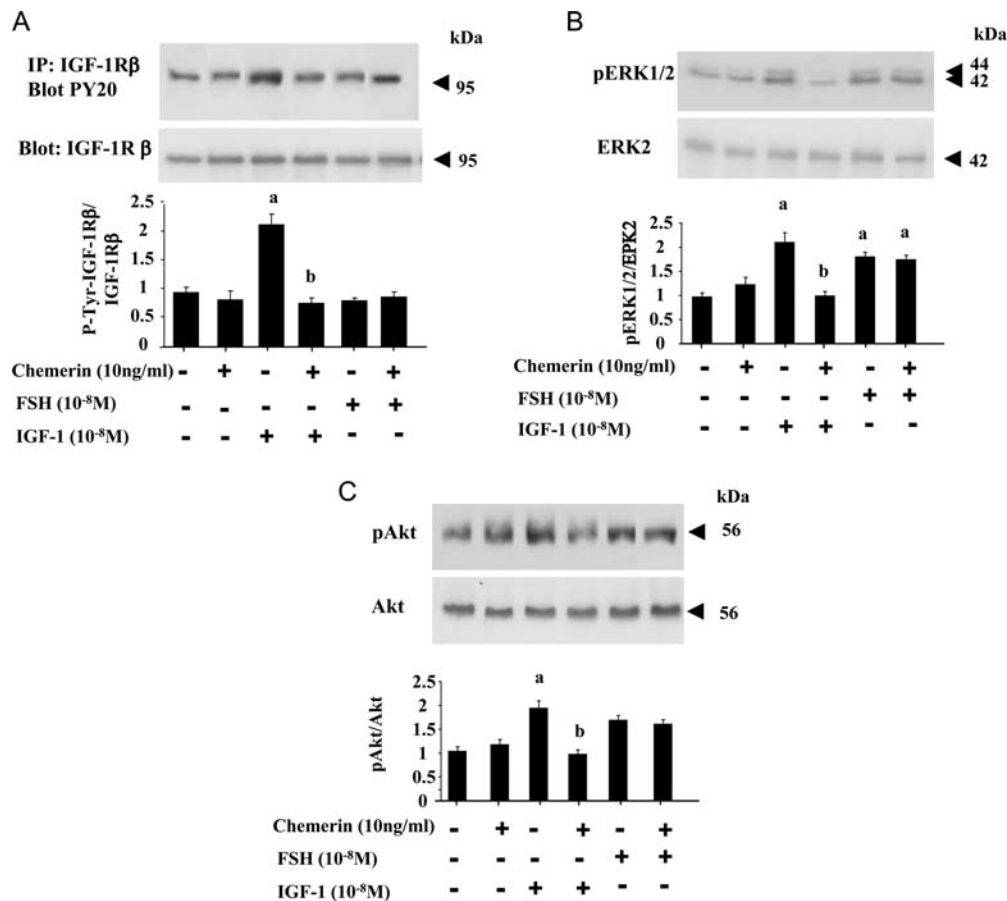
phosphorylation of AMPK (after 30 min of stimulation, Fig. 6D). Thus, human recombinant chemerin activates MAPK (ERK1/2 and P38), Akt and AMPK signalling pathways in hGCs.

### Discussion

In the present study, we report for the first time the presence of chemerin and CMKLR1 in human ovarian follicles and in the human ovarian granulosa-like tumour cell line, KGN. In primary hGCs and KGN cells, rhChem decreased IGF-I-induced thymidine incorporation and progesterone production without any variation of StAR, p450scc and 3- $\beta$ -HSD-protein levels. Chemerin also decreased IGF-I-induced E<sub>2</sub> secretion and p450 aromatase protein level. All these data were associated with an inhibition of the IGF-I-induced tyrosine phosphorylation of IGF-1R beta subunit and phosphorylation of MAPK ERK1/2 and Akt.

We found chemerin in human ovarian follicles. This result is in good agreement with data from Nagpal et al. (1997) that detected TIG2 (another name of chemerin) by northern blot in human ovary. We have found chemerin in human follicular fluid. Moreover, in 8 out of 10 patients, we observed a higher concentration in human follicular fluid than in plasma on the day before the oocyte pick-up. As a limitation, it is recognized that the ELISA assay used in our study to measure plasma and follicular fluid levels of chemerin does not distinguish between active chemerin and inactive prochemerin. Thus, total measured chemerin concentrations might not equate to the actual amount of active chemerin. Follicular fluid is in part an exudate of serum and in part the product of ovary activity. Chemerin has been found in haemofiltrate and it is particularly abundant in ascites fluid from ovarian cancer patients (1.8–7 nM) (Wittamer et al., 2003). We have showed that the messenger RNA and the protein of chemerin are expressed in human granulosa and theca cells. Thus, we can hypothesize that ovarian cells are able to produce chemerin. Other adipokines including leptin, adiponectin and resistin have already been detected in follicular fluid. Indeed, Gurbuz et al. (2005)



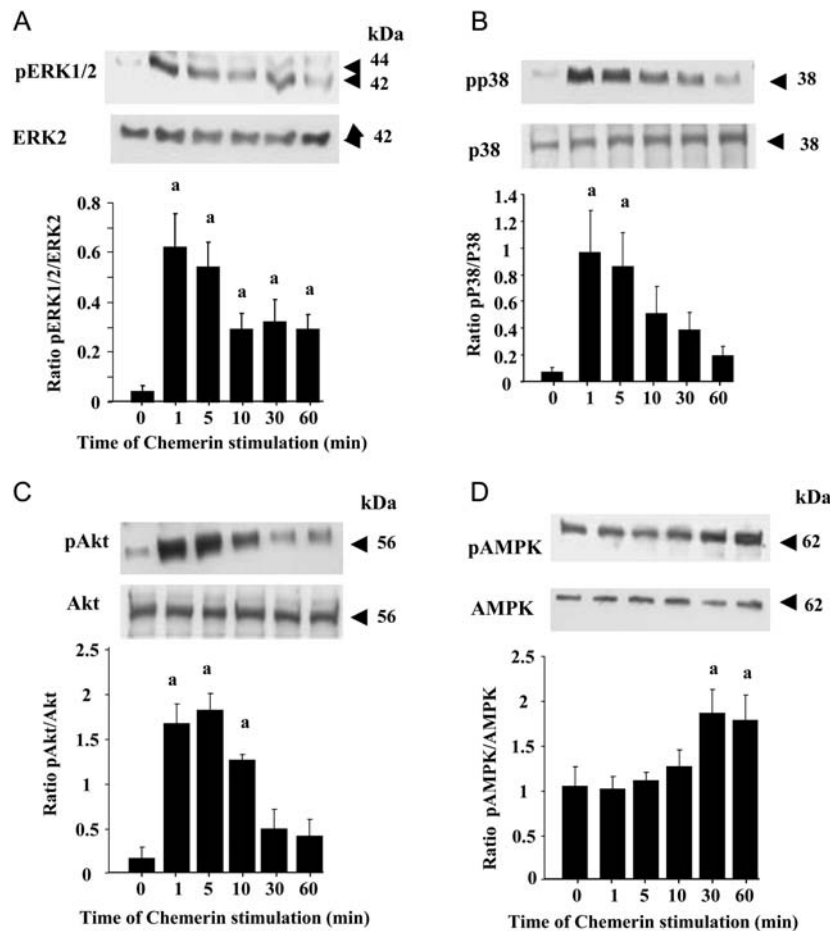


**Figure 5** The effect of human recombinant chemerin on phosphorylation of IGF-IR  $\beta$  subunit, MAPK ERK1/2 and Akt in response to IGF-I or FSH in primary hGCs. Primary hGCs were cultured in a medium with serum and then in a serum-free medium in the absence or in the presence of chemerin (10 ng/ml)  $\pm$  FSH ( $10^{-8}$  M) or IGF-1 ( $10^{-8}$  M) for 48 h (conditions used to measure progesterone and  $E_2$  production). Cells were lysed, and tyrosine phosphorylation of IGF-IR $\beta$  was determined. IGF-IR $\beta$  (A) was immunoprecipitated from whole cell lysates. Samples were then subjected to western blotting with antibodies recognizing phosphotyrosine (PY20). IGF-IR $\beta$  levels were evaluated by reprobing the membranes with IGF-IR $\beta$  antibodies. To determine MAPK ERK1/2 and Akt phosphorylation, cell lysates were directly subjected to immunoblotting with anti-phospho-MAPK ERK1/2 (B) or phospho-Akt (C) antibodies and then with anti-ERK2 or Akt protein antibodies. Representative blots from four different cultures are shown. Each culture was performed by using cells obtained from different follicles from one patient. In each culture, each treatment (chemerin in the presence or in the absence of IGF-I or FSH) was applied in duplicate. Blots were quantified and the phosphorylated protein/total protein ratio is shown. The results are represented as means  $\pm$  SEM. (i)  $P < 0.05$  compared with the untreated control, (ii)  $P < 0.05$  compared with the IGF-treated.

have observed similar levels of leptin in serum and in follicular fluid, whereas Chen *et al.* (2004) found higher follicular than serum leptin levels on the day of ovum pick up in women receiving IVF. Seow *et al.* (2005) have shown that the follicular fluid resistin levels were significantly lower than serum resistin levels in both the control and PCOS groups. In contrast, we have previously observed in six patients a higher concentration of adiponectin in human follicular fluid than in plasma on the day before the oocyte pick-up (Chabrolle *et al.*, 2009). The growth of the ovarian follicle and the oocyte maturation involve various autocrine and paracrine factors (Richards *et al.*, 2002). The fact that we observed a higher concentration of chemerin in human follicular fluid than in plasma on the day before the oocyte pick-up in 8 out of 10 patients may indicate that this hormone has a paracrine effect in the process of oocyte development in women receiving IVF. Indeed, it has been shown for other cytokines including leptin, tumour

necrosis factor- $\alpha$  and interleukin-6 that their follicular fluid levels affect the success of fertilization and early embryonic development (Brannstrom and Norman, 1993; Adashi, 1994), including in women with PCOS (Mantzoros *et al.*, 2000).

By immunohistochemistry, we found that chemerin and CMKLR1 are expressed in both granulosa and theca cells and also in KGN cells. We have previously shown that adiponectin was almost undetectable in fresh hGCs and KGN cells, whereas AdipoR1 and AdipoR2 were present (Chabrolle *et al.*, 2009; Pierre *et al.*, 2009). Karamouti *et al.* (2003) did not find leptin mRNA transcripts in primary hGCs, whereas Abir *et al.* (2005) detected them as well as its receptors. Thus, we can speculate endocrine but also autocrine and/or paracrine effects for adipokines, in hGCs. In the present study, we showed that rhChem (10 ng/ml) treatment rapidly increased MAPK ERK1/2, p38, Akt and later AMPK phosphorylation,



**Figure 6** Effect of human recombinant chemerin on phosphorylation of MAPKs (ERK1/2 and P38), Akt and AMPK in primary hGCs. Cell lysates were prepared from primary hGCs incubated with chemerin 10 ng/ml for various times: 0, 1, 5, 10, 30 or 60 min. Lysates (50  $\mu$ g) were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-phospho-MAPK ERK1/2 (**A**), anti anti-phospho-P38 (**B**), anti-phospho-Akt (**C**) or anti-phospho-AMPK (**D**) and then with anti-ERK2, P38, Akt or AMPK protein antibodies. Representative blots from three different cultures are shown. Each culture was performed by using cells obtained from different follicles from one patient. In each culture, each treatment (chemerin in the presence or in the absence of IGF-I or FSH) was applied in duplicate. Blots were quantified, and the phosphorylated protein/total protein ratio is shown. The results are represented as means  $\pm$  SEM.  $^aP < 0.05$  compared with the untreated control.

suggesting that CMKLRI is functional in primary hGCs. Similar signalling pathways have already been activated in response to adiponectin, resistin or leptin in granulosa cells of various species (Chabrolle et al., 2009; Maillard et al., 2011).

As previously reported, we have shown that IGF-I and FSH increased progesterone and  $E_2$  production by hGCs and this was improved when both IGF-I and FSH were combined (Bergh et al., 1991; Chabrolle et al., 2009). Furthermore, we observed here that rhChem inhibited IGF-I-induced progesterone and  $E_2$  secretion in the absence or in the presence of FSH. In the absence of FSH, this was associated with a decrease in the IGF-I-induced tyrosine phosphorylation of IGF-IR beta subunit, and phosphorylation of MAPK ERK1/2 and Akt. From our results, we do not know whether the effects of chemerin on IGF-IR-mediated responses are due to chemerin activation of CMKLRI or chemerin antagonism of the IGF-IR. In humans, it has been reported that higher chemerin release is associated with insulin resistance at the level of lipogenesis and

insulin-induced antilipolysis in adipocytes (Sell et al., 2009). Furthermore in this latter study, chemerin-induced insulin resistance in human skeletal muscle cells at the level of insulin receptor substrate 1, Akt and glycogen synthase kinase 3 phosphorylation and glucose uptake (Sell et al., 2009). Thus, chemerin could antagonize the action of IGF-I, which plays a key role in the development of ovarian follicles (Adashi, 1998). Several reports indicate that the MAPK ERK1/2 signalling pathway positively regulates progesterone and  $E_2$  production in cultured rat or hGCs (Seto-Young et al., 2003; Tosca et al., 2005). Thus, chemerin could decrease IGF-I-induced steroidogenesis by inhibiting MAPK ERK1/2 phosphorylation. In the present study, we observed that chemerin reduced IGF-I-induced thymidine incorporation in KGN and human primary granulosa cells. This could be due to the inhibition of the Akt signalling pathway in response to IGF-I. Indeed, this signalling pathway is involved in the proliferation of hGCs (Goto et al., 2009). We have shown that chemerin reduces IGF-I-induced progesterone secretion without affecting

the protein levels of the cholesterol carrier, StAR and of p450scc and 3- $\beta$ -HSD, two steroidogenesis enzymes. However, this could be due to an inhibition of the p450scc and 3- $\beta$ -HSD enzyme activities that we did not measure. In contrast, we observed that chemerin decreased IGF-I-induced p450 aromatase protein level, which could explain the inhibitory effect of chemerin on IGF-I-induced E<sub>2</sub> secretion.

It was reported that circulating and AT chemerin levels increased in PCOS patients versus control (Tan *et al.*, 2009). The PCOS syndrome is the most common cause of anovulation and infertility, affecting 5–10% of women of reproductive age. It is characterized by hyperandrogenism, chronic anovulation, and, occasionally, obesity. In our present study, we showed that chemerin and CMKLR1 are expressed in hGCs and less abundantly in theca cells. It will be interesting to determine the expression and the functionality of chemerin and CMKLR1 in granulosa cells from PCOS patients in order to better understand whether and how chemerin contributes to PCOS. Serum levels of chemerin are also increased in another female population at risk for metabolic and vascular disease, i.e. women with pre-eclampsia during and 6 months after pregnancy (Stepan *et al.*, 2011).

In conclusion, we demonstrated for the first time the presence *in vivo* of chemerin and CMKLR1 in the human ovarian follicle and more particularly in granulosa and theca cells and follicular fluid. Chemerin levels are significantly higher in follicular fluid than in plasma. *In vitro*, we have shown that rhChem inhibited IGF-I-induced progesterone and E<sub>2</sub> secretion and cell proliferation in hGCs and KGN cells. This was associated with a reduction in the levels of p450 aromatase and a decrease in the tyrosine phosphorylation of IGF-IR $\beta$  subunit and phosphorylation of Akt and MAPK ERK1/2 in hGCs. These findings significantly increase our understanding of the role of chemerin in hGCs. However, further investigations are necessary to understand the effects of chemerin on other human ovarian cells including theca cells and oocyte and also its potential implication in the PCOS.

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## Authors' roles

M.R. participated together with J.D. in the design of the study. The experiments were carried out by M.R., M.C., C.R., F.G., D.R. and J.D. Data analysis were performed by M.R., M.C., F.G., L.T. and J.D. The manuscript was written by M.R. All authors have read and approved the final manuscript.

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## Conflict of interest

None declared.

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