Reproductive Biology 24 (2024) 100827

Contents lists available at ScienceDirect

Reproductive Biology

journal homepage: www.journals.elsevier.com/reproductive-biology

Expression and in vitro effect of phoenixin-14 on the porcine ovarian granulosa cells

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ARTICLE INFO

Keywords:
Phoenixin-14
Granulosa
Proliferation
Oestradiol
Pig

ABSTRACT

Phoenixin-14 (PNX-14) regulates energy metabolism via the G protein-coupled receptor 173 (GPR173); elevated plasma levels have been described in patients with polycystic ovary syndrome. The aims were to investigate the ovarian expression of PNX-14/GPR173 and the in vitro effect of PNX-14 on granulosa cells (Gc) function. Transcript and protein levels of PNX-14/GRP173 were analysed by real-time PCR, western blot and immunohistochemistry in the porcine ovarian follicles at days 2–3, 10–12 and 16–18 of the oestrous. For in vitro experiments, Gc were isolated from follicles at days 10–12 of the oestrous (4–6 mm) and PNX-14 at doses 1–1000 nM was added for 24–72 h to determine Gc proliferation. Cell cycle progression, E2 secretion, expression of proliferating cells nuclear antigen, cyclins, mitogen-activated kinase (MAP3/1; ERK1/2), protein kinase B (AKT) and signal transducer and activator of transcription 3 (STAT3) were studied. The involvement of these kinases in PNX-14 action on Gc proliferation was analysed using pharmacological inhibitors. Levels of GPR173 were increased in the ovarian follicles with oestrous progression, while only PNX-14 protein was the highest at days 10–12 of the oestrous. Immuno-signal of PNX-14 was detected in Gc and theca cells and oocyte, while GPR173 was mostly in theca. Interestingly, PNX-14 stimulated Gc proliferation, E2 secretion, cell cycle progression and cyclins expression and had a modulatory effect on MAP3/1, AKT and STAT3 activation. Our study suggests that PNX-14 could be an important factor for porcine reproduction by influencing ovarian follicle growth through direct action on Gc function.

1. Introduction

Phoenixin (PNX) is a new peptide discovered in 2013 using bioinformatics tools [1]. It is produced mainly by the hypothalamus and is a cleavage product of small integral membrane protein 20 (SMIM20). In humans, the *SMIM20* gene is located on chromosome 4 at position p15.2 [2]. Proteolytic cleavage of SMIM20 leads to the production of many PNX isoforms of variable length, namely PNX-42, PNX-36, PNX-26, PNX-17, PNX-20 and PNX-14. The latter two are the predominant, most active isoforms and occur in larger amounts in many tissues [3]. Interestingly, the PNX-14 sequence is identical in humans, rats, mice and pigs [3]. Several studies have documented the presence of PNX in different tissues including the hypothalamus [3], heart [4], thymus, stomach [3], pancreas [5], lung, kidney [3], adipose tissue [6] and ovary [7] of rats, spotted scat, zebrafish and chickens. Tissue-dependent expression of PNX isoforms was also demonstrated, while PNX-20 was most common in the brain [3] more ubiquitous isoform expressed in the spinal cord, heart [4] and other peripheral tissues [5–7] is PNX-14. For example, Billert et al. [6] reported that PNX-14 was secreted from mouse adipocytes 3T3L1 cells, rat primary adipocytes and pancreatic islets in dependence of glucose concentration [5].

Interestingly, PNX plays a pleiotropic function in an organism by binding to the G protein–coupled receptor 173 (GPR173); GPR173 silencing inhibits PNX action in several tissues including the heart [8].

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https://doi.org/10.1016/j.repbio.2023.100827
Received 11 July 2023; Received in revised form 16 October 2023; Accepted 14 November 2023
Available online 27 November 2023

Moreover, PNX-20 reduces lipopolysaccharide-induced cytotoxicity by activating GPR173 in human dental pulp [9]. In addition, the function of PNX is strictly linked to the regulation of metabolism, the development of obesity, insulin (INS) resistance and the pathogenesis of inflammatory reactions [10]. Intracerebroventricular injection of PNX-14 induces food intake in rats [8]. Besides, PNX-14 regulates the expression of proinflammatory cytokines, such as tumour necrosis factor α, interleukin 1β and interleukin 6, and reduces the generation of reactive oxygen species in murine BV2 microglia [11]. Furthermore, the link between PNX and cell proliferation and endocrinology has been described [5,6]. For example, PNX-14 enhances glucose-stimulated INS secretion and proliferation in rats' pancreatic INS-1E cell line and pancreatic islets [5] and increases 3T3L1 preadipocyte proliferation and viability [6].

Interestingly, there is a strict connection between PNX/GPR173 in the reproduction at the central level and in the gonads. Indeed, PNX-14 regulates pituitary gonadotropin secretion by modulating the expression of the gonadotropin-releasing hormone receptor in rats [3]. In *Scatophagus argus*, pituitary PNX-14 stimulates the gene expression of luteinising hormone (LH) and follicle-stimulating hormone (FSH) [12]. Interestingly, immunohistochemical analysis revealed increased expression of PNX-20 and GPR173 as human ovarian follicles grow [13]. Moreover, PNX-20 enhances granulosa cell (Gc) proliferation and oestradiol (E2) secretion as well as folliculogenesis through its receptor GPR173 in human HGrC1 cells, a non-luteinised Gc line [13]. Additionally, the genes involved in the steroid production pathway including cytochrome P450 family 11 subfamily A member 1, family 17 member A1 and aromatase (CYP19A1) are upregulated in fish gonads administered PNX-20 [14]. However, knowledge regarding the effects of PNX-14 on female reproduction is still limited. Interestingly, PNX-14 levels are strictly connected to human and domestic animals' reproductive pathologies. Rybska *et al.* [15] found that the development of canine uterine disorders, including endometrial hyperplasia and pyometra, downregulates PNX-14 and GPR173 expression. Furthermore, women with polycystic ovary syndrome (PCOS) have higher serum PNX-14 levels [1] and PCOS rats have elevated PNX-14 expression in the ovary and adipose tissue [7].

Based on these previous findings, we hypothesise that PNX-14 and GPR173 are expressed in porcine ovarian follicles and directly regulate Gc function. We aimed *(i)* to describe the pattern of SMIM20/PNX-14 and GPR173 messenger RNA (mRNA) and protein expression in the porcine ovarian follicle during the oestrous cycle, as well as their immunolocalisation; *(ii)* to investigate in vitro direct effect of PNX-14 on Gc proliferation, E2 secretion, cell cycle progression and the levels of proliferating cell nuclear antigen (PCNA) and cyclins (A, B, D, E); *(iii)* to evaluate the effect of PNX-14 on the phosphorylation of mitogen activated kinase (MAP3/1; ERK/1/2), protein kinase B (AKT) and signal transducer and activator of transcription 3 (STAT3); and *(iv)* to determine the involvement of these kinases in the effect of PNX-14 on Gc proliferation. Conducted research will allow for a better understanding of porcine ovarian physiology and, thanks to studying the molecular mechanism of PNX-14 action, this peptide may in the future constitute a molecular target for pharmacological interventions treating infertility caused by disorders linked with improper ovarian cell proliferation.

2. Materials and methods

2.1. Reagents

Medium 199 (M199; product no. M2154), PNX-14 (product no. SRP4651), FSH (product no. F4021), LH (product no. L5259), INS (product no. I3505), insulin-like growth factor type 1 (IGF1) (product no. I3769), AG490 (product no. T3434), Laemmli buffer (product no. 38733), antibiotic-antimycotic solution (product no. A5955), sodium dodecyl sulphate (SDS), Tris and Tween 20, Na-deoxycholate, Nonidet NP-40, protease inhibitors (ethylenediaminetetraacetic acid-free), dithiothreitol (DTT), bromophenol blue and 1 bromo-3-chloropropane were bought from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) (product no. 16140071), phosphate-buffered saline (PBS) (product no. 14040174), electrophoresis markers and the TaqMan Gene Expression Cells-to-CT Kit (product no. AM1728) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). PD98059 (product no. 1213) was purchased from Tocris (Bristol, UK). WesternBright Quantum HRP substrate (product no. K-12043 D200) was purchased from Advansta Inc. (Menlo Park, CA, USA). LY294002 (product no. 9901) was obtained from Cell Signalling Technology (Danvers, MA, USA).

2.2. Material collection

Ovaries were collected from sexually mature crossbreed pigs (Large White × Polish Landrace) aged 6–8 months old and weighing 140–150 kg at a local abattoir under veterinarian control. Sows were euthanised according to European Legislation (EFSA, AHAW/04–027). Consent of the bioethics committee is not required because the ovaries are by-products of the slaughter. Ovarian follicles were collected on days 4–6 (2–4 mm), 10–12 (4–6 mm) and 16–18 (8–12 mm) of the oestrous cycle based on morphological examination of the ovaries [16].

Experiment 1: After excision from the ovary, some ovarian follicles were immediately frozen in liquid nitrogen and stored at − 70 °C for future quantification of *SMIM20* and *GPR173* mRNA expression (n = 6/group). To analyse PNX-14 and GPR173 protein expression, some ovarian follicles (n = 6/group) were homogenised twice in ice-cold lysis buffer. The lysates were cleared by centrifugation at 15,000 *g* at 4 °C for 30 min, and the protein concentration was determined by the Bradford protein assay using bovine serum albumin as the standard.

Experiment 2: Additionally, some ovarian follicles at days 10–12 of the oestrous cycle (n = 3) were fixed in 4% paraformaldehyde, dehydrated in an increasing gradient of ethanol and then embedded in paraplast to analyse PNX-14 and GPR173 immunolocalisation.

2.3. In vitro culture of Gc

Cultures of Gc were prepared according to the technique established by Stoklosowa *et al.* [17] from follicles at days 10–12 of oestrous cycle. As Stoklosowa *et al.* [17] showed porcine Gc are able to secrete E2 with no additional androgens stimulation due to CYP19A1 activity [17]. Briefly, Gc were scrubbed from the follicular wall with tweezers round-tipped and rinsed with PBS. After that isolated Gc were exposed to DNAse I (500 U for 1 min), washed in M199, collected and resuspended in M199 with 10% FBS [v/v]. The viability of the cells (90 %) was measured using the trypan blue exclusion test. Then, Gc were seeded in 96-well culture plates in M199 medium with 10 % FBS [v/v] at a concentration of 5×10^4 viable cells per well. After 24 h of preincubation, the medium was changed to M199 with 1 % FBS [v/v]. All cultures were maintained at 37 °C in a humidified atmosphere consisting of 5 % CO_2 and 95 % O_2.

Experiment 3: to determine the time- and dose-dependent effects of PNX-14 on cell proliferation, Gc were incubated for 24, 48 or 72 h in M199 contained 1 % FBS [v/v] as a control, or with PNX-14 at a dose of 1, 10, 100 or 1000 nM. The PNX-14 doses were chosen based on the literature [1,5] and their plasma level in rats [7] and humans [18]. Subsequently, the alamarBlue reagent (product no. DAL1100, Invitrogen, Carlsbad, CA, USA) was added for 2 h to evaluate Gc proliferation as described previously [19]. Next, to study the effect of PNX-14 on the expression of PCNA and CYP19A1 Gc were incubated for 48 h in M199 containing 1 % FBS [v/v] alone or with 100 nM PNX-14. The PNX-14 dose was based on the results from the proliferation experiment. Then Gc were washed in PBS and stored at − 70 °C to quantify mRNA levels or boiled in Laemmli buffer for 4 min to quantify protein expression. While, to investigate the effect of PNX-14 on E2 secretion, Gc were incubated for 48 h in M199 containing 1 % FBS [v/v] alone or with 1, 10, 100 or 1000 nM PNX-14. The culture medium was collected and

stored at − 20 °C to measure the E2 concentration by enzyme-linked immunosorbent assay (ELISA).

Experiment 4: to study interactions between PNX-14 and FSH, LH, INS or IGF1 in Gc proliferation, Gc were incubated for 24, 48 or 72 h in M199 contained 1 % FBS [v/v] as a control, or with PNX-14 at 100 nM alone, or with 100 nM PNX-14 combined with LH and FSH at 100 ng/mL or INS and IGF1 at 50 ng/mL, which induce Gc proliferation [20,21]. The LH, FSH, INS and IGF1 doses are based on our previous papers [22]. Next, cell proliferation was measured as described in experiment 3.

Experiment 5: for cell cycle analysis, Gc were incubated with 100 nM PNX-14 for 48 h and then fixed with 70 % cold ethanol at 4 °C for 60 min and stored at − 20 °C for flow cytometry analysis as described previously [23]. While, to study the effect of PNX-14 on the expression cyclins A, B, D and E, Gc were incubated for 48 h in M199 containing 1% FBS [v/v] alone or with 100 nM PNX-14 and proceeded as described in experiment 3.

Experiment 6: to measure the effect of PNX-14 on the phosphorylation of several kinases pMAP3/1, MAP3/1, pAKT, AKT, pSTAT3, STAT3 Gc were incubated with 100 nM PNX-14 for 1, 5, 15, 30, 45 and 60 min. Then collected Gc were boiled in a Laemmli buffer for 4 min to evaluate protein expression. To assess the involvement of MAP3/1, phosphoinositide 3-kinases (PI3K)/AKT and Janus kinase (JAK)/STAT3 in the effect of PNX-14 on Gc proliferation, Gc cultured in M199 supplemented with 1% FBS [v/v] were pre-treated for 1 h with a pharmacological inhibitor of MAP3/1 (PD98059, at a dose of 25 μM), PI3K/AKT (LY294002 at 5 μM) or JAK/STAT3 (AG490 at 5 μM). The inhibitor doses were chosen based on preliminary experiments and the literature [24,25]. The specificity of the inhibitors was confirmed based on the literature [26–28]. Subsequently, 100 nM PNX-14 was added for the next 48 h. The alamarBlue reagent was added for 2 h to evaluate Gc proliferation.

2.4. Real-time polymerase chain reaction (PCR)

To determine *SMIM20* and *GPR173* mRNA expression, RNA was extracted from the ovarian follicles using the TRIzol reagent according to the manufacturer's procedure (Merck, Germany). RNA was reverse-transcribed to complementary DNA (cDNA) as described previously [23]. Amplification was performed using the MYIQ Cycler real-time PCR system (Bio-Rad, Prague, Czech Republic) following a published protocol [29]. Briefly, real-time PCR was performed in a 20 μL final volume containing 10 μL iQ SYBR Green supermix (Bio-Rad, Hercules, CA, USA), 0.25 μL of each primer (10 μM), 4.5 μL of water and 5.0 μL of template at the following conditions: 1 cycle for 5 min at 95 °C to denature the sample and then 40 cycles, 1 min at 95 °C for denaturation, 1 min at 60 °C for hybridisation, 1 min at 72 °C for stretching and finally 1 cycle for 5 min at 72 °C for final elongation. The primers were synthesised by Sigma-Aldrich; their sequences are presented in Table 1 and were selected using BLAST (National Library of Medicine, USA). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), β-actin (*ACTB*) and cyclophilin A (*PPIA*) served as reference genes. All gene expression data were normalised to the geometric mean of the reference genes and are presented as arbitrary units [30].

To determine the mRNA levels of *PCNA*, *CYP19A1* and cyclins, the TaqMan Gene Expression Cells-to-CT Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for RNA isolation and cDNA synthesis following the manufacturer's protocols. Subsequently, RNA and cDNA quantity were determined by measuring the absorbance at 260 nm and 280 nm by spectrophotometry. Then TaqMan Gene Expression Master Mix and specific primers (Thermo Fisher Scientific, Waltham, MA, USA) for *PCNA*, *CYP19A1* and cyclins A, B, D and E (Table 1) were used and amplifications were performed using the StepOnePlus system (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's instructions and as we described previously [31]. As a reference gene, *PPIA* was used. Briefly, 100 ng cDNA was used for reaction with 1 μL TaqMan Gene Expression primers, and 10 μL TaqMan PCR master mix (Applied Biosystems, Waltham, MA, USA). The thermal cycling conditions to determine the cycle threshold number (Ct) for quantitative measurement were: 2 min at 50 °C; 10 min at 95 °C; and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The relative mRNA expression levels of the studied genes were determined using the $2^{-\Delta\Delta Ct}$ method.

2.5. Western blotting

Western blotting was performed as described previously [23]. Briefly, 30–50 μg of protein was separated by gel electrophoresis and then transferred to a membrane. The primary and secondary antibodiesto detect PNX-14 and receptor GPR173, proliferation marker PCNA, CYP19A1, cyclins (A, B, D and E) and signalling pathway (MAP3/1, AKT and STAT3) are described in Table 2. ACTB or α-tubulin (TUBA) was used as a loading control, depending on the molecular weight of the studied protein. The Western Bright Quantum HRP substrate and the Chemidoc XRS + System (Bio-Rad, Hercules, CA, USA) were used to visualise the protein bands. ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to quantify the optical density of the protein bands.

2.6. Immunohistochemistry

To determine PNX-14 and GPR173 localisation in the ovary, immunohistochemistry was used as described previously [23]. Section (5 μm thick) were mounted onto APES-coated slides, deparaffinised in xylene

Table 1
Primers used in real-time PCR.

Gene	*Sequence/ product no.*
SMIM20	Forward: 5′-ATCTACTTCCGGCCCCTAATG-3′ Reverse: 5′-GCCCGGTTTATGGCCTGT-3′
GPR173	Forward: 5′-TGGATCTTTGATGCGGCCTT-3′ Reverse: 5′-GGGAATCACGAAGCTCACCA-3′
GAPDH	Forward: 5′-GCACCGTCAAGGCTGAGAAC-3′ Reverse: 5′-ATGGTGGTGAAGACGCCAGT-3′
ACTB	Forward: 5′-TCCCTGGAGAAGAGCTACG-3′ Reverse: 5′-GTAGTTTCGTGGATGCCACA-3′
PPIA	Forward: 5′-GCATACAGGTCCTGGCATCT-3′ Reverse: 5′-TGTCCACATGCAGCAATGGT-3′
PCNA	product no. Ss03377029_g1
CYP19A1	product no. Ss03384876_u1
Cyclin A	product no. Ss06866662_m1
Cyclin B	product no. Ss03382740_u1
Cyclin D	product no. Ss06884487_m1
Cyclin E	product no. Ss06921885_g1
PPIA	product no. Ss03394782_g1

Table 2
Antibodies used in Western blot.

Antibody	*Host*	*Dilution*	*Catalog number and vendor*
PNX-14	Mouse	1:500	G-079–01, Phoenix Pharmaceuticals
GPR173	Rabbit	1:300	PA5–50967, Thermo Fisher Scientific
PCNA	Mouse	1:250	13–3900, Thermo Fisher Scientific
Cyclin A	Mouse	1:10 000	ab38, Abcam
Cyclin B	Rabbit	1:20 000	ab32053, Abcam
Cyclin D	Rabbit	1:1000	ab134175, Abcam
Cyclin E	Rabbit	1:1000	ab3927, Abcam
CYP19A1	Rabbit	1:200	PA1–21398, Thermo Fisher Scientific
p-STAT3	Rabbit	1:1000	9131 S, Cell Signalling Technology
STAT3	Rabbit	1:2000	4904 S, Cell Signalling Technology
p-AKT	Rabbit	1:1000	9271 S, Cell Signalling Technology
AKT	Rabbit	1:1000	9272 S, Cell Signalling Technology
p-MAP3/1	Rabbit	1:1000	9101 S, Cell Signalling Technology
MAP3/1	Rabbit	1:1000	9102 S, Cell Signalling Technology
ACTB	Mouse	1:1000	A5316, Sigma-Aldrich
TUBA	Mouse	1:1000	T6074, Proteintech
Anti-rabbit	Mouse	1:1000	#7074, Cell Signalling Technology
Anti-mouse	Rabbit	1:1000	#7076, Cell Signalling Technology

and then gradually rehydrated through a graded series of ethanol. The sections were immersed in 0.01 M citrate buffer and heated in a microwave oven for antigen retrieval. Endogenous peroxidase activity and nonspecific protein binding were blocked. The sections were incubated overnight at 4 °C with the anti-PNX-14 or anti-GPR173 antibody at 1:50 and then washed in Tris-buffered saline with 0.1 % Tween 20 (TBST). Next, they were incubated with biotinylated goat anti-rabbit IgG (1:400; Vector Laboratories, CA, USA) followed by avidin–biotin–peroxidase complex (1:1:100; Strept ABC complex/HRP, DAKO/AS). The sections were dehydrated, mounted in DPX (Fluka, Chemie GmbH, Buchs, Switzerland) and then photographed using the Nikon Eclipse E200 microscope attached to the Coolpix 5400 digital camera (Nikon, Tokyo, Japan) with the corresponding software.

2.7. AlamarBlue assay

To determine Gc proliferation, the alamarBlue reagent (Invitrogen, Carlsbad, CA, USA) was aseptically added to the well in an amount equal to 10 % [v/v] of the incubation volume. After incubation for 2 h, the fluorescence was measured at 530 and 590 nm using a Varisokan Lux plate reader (ThermoFisher Scientific, CA, USA).

2.8. ELISA

A commercially available E2 ELISA kit (product no. EIA-2693, DRG, Germany) was used to analyse E2 secretion in the culture medium as described previously [32]. The sensitivity of the assay is 9.714 pg/mL. The inter- and intra-experimental coefficients of variation are < 9.39 % and < 6.81 %, respectively. Each sample was run in duplicate.

2.9. Flow cytometry

Flow cytometry was used to evaluate cell cycle progression. First, Gc were washed twice in 1 mL PBS. Then, the cell pellet was resuspended in 300 µL of the propidium iodide/RNase staining buffer (BD Biosciences, San Jose, CA, USA) and incubated in the dark for 30 min. The red fluorescence of propidium iodide was measured by FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Ten thousand cells were examined per sample. The percentage of the cell population in each phase of the cell cycle – G0/G1, S and G2/M – was calculated from the DNA content histograms using the WinMDI 2.8 software.

2.10. Statistical analysis

GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA) was used for all statistical analysis. The data are presented as the mean ± standard error of the mean (SEM). For one set of experiments Gc were collected from 10 ovaries (one ovary from a pig). Each in vitro experiment was repeated a minimum of four times (n = 4). A total of 50 pigs were used for in vitro experiments. The normality of the data was evaluated with the Shapiro–Wilk test. To compare between groups, one- or two-way analysis of variance (ANOVA) followed by post hoc tests for multiple group comparisons or Student's t-test was used. The details regarding the tests used are provided in the figure legends. Statistical significance is indicated by different letters ($P < 0.05$) or asterisks (* $P <$ 0.05, ** $P < 0.01$, *** $P < 0.001$).

3. Results

3.1. Expression of SMIM20/PNX-14 and GPR173 in the porcine ovarian follicle during the oestrous cycle

In porcine ovarian follicles, *SMIM20* mRNA levels were stable during the oestrous cycle, while *GPR173* mRNA levels increased as the oestrous cycle progressed: by 2.2-fold at days 10–12 and by 4.6-fold at days 16–18 compared with days 2–3 (Fig. 1A, $P < 0.05$). It was confirmed on protein level for GPR173, while PNX-14 protein expression was highest at days 10–12 of oestrous cycle (Fig. 1B, $P < 0.05$). We observed PNX-14 localised in the oocyte, cumulus cells, Gc and theca cell cytoplasm and GPR173 mostly in the theca cell cytoplasm (Fig. 1C).

3.2. Effect of PNX-14 on Gc proliferation and PCNA level

As presented in Fig. 2A PNX-14 at all investigated doses increased porcine Gc proliferation after 24, 48 and 72 h of in vitro culture (Fig. 2A, $P < 0.05$). We confirmed the mitogenic properties of PNX-14 by showing its stimulatory effect on the expression of PCNA, which is a main marker of cell proliferation; its accumulation has been detected in the proliferator phase S of the cell cycle [33]. Briefly, we showed that PNX-14 at dose 100 nM elevated *PCNA* transcript level by 2.33 fold compared to control (Fig. 2B, $P < 0.05$) and stimulated PCNA protein expression (Fig. 2C, $P < 0.001$).

3.3. Effect of PNX-14 on E2 secretion and CYP19A1 expression

In the next part, we focussed on PNX-14 role in E2 secretion and expression of the enzyme, which participated in its synthesis- CYP19A1. It is well known that E2 is a strong mitogenic factor in Gc: it regulates important events that occur during the normal menstrual cycle in women and domestic animals, especially the sequence of ovarian follicle growth and maturation [34]. Thus, abnormalities in E2 secretion lead to abnormal folliculogenesis and failed dominant follicle selection [35], which are often linked to proliferation inhibition [36]. We showed, that, PNX-14 at dose 10 and 1000 nM significantly stimulated E2 secretion by Gc at 39.53 ± 5.96 pg/mL and 33.70 ± 1.50 pg/mL, respectively vs 19.06 ± 1.13 pg/mL in control (Fig. 3A, $P < 0.05$, $P < 0.01$). Also, we observed that PNX-14 (100 nM) upregulated CYP19A1 mRNA level by 3.24 fold compared to control (Fig. 3B, $P < 0.05$) and stimulated CYP19A1 protein expression (Fig. 3C, $P < 0.001$).

3.4. Effect of PNX-14 on LH-, FSH-, IGF1-, and INS-induced Gc proliferation

Literature data indicated that LH, FSH, IGF1 and INS stimulate, Gc proliferation; for example, FSH regulates the number of ovarian follicles that mature as well as Gc division [20], while mice with *Igf1* gene deletion are infertile; they have ovarian follicles arrested at an early stage of development because Gc proliferation is inhibited [21]. We observed that PNX-14 stimulated FSH-induced Gc proliferation after 48 h of incubation, while there was an inhibitory effect after 72 h of incubation (Fig. 4A, $P < 0.05$). After 48 and 72 h of in vitro culture, PNX-14 decreased IGF1-induced Gc proliferation (Fig. 4C, $P < 0.05$) but had no effect on LH- or INS-induced Gc proliferation (Fig. 4B and D).

3.5. Effect of PNX-14 on cell cycle progression and the expression of cyclins A, B, D and E in Gc

Cell proliferation is closely linked with cell cycle progression; it has been also very well established that cyclins are regulators of key events during the progression of the cell cycle [37] and are markers of porcine ovarian cell proliferation, growth and development [38,39]. Briefly, cyclin A begins to appear towards the end of G1 and its protein levels continue to rise throughout the S phase [40], while cyclin B begins to appear during the S phase. This elevation continues into the G2 phase and then rapidly falls during the G2/M phase [40]. Cyclin E peaks in the S phase, while cyclin D remains elevated in the S and G2/M phases [40]. We noted that PNX-14 at dose 100 nM increased the number of cells in the S, proliferator phase of the cycle but had no effect on the number of cells in the G0/G1 or G2/M phase of the cell cycle compared with the control (Fig. 5A, $P < 0.05$). In addition, PNX-14 (100 nM) increased the cyclin B mRNA level by 3.14 fold, decreased the cyclin D mRNA level by 2.53 fold and had no effect on cyclin A and E mRNA levels (Fig. 5B,

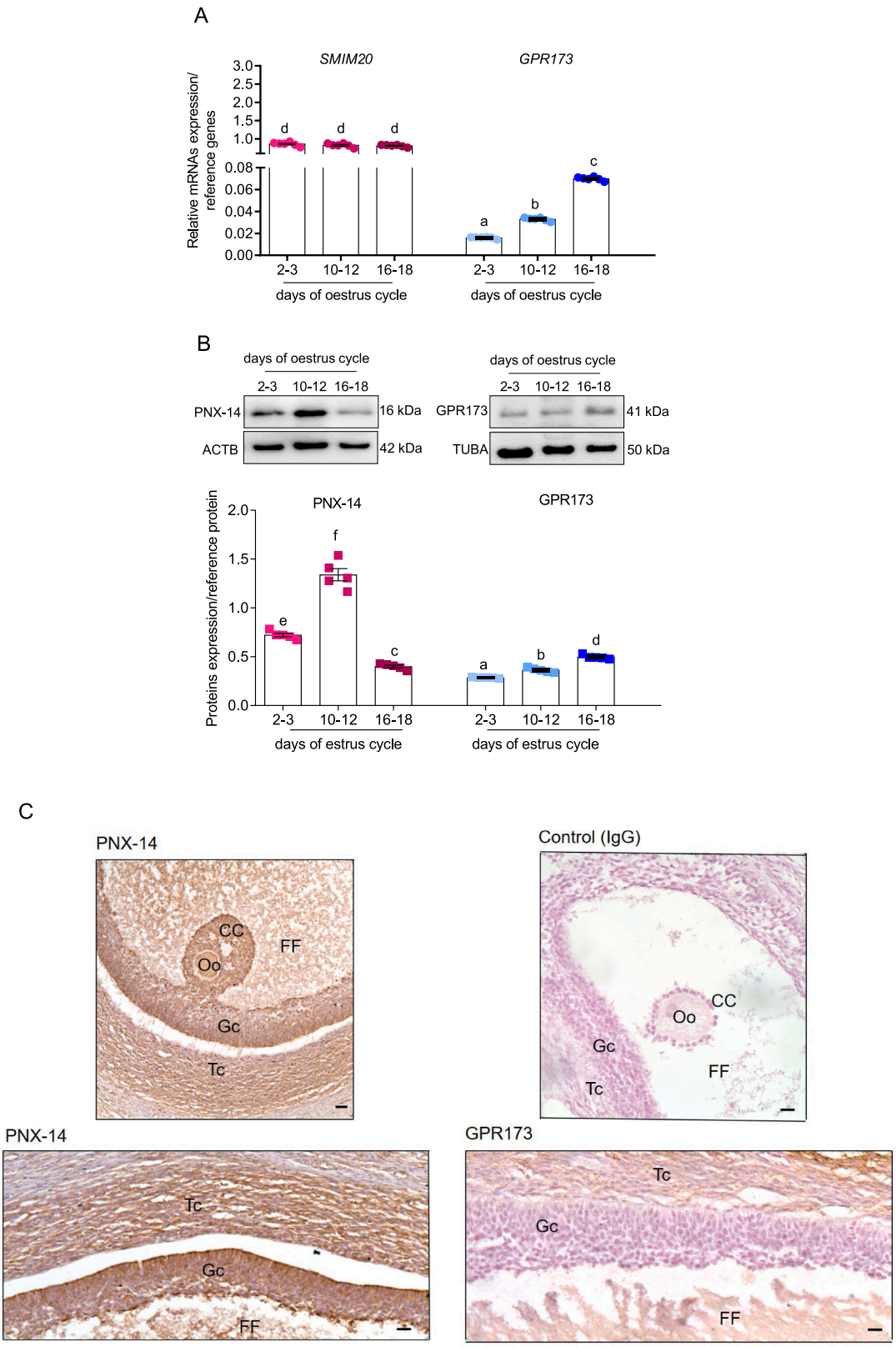

Fig. 1. Comparison of small integral membrane protein 20 (SMIM20)/phoenixin-14 (PNX-14) and probable G-protein coupled receptor 173 (GPR173) mRNA (A) and protein (B) level in porcine ovarian follicle during oestrous cycle (n = 6), as well as it's immunolocalization (C) (n = 3). Representative blots and immunohistochemistry photos are shown (scale bar 50 μM). Genes expression level was normalised to cyclophilin A (PPIA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin (ACTB) geometric mean, while proteins level was normalised to ACTB (PNX-14) or α-tubulin (TUBA, GPR173). Data are plotted as the mean ± SEM. One-way ANOVA, followed by post hoc tests was used for statistical analysis (GraphPad Prism 8). Significance between groups is indicated by different letters ($P < 0.05$). Oo: oocyte, FF: follicular fluid, CC: cumulus cells, Gc: granulosa cells, Tc: theca cells.

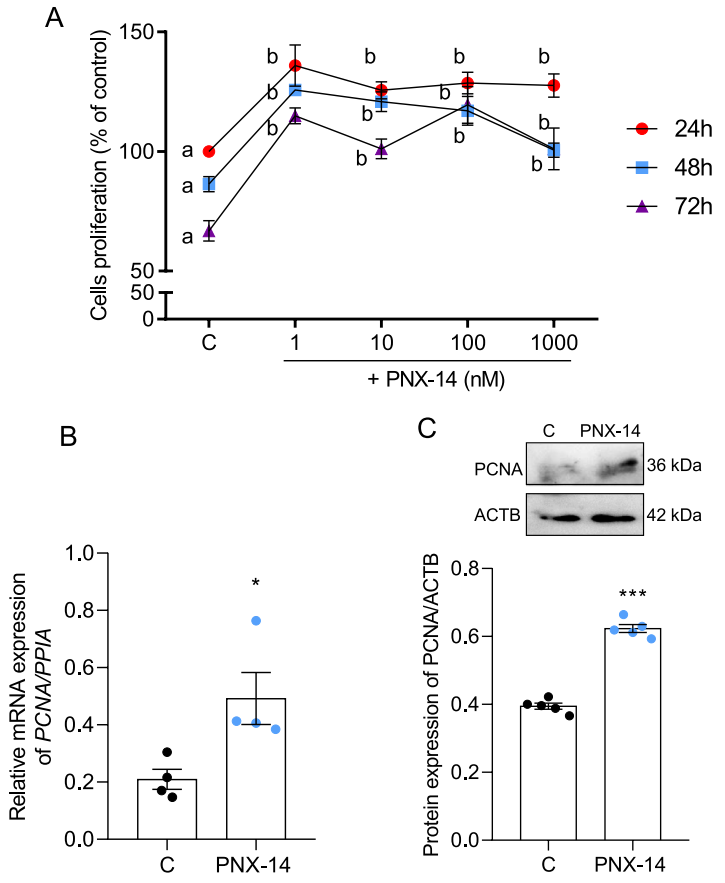

Fig. 2. Effect of phoenixin-14 (PNX-14) on granulosa cells proliferation (A) and proliferating cells nuclear antigen (PCNA) mRNA (B) and protein (C). Representative blots are shown. Gene expression level was normalised to cyclophilin A (PPIA), while protein to β-actin (ACTB). Data are plotted as the mean ± SEM. Two- way ANOVA, followed by post hoc tests was used for statistical analysis of cells proliferation, while T-student tests for PCNA levels (GraphPad Prism 8). Significance between groups is indicated by different letters at $P < 0.05$ or * $P < 0.05$, * ** $P < 0.001$ ($n = 5$). C: control.

$P < 0.05$, $P < 0.001$). On the protein expression, we showed the stimulatory effect of PNX-14 (100 nM) on the levels of all investigated cyclins (Fig. 5C, $P < 0.001$).

3.6. Effect of PNX-14 on the phosphorylation of MAP3/1, AKT and STAT3 kinases pathways and their involvement on PNX-14 action on Gc proliferation

The changes in the phosphorylation of kinases pathways are a quick answer to changing environments often connected with the regulations of cell proliferation. Briefly, MAP3/1 kinase participates in oocyte maturation, and follicle rupture [41], AKT provides cells with a survival signal that allows them to withstand apoptotic stimuli [42], while JAK/STAT3 activation stimulates cell proliferation, differentiation and migration [43]. Fig. 6A showed that 100 nM PNX-14 decreased MAP3/1 kinase phosphorylation after 5, 15, 30, 45 and 60 min of in vitro culture and STAT3 phosphorylation at all investigated time points. Conversely, PNX-14 (100 nM) stimulated phosphorylation of AKT in Gc after 5 and 15 min (Fig. 6A, *P < 0.05*). Finally, we observed that the positive effect of PNX-14 (100 nM) on Gc proliferation was reversed to the control level after pharmacological inhibition of the MAP3/1, PI3K/AKT and JAK/-STAT3 pathways. None of the tested inhibitors, added alone, changed Gc proliferation (Fig. 6B, *P < 0.05*).

4. Discussion

To our knowledge, this is the first study to describe the expression of PNX-14 and its receptor GRP173 in porcine ovarian follicles during the oestrous cycle and the in vitro impact of PNX-14 on Gc proliferation, E2 secretion, the cell cycle and phosphorylation of MAP3/1, AKT and STAT3. Although the function of PNX-20 in the human ovary has been described [13], we decided to supplement knowledge about expression and role of PNX-14 in regulating porcine ovary function, because pigs are economically important and increasing the fertility of these animals would provide greater economic benefits than improving their growth rate. In addition, pigs struggle with numerous reproductive pathologies caused by an incorrect energy balance – for example, sows with low weight require a longer time to the first oestrous and they have fewer offspring [44]. Besides, a negative energy balance in pigs impacts follicle size, the follicular fluid steroid profile and oocyte and zygote developmental competence [45]. Therefore, understanding how PNX-14 regulates food intake and energy metabolism [10] may improve the fertility of female pigs Fig. 7.

We found that the relative abundance of *SMIM20* mRNA did not change in ovarian follicles during the oestrous cycle, while PNX-14 protein expression was highest at days 10–12 of the oestrous cycle. The differences between the transcript and protein levels may be explained by complex and diverse post-transcriptional mechanisms involved in converting mRNA to the protein that has not yet been sufficiently defined to be able to calculate protein concentrations from mRNA [46]. This may also be related to the function of *SMIM20*, inner mitochondrial membrane protein, a component of the mitochondrial translation regulation assembly intermediate of the cytochrome c oxidase complex, which is involved in the biogenesis of cytochrome c

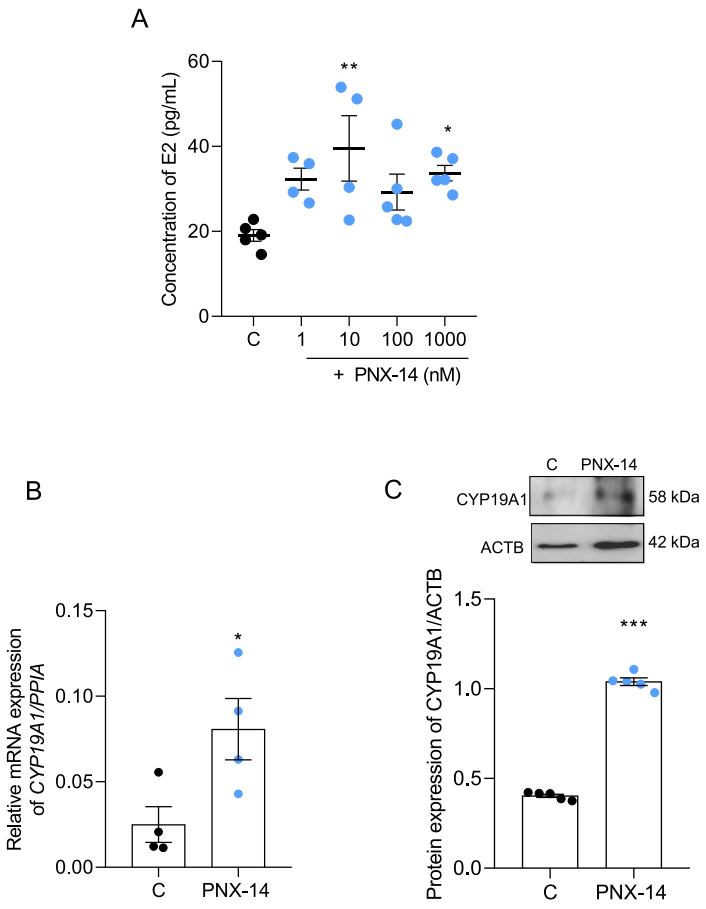

Fig. 3. Effect of phoenixin-14 (PNX-14) on oestradiol (E2) secretion (A) and aromatase (CYP19A1) mRNA (B) and protein (C) expression. Representative blots are shown. Gene expression level was normalised to cyclophilin A (PPIA), while protein to β-actin (ACTB). Data are plotted as the mean ± SEM. One-way ANOVA was used for E2 secretion statistical analysis, while T-student tests for CYP19A1 levels (GraphPad Prism 8). Significance between groups is indicated by different letters at $P < 0.05$ or * $P < 0.05$, * * $P < 0.01$, * ** $P < 0.001$ ($n = 5$). C: control.

oxidase, thus its expression may be stable during the cycle [47]. Additionally, the differences in the abundance of proteins between individual ovarian follicles are probably controlled by steroid hormones produced or secreted by follicular cells, the concentrations of which change during the oestrous cycle. Interestingly, Ullah *et al.* [1] observed correlations between PNX-14 and progesterone (P4) and LH in human serum. Moreover, researchers have described increased serum PNX-14 levels in patients with PCOS, which is associated with elevated LH and androgen concentrations [18], as well as in ovaries of PCOS rats [7]. On the other hand, Ullah *et al.* [1] noted a negative correlation between PNX-14 and E2. During the oestrous cycle, E2 secretion is increased before ovulation [48], a phenomenon that may explain the decrease in PNX-14 protein at days 16–18 of the oestrous cycle. However, it may depend on the species studied and requires further research. Additionally, studying the direct effect of LH, P4 or E2 on the level of PNX-14 requires further experiments. So far, it has been shown that FSH, LH, P4 and E2 stimulate the level of resistin in the pig ovarian follicles [22]. Moreover, Kulinska *et al.* [49] demonstrated that PNX-14 decreases *SMIM20* mRNA expression with no change in *GPR173* levels in Z12 endometrial cells, which suggests regulation of ligand–receptor expression.

We found that GPR173 mRNA and protein expression increased in ovarian follicles as the oestrous cycle progressed. Our results are consistent with the study by Nguyen *et al.* [13], who observed that GPR173 expression increases in human follicular Gc and HGrC1 cells as the follicular stage develops. These authors suggested that E2 is involved in the regulation of GPR173. Previously published data by Bauman *et al.* [50] provided evidence that E2 increased expression of *Gpr173* in the rat's hypothalamus.

We demonstrated the immunolocalisation of PNX-14 and GPR173 in the cytoplasm of Gc and theca cells and oocytes. Our findings are consistent with reports describing PNX-14 and GPR173 expression in the same structures in rats [7] and human ovaries [13]. In rats, researchers noted strong PNX-14 immunostaining in antral follicle Gc and a very weak signal for PNX-14 in theca cells and oocytes, similar to our observation. Interestingly, the authors noted no PNX-14 signal in the ovaries of PCOS rats [7]; this finding underscores the important role of PNX-14 in PCOS development. Moreover, they found strong GPR173 immunostaining in theca cells and stromal cells close to prenatal and small antral follicles [7]. We also noted the strongest GPR173 signal in theca cells rather than Gc. Interestingly, in a human model, researchers found that both PNX-20 and GPR173 immunostaining intensity increase with follicle growth and they found the most intense PNX-20 immunostaining in antral follicle Gc. The authors also noted prominent PNX-20 and GPR173 immunostaining in oocytes [13]. In that study, theca cells of the secondary and antral follicles showed weak PNX-20 and GPR173 staining [13], opposite to our observation, which indicates that expression is differentially regulated depending on the model studied.

Overall, the expression of both components (ligand and receptor) of the PNX-14 signalling system allows for analysis of the direct role of PNX-14 in the function of porcine ovarian follicles, specifically Gc proliferation and E2 production. Understanding the role of PNX-14 in ovarian physiology is very important considering that the studies conducted so far have indicated increased serum PNX-14 levels and increased PNX-14 protein expression in the ovaries of PCOS rats [7].

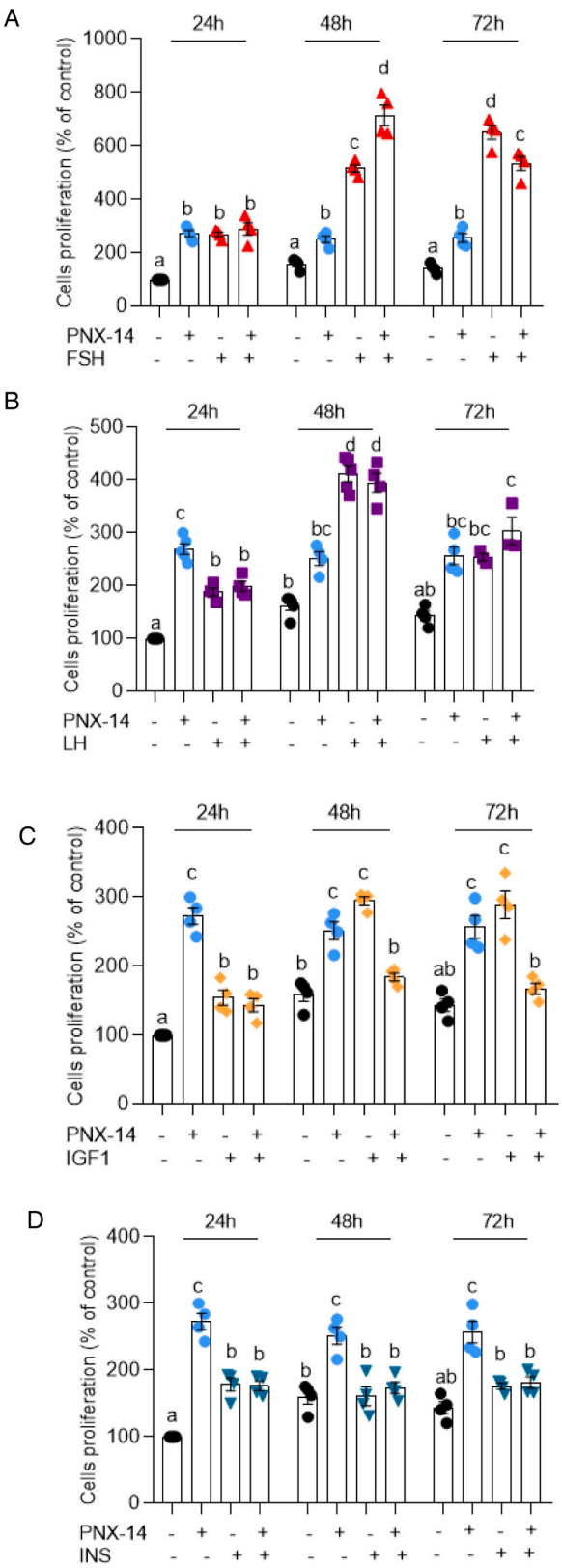

Fig. 4. Effect of phoenixin-14 (PNX-14) added alone or in combination with luteinizing hormone (LH), follicle stimulating hormone (FSH), insulin-like growth factor type 1 (IGF1), or insulin (INS) on granulosa cells proliferation (A-D). Data are plotted as the mean ± SEM. Two-way ANOVA, followed by post hoc tests was used for statistical analysis of cells proliferation (GraphPad Prism 8). Significance between groups is indicated by different letters at $P < 0.05$ ($n = 4$). C: control.

Moreover, Gc proliferation is a major physiological process in ovarian function; it is necessary for proper folliculogenesis and oocyte growth [36]. Briefly, during follicle formation, the morphological and physiological properties of Gc change and are connected to the capacity of cells to divide continuously [36]. For the first time, our data have documented the stimulatory effect of PNX-14 on Gc proliferation confirmed by its positive action on the PCNA level. However, Nguyen *et al.* [13] previously showed that another PNX isoform – PNX-20 –induces the proliferation of HGrC1 cells. A study on human Gc showed that PNX-20 increases the expression of the FSH and LH receptors, which are necessary for Gc differentiation and proliferation [51], and KIT ligand, which promotes primordial to primary follicle transition and increases the diameter of oocytes from growing primary follicles [52]. Additionally, PNX-14 elevated proliferation in other tissues including rat pancreatic INS-1E cells [5], human endometrial cells [49] and rat preadipocytes [6]. Moreover, as Nguyen *et al.* [13] showed in human Gc, PNX-20-stimulated cell proliferation is linked to increased E2 secretion. Similarly, in our study, we also observed that PNX-14 increased both E2 secretion and CYP19A1 expression. Moreover, modulation of Gc proliferation by adipokines or neuropeptides is strictly linked to changes in E2 synthesis. For example, apelin [29] and vaspin act positively on Gc proliferation [25] and E2 synthesis [31] in porcine Gc. Thus, our observations indicate that PNX-14 directly stimulates Gc proliferation and also indirectly influences this process by elevating E2 synthesis, which is a strong mitogenic factor [34]. Interestingly we did not observe significantly relevant effects of PNX-14 at dose 100 nM on E2 secretion, while a positive effect on CYP19A1 level was noted. One hypothesis to explain these results is that PNX-14 could modulate the activity of steroid enzymes, which directly influence hormone synthesis. However, this needs to be future investigated. So far, literature data indicated on differences between the steroidogenic enzymes activity and their expression; for example ciprofibrate decreases 3β-Hydroxysteroid dehydrogenase activity without affecting either protein or mRNA expression in rat testis [53].

Hormonal interactions of the hypothalamic–pituitary–ovarian axis are necessary for proper ovarian activity. We observed that PNX-14 decreased FSH- and IGF1-induced Gc proliferation. Interestingly, there is a close association between PNX-14 and FSH levels, which may influence cell proliferation. Previously, Ullah *et al.* [1] showed that in humans, serum PNX-14 concentrations increase as FSH increases. Similarly, Wang *et al.* [12] reported elevated *Fsh* mRNA expression in the pituitary of *Scatophagus argus* after injection with both PNX-14 and PNX-20. Further investigation is required to understand the molecular mechanisms by which PNX-14 interacts with different hormones such as gonadotropin and IGF1 to affect the function of ovarian cells, however, we suggest that it may be a protective mechanism against excessive Gc proliferation exposed to PNX-14. A similar observation was noted previously, where chemerin inhibited IGF1- induced human Gc proliferation [54].

For the in vitro experiments, we chose PNX-14 because its sequence is identical in humans, rats, mice and pigs [3]. We observed that PNX-14 in porcine Gc acts the same as PNX-20 in human Gc regarding proliferation and E2 secretion [13]. It is often noted in the literature that different isoforms of peptides act similarly in different cell types. For example, both apelin-13 and apelin-36 increase Ca^{2+} levels in a human embryonic kidney cell line probably because they can both bind the same receptor [55]. Moreover, there is evidence supporting the idea that isoforms as well as other ligands may act similarly to regulate physiological processes: as was described previously both apelin and ELABELA, a ligand for the apelin receptor, increase cardiac contractility in the rat heart [56,57].

The mitogenic effect of PNX-14 in porcine Gc occurred in parallel with the promotion of cell cycle progression into the S phase. We also noted that PNX-14 stimulated cyclin B but inhibited cyclin D mRNA expression. Of note, PNX-14 upregulated cyclin A, B, D and E protein expression, and it is the cyclin protein levels that are most critical in

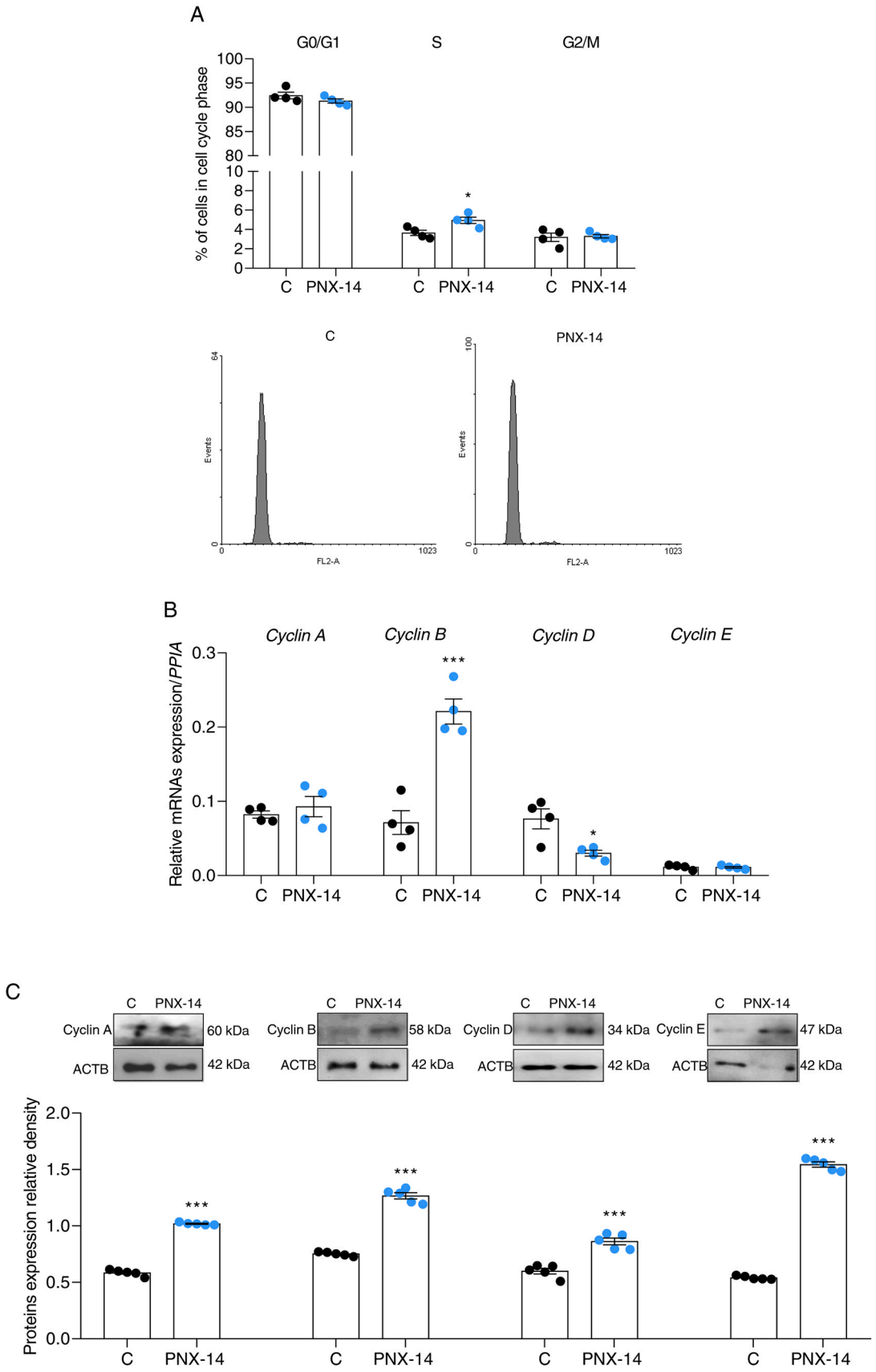

Fig. 5. Effect on phoenixin-14 (PNX-14) on cell cycle progression (A) and cyclins A, B, D, E mRNA (B) and protein level (C). Representative blots are shown. Genes expression level was normalised to cyclophilin A (PPIA), while protein to β-actin (ACTB). Data are plotted as the mean ± SEM. T-student, followed by post hoc tests was used for statistical analysis (GraphPad Prism 8). Significance between groups is indicated at * $P < 0.05$, * ** $P < 0.001$ ($n = 4$). C: control.

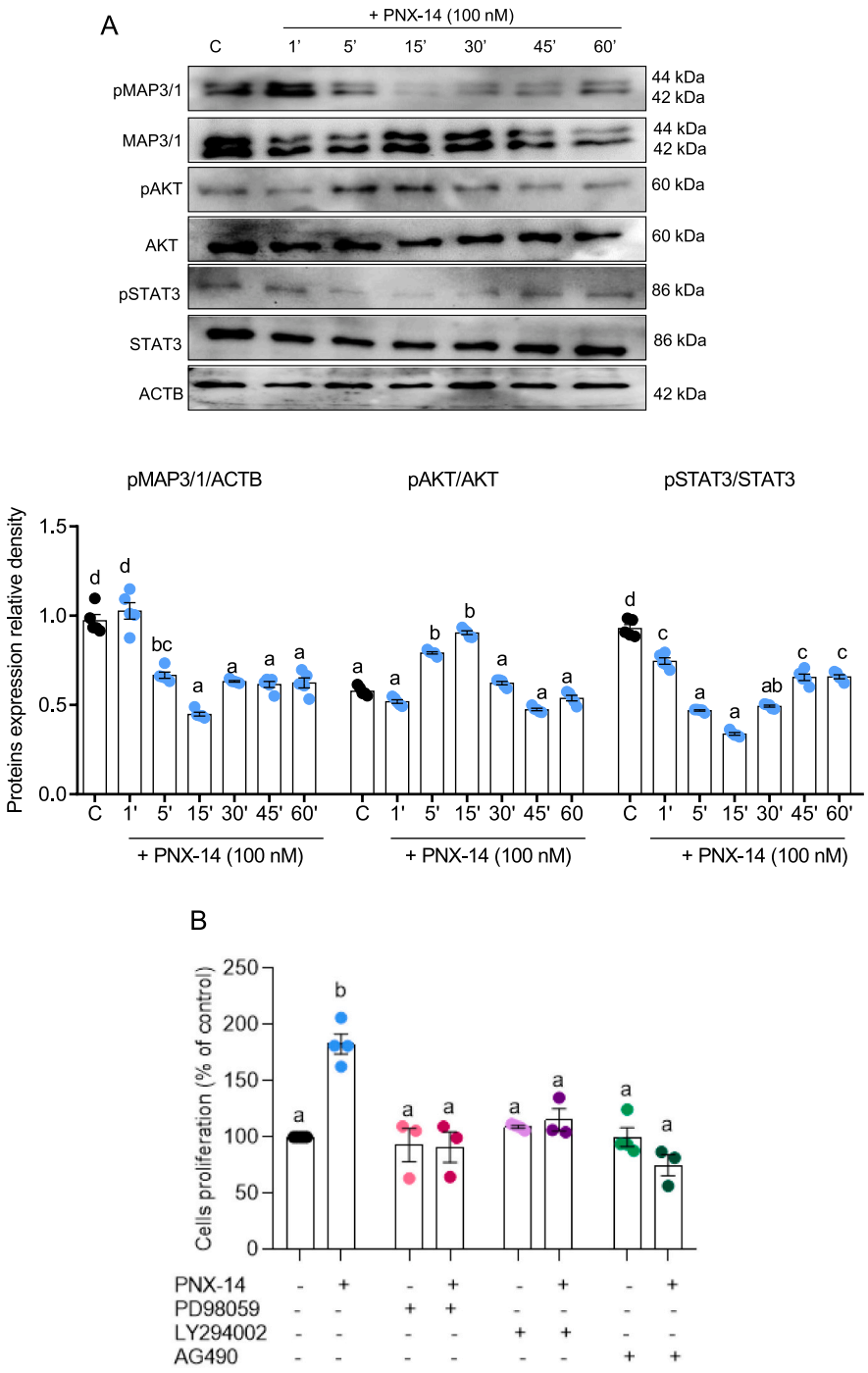

Fig. 6. Effect of phoenixin-14 (PNX-14) on phosphorylation of mitogen activated kinase (MAP3/1), protein kinase B (AKT) and signal transducer and activator of transcription 3 (STAT3) (A) and their involvement in PNX-14 mediated action on Gc proliferation (B). Representative blots are shown. Protein levels were normalised to the β-actin (ACTB) for MAP3/1 kinase or total form of kinase for AKT and STAT3. Data are plotted as the mean ± SEM. One-way ANOVA, followed by post hoc tests was used for statistical analysis (GraphPad Prism 8). Significance between groups is indicated by different letters at $P < 0.05$ (n = 4). C: control; PD98059: MAP3/1 inhibitor, LY294002: AKT inhibitor, AG490: STAT3 inhibitor.

regulating cell physiology. As described previously, resistin has an anti-apoptotic effect in porcine ovarian follicle cells; in addition, it stimulates the expression of the survival-promoting BCL2 protein without affecting its mRNA levels [24]. Thus, our findings indicate that PNX-14 stimulates DNA synthesis (S phase) and mitosis required for cell division (G2/M phase) by directly influencing the expression of cyclins. Our results are consistent with Han *et al.* [58], who showed that FSH increases cyclin D protein expression, which is connected with the stimulation of proliferation in rat Gc, while vaspin proliferative effect in porcine Gc was connected with stimulation in cyclins D and A level [25]. Besides, PNX-14 resolves the morphine-caused cell cycle arrest in neuronal cells with significant changes in the expression levels of cyclin-dependent kinase 6, which promotes entry to the S phase of the cell cycle [59]. Interestingly, researchers have also indicated that PNX-14 is an important regulator of apoptosis, which is strictly connected to ovarian follicle remodelling during folliculogenesis [60]. For example, in human umbilical vein endothelial cells, PNX-14 inhibits cell apoptosis and decreases proapoptotic BAX protein expression [61]. Additionally, PNX-14

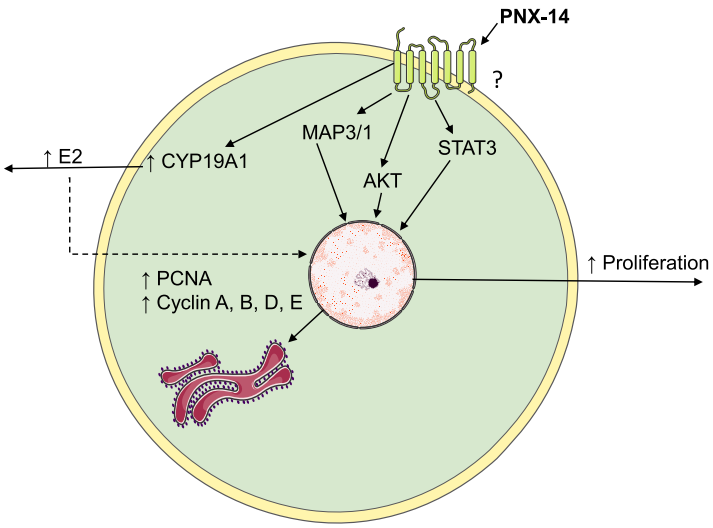

Fig. 7. Summary of phoenixin-14 (PNX-14) action in granulosa cells (Gc). PNX-14 via mitogen activated kinase (MAP3/1), protein kinase B (AKT) and signal transducer and activator of transcription 3 (STAT3) stimulated porcine Gc proliferation. PCNA: proliferating cells nuclear antigen, E2: oestradiol, CYP19A1: aromatase.

facilitates apoptosis in vascular smooth muscle cells [62]. This suggests that PNX-14 may also regulate apoptosis in the ovarian cells. Therefore, further investigation is needed to understand the role of PNX-14 in ovarian apoptosis to better understand its effect on follicular development. Previous data have also clearly shown that other hormones – such as resistin [22] and vaspin [25] in porcine Gc – stimulate proliferation and exert an inhibitory effect on apoptosis.

The changes in the phosphorylation of kinase pathways are a quick answer to changing environments even in ovarian follicles often connected with the regulations of cell proliferation. In the present study, we observed that PNX-14 stimulated AKT and inhibited both MAP3/1 and STAT3 phosphorylation in a time-dependent manner. A previous study demonstrated that PNX-14 decreases AKT phosphorylation in murine BV2 cells [61], so we suggest that the effect of PNX-14 on the phosphorylation of kinases, and thus the modulation of signalling pathways, may be dependent on the cell type. For example, vaspin stimulates STAT3 phosphorylation in follicular cells [31] but has no effect in luteal cells [62] in pigs. Additionally, we investigated the involvement of PI3K/AKT, MAP3/1 and JAK/STAT3 in PNX-14-induced Gc proliferation. We showed after pharmacological inhibition of these kinases, cell proliferation was abolished. Billert *et al.* [6] demonstrated that PNX-14 stimulates rat preadipocyte differentiation independently of the AKT signalling pathway, but increases cyclic adenosine monophosphate (cAMP) levels in 3T3L1 cells. Potentiation of cell growth and *Ins* mRNA expression in rats is mediated by the MAP3/1 and AKT signalling pathway [5]. Moreover, exposure of the rat heart to exogenous PNX-14 reduces contractility and relaxation, changes accompanied by increased MAP3/1 and AKT phosphorylation [4]. Interestingly, the MAP3/1 pathway and PI3K/AKT pathway interact at multiple points in cell cycle progression regulation including cross-activation, cross-inhibition, and pathway convergence on substrates [63]. However, such interactions occur both under physiological conditions, after an action of different hormones, or even may occur after blocking these kinases. A good argument for the selective, specific effect of chosen inhibitors is our previous work, where we showed that PCNA expression in pig luteal cells is regulated by the MAP3/1 kinase pathway, but not by PI3K/AKT [64]. Additionally, the literature showed that LY294002 blocks the phosphorylation of AKT or pyruvate dehydrogenase kinase 1 in prostate cancer cells [28].

In conclusion, our study provides the first evidence that the expression of PNX-14 and its receptor GPR173 in porcine Gc depends on oestrous cycle progression. We also showed that PNX-14 stimulates Gc proliferation via the MAP3/1, JAK/STAT3 and PI3K/AKT signalling pathways. In light of these findings, PNX-14 appears to be a novel modulator of porcine Gc physiology and could represent a relevant player in ovarian follicle growth.

Consent to publish

All authors agree to publish this manuscript.

Funding

This research was supported by NCN, project Preludium 2020/37/N/NZ9/00981 (PI: Ewa Mlyczyńska).

CRediT authorship contribution statement

The conception and design of the study P.K., E.M., A.R.; acquisition of data P.K., E.M. J.W., K.K., P.G., K.P., O.S., A.G., M.O., J.D; analysis and interpretation of data P.K., E.M., J.W., K.K., P.G., A.R.; drafting the article P.K., A.R.; revising article for important intellectual content A.R., J.D; final approval of the version to be submitted P.K., A.R.

Declaration of Competing Interest

No conflict of interest exist in the submission of this manuscript, and the manuscript is approved by all authors for publication.

Data availability

The data that supports the findings of this study are available in the method part and on request from the correspondence author.

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