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Neuregulin 1 (NRG1) modulates oocyte nuclear maturation during IVM and improves post-IVF embryo development

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Abstract

Oocyte in vitro maturation (IVM) is still a major challenge in human and animal assisted reproduction. Gradual instead of abrupt activation of the ovulatory cascade during IVM has been proposed to enhance nuclear-cytoplasmic synchrony and cumulus-oocyte communication, thus favoring oocyte developmental competence. Herein, we assessed the effects of neuregulin 1 (NRG1), an EGF-like factor that modulates EGFR signaling, on oocyte nuclear maturation dynamics, cumulus expansion and expression of mRNAs regulating these processes during IVM, as well as on post-IVF embryo development following AREG- stimulated IVM in cattle. In experiment 1, cumulus-oocyte complexes (COCs) were subjected to IVM with graded doses of NRG1 (1, 10 or 100 ng/mL) for 6, 9, 12, 20, and 24 h, after which oocyte nuclear status and cumulus mRNA expression were assessed. At 6 h of IVM, NRG1 at 1 ng/mL significantly decreased the percentage of GVBD (germinal vesicle breakdown) oocytes without altering later meiotic dynamics or the percentage of oocytes achieving meiosis II. In experiment 2, adding NRG1 (1 ng/mL) to the IVM medium did not affect cumulus expansion but increased the percentage of expanded and hatched blastocysts, and blastocyst total cell number following IVF/IVC. NRG1 decreased EGFR mRNA abundance while increasing NPR2 and PTX3 mRNA levels at 9 h, and TNFAIP6 mRNA abundance at 20 h of IVM. This is the first study that reports the modulatory effect of NGR1 during oocyte maturation in a mono-ovulatory species and demonstrates that this action may be applied during IVM to improve post- IVF embryo development.

Introduction

In vitro maturation (IVM) of cumulus-oocyte complexes (COCs) is a crucial and limiting step for in vitro embryo production (IVP) applied to animal production and still a challenge in human reproductive medicine to render infertility treatments more accessible and safer [1,2]. In vitro matured oocytes are less competent to be fertilized and to reach the blastocyst stage than in vivo matured counterparts [3], suggesting that IVM efficacy can be improved by culture strategies mimicking more closely the physiological environment where oocyte maturation takes place.

The efficacy of IVM is also determined by oocyte developmental competence, which relies on the coordination of mechanisms controlling nuclear and cytoplasmic maturation [2,4,5]. This fine- tuning largely depends on the delivery of cumulus-derived me- tabolites and regulatory factors into the oocyte, a process mediated by gap junctions communicating the tip of cumulus cells transzonal projections with the ooplasm [6,7]. Interestingly, through a synapse-like mechanism, cumulus cells transzonal projections also deliver cumulus-derived polyadenylated mRNA, which appears crucial for regulating gene expression and successful meiotic completion [7e9].

In most mammalian species, oocyte meiosis begins still during fetal life, being subsequently arrested at the diplotene stage of prophase [4]. In vivo, meiotic resumption is triggered by the LH preovulatory surge. After achieving metaphase II (MII), meiosis is again interrupted and only completed if fertilization occurs [10]. LH stimulates the expression of epidermal growth factor (EGF)-like molecules, namely amphiregulin (AREG), epiregulin (EREG) and betacellulin (BTC) in granulosa cells [11,12], which then trigger an extensive network of genes in mural and cumulus granulosa cells, leading to cumulus expansion, meiotic resumption and ovulation

[13]. Meiotic resumption is specifically triggered by the interrup- tion of cumulus-oocyte transfer of cyclic guanosine monophosphate (cGMP), a molecule produced by cumulus cells under the stimula- tion of the natriuretic peptide type C (NPPC) receptor (NPR2), that prevents meiotic resumption by inhibiting the degradation of cAMP by phosphodiesterase 3 (PDE3) [1,14,15]. The interruption of cGMP delivery appears to result from both gap junction closure due to connexin phosphorylation [16], and retraction of transzonal pro- jections triggered by AREG/EREG-induced ERK1/2 signaling [17].

Neuregulin 1 (NRG1) is another member of the EGF-like family stimulated by the LH surge in granulosa cells, previously suggested to function as a modulator of the ovulatory cascade [18e20]; NRG1 reduced intracellular responses to AREG and the speed of meiotic progression in mice [18,20]. In addition, NRG1 supplementation during IVM enhanced the expression of TNFAIP6, a gene crucial for extracellular matrix organization during cumulus expansion, and increased the percentage of oocytes reaching the cleavage stage following IVF in mice [19].

We have recently proposed a new IVM strategy based on physiological parameters, namely “the follicular system”, in which oocyte maturation is promoted with AREG, combined with intra- follicular concentrations of IGF-1, FSH, and steroids [21]. In the present study, we tested the hypothesis that supplementation of the so called “follicular system” with NRG1 would modulate nu- clear maturation dynamics during culture and improve post-IVF embryo development in cattle. In addition, to shed light on the mechanisms through which NRG1 may influence meiotic pro- gression and oocyte developmental competence, we assessed the effects of NRG1 on the expression of genes regulating the final differentiation of cumulus cells.

2. Material and methods

All products used in the study were purchased from Sigma- Aldrich (St. Louis, MO, USA) unless otherwise specified.

The study was divided into 2 Experiments, herein described as Experiments 1 and 2 shown in the experimental design section.

2.1. Experimental design

2.1. 1. Experiment 1: effects of NRG1 supplementation on oocyte maturation dynamics and gene expression during AREG-stimulated IVM

To assess the effects of NRG1 supplementation during AREG- stimulated IVM on oocyte nuclear maturation, based on previous studies in mice and pigs in which NRG1 effects were observed at 10 and 20 ng/mL, respectively [19,22], 4 treatments with graded doses of NRG1 (0, 1, 10, or 100 ng/mL) were compared (rh-Neuregulin 1, R&D Systems, Minneapolis, MN, USA). The “follicular system” was used as the base IVM medium [TCM199 containing Earle's salts supplemented with 4 mg/mL fatty acid-free bovine serum albumin (BSA), 75 mg/mL amikacin, 22 mg/mL sodium pyruvate, 1 mmol cysteamine, 0.01 UI/mL recombinant human FSH (rh-FSH, Gonal- f®, Merck Serono S.A., Aubonne, Switzerland), 50 ng/mL 17b- estradiol, 150 ng/mL progesterone, 10 ng/mL IGF-1 and 100 ng/mL AREG (rh-Amphiregulin, R&D Systems)] [21]. Five experimental replicates, each containing 4 pools of 20e25 COCs treated with the graded doses specified above, were performed. Meiotic progression was evaluated through the assessment of chromatin configuration at 0, 6, 9, 12, 20, and 24 h of IVM. Cumulus cells were recovered at 6, 9, and 20 h of IVM to assess the effects of NRG1 on the relative

abundance of mRNA regulating oocyte maturation and cumulus differentiation. Only pools treated with 0 and 1 ng/mL were selected to assess cumulus gene expression as 1 ng/mL was the lowest and only dose to significantly alter oocyte maturation.

2.1. 2. Experiment 2: effects of NRG1 supplementation during AREG- stimulated IVM on embryo production and cumulus expansion

To investigate whether the modulatory action of NGR1 on meiotic dynamics could benefit COC developmental competence, a second experiment was designed to test the effects of NRG1 on cumulus expansion and post-IVF embryo development. For this follow-up experiment, the lowest NRG1 concentration effective to delay GVBD (1 ng/mL NRG1) in Experiment 1 was chosen. COCs were subjected to IVM in the follicular system without additives (Control Group) or in the follicular system supplemented with 1 ng/ mL NRG1 (NRG1 Group). At 24 h of IVM cumulus expansion was visually assessed, after which COCs were fertilized, and presump- tive zygotes were cultured as described below. Five experimental replicates were performed, each of them with 2 pools of 20e25 COCs subjected to the Control or NRG1 treatment. Control and NRG1 groups were compared with regard to the percentage of COCs exhibiting full expansion, total blastocyst yield, expanded and hatched blastocyst rates, as well as total blastocyst cell number.

2.2. IVM and cumulus expansion assessment

Ovaries of adult cows were obtained from nearby slaughter- houses and transported to the laboratory in sterile saline solution (0.9% NaCl) at 37 C. COCs were aspirated from 2 to 8 mm

diameter follicles with an 18-gauge needle and pooled in a 15 mL conical tube. After sedimentation, COCs were recovered and selected using a stereomicroscope (Nikon, SMZ800, Tokyo, Japan). Only COCs with homogenous cytoplasm and at least three compact layers of cumulus cells were used (grades I and II) in the study [23].

The selected COCs (20e25 COCs/group) were washed in three drops (50 mL) of washing medium (TCM199 with Earle's salts and 25 mmol HEPES, supplemented with 75 mg/mL amikacin and 4 mg/ mL BSA) and three drops (50 mL) of IVM medium. After washing, COCs were cultured in 500 mL of the serum-free “follicular system” IVM medium, with or without NRG1 as detailed above in the “experimental design”, in four-well plates at 38.5 C and 5.5% CO2 in humidified air. COCs were submitted to cumulus expansion analysis and then transferred to IVF medium or collected for meiotic pro- gression assessment. Cumulus expansion was visually assessed after 24 h of IVM as previously described [24,25], and treatment-groups were compared regarding the percentage of COCs achievement maximal/full expansion.

2.3. In vitro fertilization and embryo culture

IVF and embryo culture were performed as previously described [21], with small modifications. Briefly, matured COCs were washed in IVF medium drops (50 mL) and placed in four-well plates with 300 mL commercial IVF medium (BotuFIV®, BotuPharma, Botucatu, Sa~o Paulo, Brazil). Cryopreserved sperm from a single Nelore bull (Bos indicus) and batch were used throughout the study. Semen straws were thawed at 37 C for 30 s, and spermatozoa were selected in a 45e90% commercial gradient (BotuFIV® Select SPERM gradient,

BotuPharma, Botucatu, Sa~o Paulo, Brazil). Sperm sample volume was calculated and added to each IVF well to reach the final concentration of 2 106 spermatozoa/mL. COCs and spermatozoa were co-incubated at 38.5 C in humidified air containing 5.5% CO2 for 18 h (Day 0). Subsequently, the presumptive zygotes were denuded with a vigorous shaker (Phoenix Luferco AP59, Arara- quara, Sa~o Paulo, Brazil) in washing medium.

Embryo culture was then started in four-well plates containing 500 mL commercial IVC medium (BotuFIV® IVC medium, Botu- Pharma, Botucatu, Sa~o Paulo, Brazil) supplemented with 2.5% FBS (Cripion, Andradina, Sa~o Paulo, Brazil) for 7 days (Day 1 to Day 8) at 38.5 C in humidified air containing 5% CO2, 5% O2, and 90% N2. Embryo development was assessed on Day 8 (Nikon Stereomicro- scope, SMZ800, Tokyo, Japan), blastocysts were identified and morphologically categorized as non-expanded, expanded, and hatched blastocysts [26]. Blastocysts were then fixed in 60% methanol, stained in 1 mg/mL Hoechst 33342 (Invitrogen, Waltham, MA, USA), and the total cell number was counted in a fluorescence microscope (400 magnification, Nikon, Eclipse 80i, Tokyo, Japan).

2.4. Assessment of meiotic progression

Oocytes were denuded by repeated pipetting in washing me- dium, fixed in 60% methanol, and stained with 1 mg/mL Hoechst 33342 (Invitrogen, Waltham, MA, USA). Chromatin status and meiotic stages were determined by fluorescence microscopy (Nikon, Eclipse 80i, Tokyo, Japan). At 6 h of IVM, oocytes were classified as GV or GVBD oocytes if before or after germinal vesicle breakdown, respectively. Meiotic progression was evaluated from 9 to 24 h of IVM, when oocytes were classified as MI or MII oocytes [27,28].

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193 **2.5. Assessment of mRNA relative abundance in cumulus cells**

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195 After mechanical isolation from 20 to 25 COCs cultured in each experimental replicate,
196 cumulus cells were pooled, washed, sub- jected to total RNA extraction (ArcturusTM
197 PicoPureTM RNA Isolation Kit, Applied Biosystems, Waltham, MA, USA), and total RNA con-
198 centration was measured by spectrophotometry (NanoDropTM 2000, Thermo Scientific,
199 Waltham, MA, USA). The entire RNA sample was incubated with DNAse I (1 IU/mg; Invitrogen,
200 Waltham, MA, USA) and reverse transcription was performed using random primers (High-
201 Capacity kit, Applied Biosystems, Waltham, MA, USA).

202

203 Messenger RNA relative abundance was assessed by real-time RT-qPCR using bovine specific
204 primers listed in Table 1 and the Power SYBRTM Green PCR Master Mix (Applied Biosystems,
205 Wal- tham, MA, USA) at the final volume of 20 mL. PCR was performed in duplicates in the
206 StepOnePlusTM Real-Time PCR System (Applied Biosystems, Waltham, MA, USA); cycling
207 conditions were 95 C for 10 min (1 cycle), denaturing at 95 C for 15 s followed by annealing at
208 60 C for 1 min (40 cycles). Threshold cycle (Ct) values were obtained by adjusting the raw
209 fluorescence values with the Lin- RegPCR software [29]. Relative expression values were then
210 calculated with the DDCt method [30], and data were normalized with two reference genes
211 [H2A histone family member Z (H2AFZ) and peptidylprolyl isomerase A (PPIA)] previously
212 tested in our laboratory [25,31].

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214 **2.6. Statistical analysis**

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Data in percentages were arcsine transformed and all the data were first tested for normality with the Shapiro-Wilk test before assessing treatment effects. The effects of NRG1 on meiotic pro- gression were tested by ANOVA, followed by group comparisons with the TukeyeKramer test. The effects of NRG1 on cumulus expansion, embryo development rates, embryo cell number, and mRNA relative abundance were tested with the Student's t-test (time points providing parametric data) or Wilcoxon test (time points providing non-parametric data). Outlier relative mRNA values were identified by the extreme studentized deviate method (ESD) available in the GraphPad PRISM software (GraphPad Soft- ware, Inc., San Diego, CA, USA) and were excluded. Data are pre- sented by mean ± SEM, and differences were considered significant when $P < 0.05$. The analyses were performed using the JMP® software (SAS Institute, Cary, NC, USA).

3. Results

Supplementation of the IVM medium with NRG1 at 1 ng/mL, but not at 10 or 100 ng/mL, significantly decreased the percentage of COCs achieving GVBD at 6 h of IVM (P 1⁄4 0.0244; Fig. 1). Differently, meiotic progression was not affected by NRG1 at later time-points of IVM (Fig. 1). In parallel with its effects on GVBD, NRG1 did not alter the expression of genes involved in meiotic maturation at 6 h but decreased EGFR mRNA abundance (P 1⁄4 0.0159), while increasing that of NPR2 (P 1⁄4 0.0438) at 9 h. Abundance of AREG, EREG, EGFR, NPR2, and FSHR mRNA was not affected 20 h after the addition of NRG1 to the IVM medium (Fig. 2). The percentage of COCs achieving full cumulus expansion at the end of IVM was not altered by NRG1 (Fig. 3). However, NRG1 increased mRNA levels of PTX3 (P 1⁄4 0.0417) and TNFAIP6 (P 1⁄4 0.0225) at 9 and 20 h of IVM, respectively (Fig. 3).

Although NRG1 supplementation during IVM did not alter total blastocyst yield following IVF and IVC, it caused a 20% increase in the production of transferable blastocysts (expanded and hatched) in relation to total blastocysts (P 1⁄4 0.0384), and a 25% increase in the production of transferable blastocysts in relation to total oo- cytes (P 1⁄4 0.0301). In addition, NRG1 addition to the IVM medium led to a 25% increase in blastocyst total cell number (P 1⁄4 0.0273; Fig. 4).

4. Discussion

The detrimental impact of nuclear and cytoplasmic desynchro- nization during oocyte maturation on developmental competence has been discussed for more than 30 years [32]. Overcoming this challenge is crucial to increase developmental competence following IVM and thus IVP efficiency [2,5,33]. Previous studies have suggested that granulosa-derived NRG1 acts on cumulus cells to modulate EGF-like signaling and meiotic resumption after the LH surge [19,20]. Herein we provide novel evidence that NRG1 regu- lates the dynamics of oocyte nuclear maturation in cattle and may be utilized during EGF-induced IVM to enhance oocyte develop- mental competence, thus representing a potentially valuable tool to improve IVM/IVF outcomes.

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As hypothesized, NRG1 did modulate oocyte nuclear maturation during IVM in the present study. Interestingly, NRG1 specifically delayed GVBD without altering subsequent meiotic progression. While in the present study, the lowest dose of NRG1 tested (1 ng/ mL) was sufficient to delay GVBD during AREG-stimulated IVM, in mice, the same was only observed with NRG1 supplementation at 10 ng/mL [19,20]. Although this may simply reflect a variation in biological activities of the different NRG1 sources used in mice and As hypothesized, NRG1 did modulate oocyte nuclear maturation during IVM in the present study. Interestingly, NRG1 specifically delayed GVBD without altering subsequent meiotic progression. While in the present study, the lowest dose of NRG1 tested (1 ng/ mL) was sufficient to delay GVBD during AREG-stimulated IVM, in mice, the same was only observed with NRG1 supplementation at 10 ng/mL [19,20]. Although this may simply reflect a variation in biological activities of the different NRG1 sources used in mice and

Since, on the one hand, a drastic decrease in gap junction- mediated communication preventing the delivery of cGMP into the oocyte appears crucial for the induction of GVBD [21,36e38], and on the other, ERK1/2 and PKC-induced connexin 43 (Cx-43) phosphorylation causes gap junction closure, it has been suggested that NRG1 inhibitory effect on oocyte nuclear maturation may be a consequence of its inhibitory influences on EGF-induced ERK1/2 and PKC activity [18,20,39,40]. Indeed, while Cx-43 phosphoryla- tion has been predominantly

attributed to ERK1/2 in cumulus cells it also presents sites for phosphorylated PKC and depends on the availability of intracellular calcium [20,41,42]. Therefore, in parallel to prolonged gap junction-mediated transfer of cGMP, NGR1 would also increase/prolong the delivery of other cumulus-derived me- tabolites such as pyruvate and NAPDH, which are crucial for oocyte homeostasis and thus developmental competence [7].

The present data indicate that the effects of NGR1 on GVBD are not mediated at the transcription level of crucial genes regulating the ovulatory cascade; NGR1 treatment only reduced EGFR and increased NPR2 mRNA levels after the effect on GVBD was observed. It is, however, fair to speculate that even without impacting GVBD these changes may have attenuated the rhythm of cumulus-oocyte communication loss over culture, thus contributing for enhanced oocyte developmental competence.

The major findings of the present study concern the effects of NRG1 supplementation during IVM on post-IVF embryo develop- ment. Although NRG1 did not alter total blastocyst rate, it did in- crease the rates of expanded and hatched blastocysts, both in relation to total oocytes subjected to IVM/IVF and total embryos produced, implying in an approximately 25% increase in the pro- duction of transferable embryos (non-expanded blastocysts recovered on day 7 are usually discarded) [43e45]. In addition, in relation to the control group, IVM with NGR1 generated embryos with a higher number of blastomeres, which has been often utilized as an indicator of embryo developmental competence [26]. These findings agree with a previous study in which treatment with NRG1 (20 ng/mL) during EGF-induced IVM increased blastocyst rates in pigs [22].

Although the addition of NRG1 to the IVM medium did not alter the degree of cumulus expansion in the present study, it did in- crease the abundance of PTX3 (pentraxin 3) and TNFAIP6 (tumor necrosis factor-inducible gene 6 protein) mRNA in cumulus cells, two genes encoding proteins essential for the structural organiza- tion of the extra-cellular matrix. The stimulatory effect of NRG1 on TNFAIP6 transcription is consistent with previous findings in the mouse [19]. Interestingly, it is thus possible that NRG1 regulates the quality of the extracellular matrix without promoting observable alterations in the magnitude of the expanded cumulus. This is suggested by our previous study in which increased expression of TNFAIP6 induced by fibroblast growth factor 2 (FGF2) was associ- ated with decreased cohesion of the cumulus matrix in bovine COCs subjected to IVM [46].

Conclusion

In conclusion, we report for the first-time evidence that NRG1 modulates oocyte nuclear maturation in mono-ovulatory mammals and, more importantly, that NRG1 may be utilized in IVM to enhance oocyte developmental competence, thus improving post- IVM/IVF embryo development in cattle. Therefore, while contrib- uting to a better understanding of oocyte biology, the present data provide novel and valuable references for the improvement of IVM/IVF practice.

Author's contributions

TTD: experiments, data analysis and manuscript writing; RAV and LCZJ: contribution during the experiments; MDC, MMR, VL, and AML: data interpretation and manuscript review; JB study coordi- nation, data analysis and manuscript preparation.

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Declaration of competing interest

The authors declare that they have no conflicts of interest.

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514

515 **Figure legend**

516

517 **Fig. 1.** Effects of NRG1 during AREG-stimulated IVM on meiosis progression. COCs were
518 cultured in the follicular system-IVM medium supplemented with 0 (Control), 1, 10, 100 ng/
519 mL NRG1 for 6, 9, 12, 20 or 24 h. GV: germinal vesicle (immature oocyte); GVBD: germinal
520 vesicle breakdown; MI: metaphase I; MII: metaphase II. (means ± SEM; n 1⁄4 5). Different
521 letters indicate statistically significant differences ($P < 0.05$).

522

523 **Fig. 2.** Effects of NRG1 during AREG-stimulated IVM on mRNA abundance in cumulus cells of
524 genes involved in the regulation of oocyte maturation. COCs were cultured in the follicular
525 system-IVM medium without additives (Control) or supplemented with 1 ng/mL NRG1 for a)
526 6 h, b) 9 h or c) 20 h (Data indicate fold change of mRNA levels relative to reference genes
527 H2AFZ and PPIA; n 1⁄4 5). *Statistically significant differences ($P < 0.05$).

528

529 **Fig. 3.** Effects of NRG1 during AREG-stimulated IVM on cumulus expansion. COCs were
530 cultured in the follicular system-IVM medium without additives (Control) or supplemented
531 with 1 ng/mL NRG1. a) Representative images of Control and NRG1 groups after 24 h of IVM.
532 b) Percentage of COCs with complete or nearly complete expansion (mean ± SEM, n 1⁄4 5).
533 Messenger RNA levels of genes involved in cumulus expansion after 9 h (c) or 20 h (d) of IVM
534 (Data indicate fold change of mRNA levels relative to reference genes H2AFZ and PPIA; n 1⁄4
535 5) *Statistically significant differences ($P < 0.05$).

536

Fig. 4. Effects of NRG1 during AREG-stimulated IVM on embryo production and quality. COCs were cultured in the follicular system-IVM medium without additives (Control) or supplemented with 1 ng/mL NRG1. a) Blastocyst production rate in relation to total oocytes. b) Expanded and hatched blastocysts in relation to total blastocysts. c) Expanded and hatched blastocysts in relation to total oocytes (mean ± SEM, n 1⁄4 5). d) Representative images (400 times magnification) of blastocysts evaluated by fluorescence microscopy to assess total cell number. *Statistically significant differences ($P < 0.05$).

Tables:

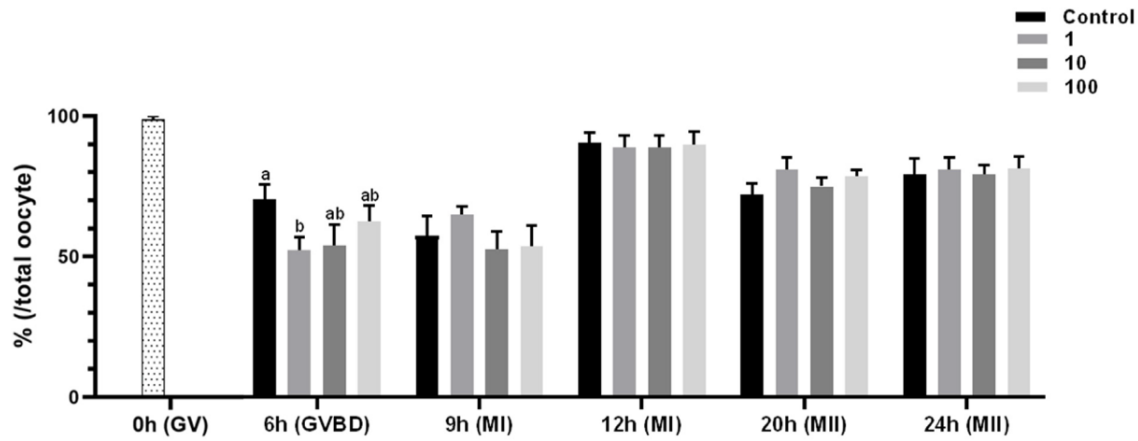
Table 1

Genes analyzed in cumulus cells samples by RT-qPCR.

GENE	SEQUENCE	CATEGORY	ID NUMBER	REFERENCE
PPIA	F: 5′-GCC ATG GAG CGC TTT GG-3′ R: 5′-CCA CAG TCA GCA ATG GTG ATC T-3′	Reference	NM_178320.2	[31]
H2AFZ	F: 5′-GAG GAG CTG AAC AAG CTG TTG-3′ R: 5′-TTG TGG TGG CTC TCA GTC TTC-3′	Reference	BC109743.1	[31]
FSHR	F: 5′-AGC CCC TTG TCA CAA CTC TAT GTC-3′ R: 5′-GTT CCT CAC CGT GAG GTA GAT GT-3′	Cell growth regulation	NM_174061.1	[47]
EGFR	F: 5′-AAA GTT TGC CAA GGG ACA AG-3′ R: 5′-AAA GCA CAT TTC CTC GGA TG-3′	Cell growth regulation	XM_002696890.5	[47]
PTX3	F: 5′-CCT CAG CTA TCG GTC CAT AA-3′ R: 5′-ATT GAA GCC TGT GAG GTC TGC-3′	*Cumulus* expansion	NM_001076259.2	[47]
COX2	F: 5′-AAG CCT AGC ACT TTC GGT GGA GAA-3′ R: 5′-TCC AGA GTG GGA AGA GCT TGC ATT-3′	*Cumulus* expansion	NM_174445.2	[25]
HAS2	F: 5′-ACA CAG ACA GGC TGA GGA CAA CTT-3′ R: 5′-AAG CAG CTG TGA TTC CAA GGA GGA-3′	*Cumulus* expansion	NM_174079.2	[25]
TNFAIP6	F: 5′-GCA AAG GAG TGT GGT GGT GTG TTT-3′ R: 5′-ACT GAG GTG AAT GCG CTG ACC ATA-3′	*Cumulus* expansion	NM_001007813.2	[25]
AREG	F: 5′-CTT TCG TCT CTG CCA TGA CCT T-3′ R: 5′-CGT TCT TCA GCG ACA CCT TCA-3′	Meiotic resumption	NM_001099092.1	[25]
EREG	F: 5′-ACT GCA CAG CAT TAG TTC AAA CTG A-3′ R: 5′-TGT CCA TGC AAA CAG TAG CCA TT-3′	Meiotic resumption	XM_019962926.1	[25]
NPR2	F: 5′-ATG ACA GCA TCA ACC TGG ACT GGA-3′ R: 5′-AGC ACG AAA CGA CTA TCC ACC ACA-3′	Meiotic arrest	NM_174126.2	[48]

F: forward primer; R: reverse primer.

551 FIGURE 1



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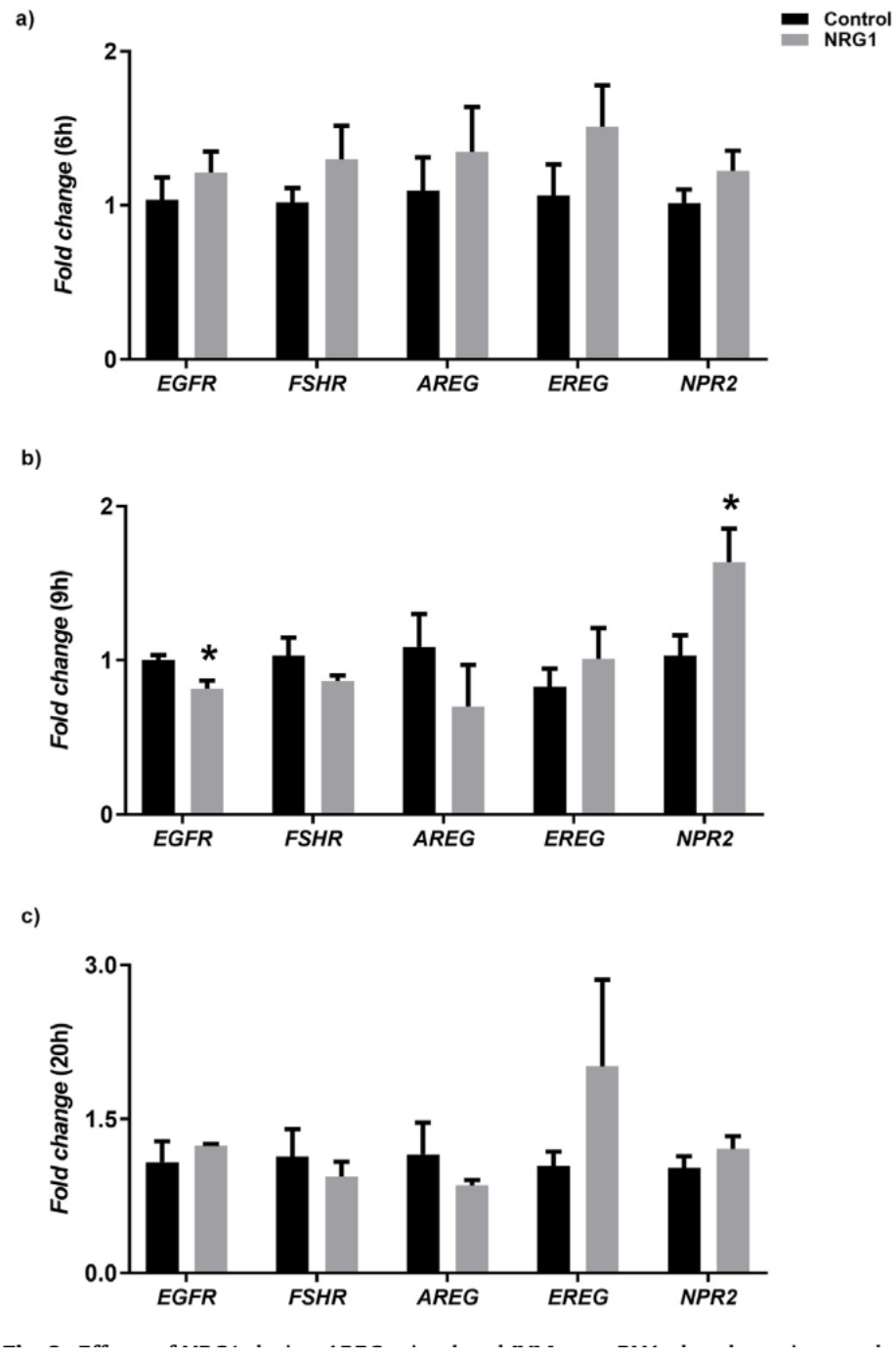
555 FIGURE 2



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559 FIGURE 3

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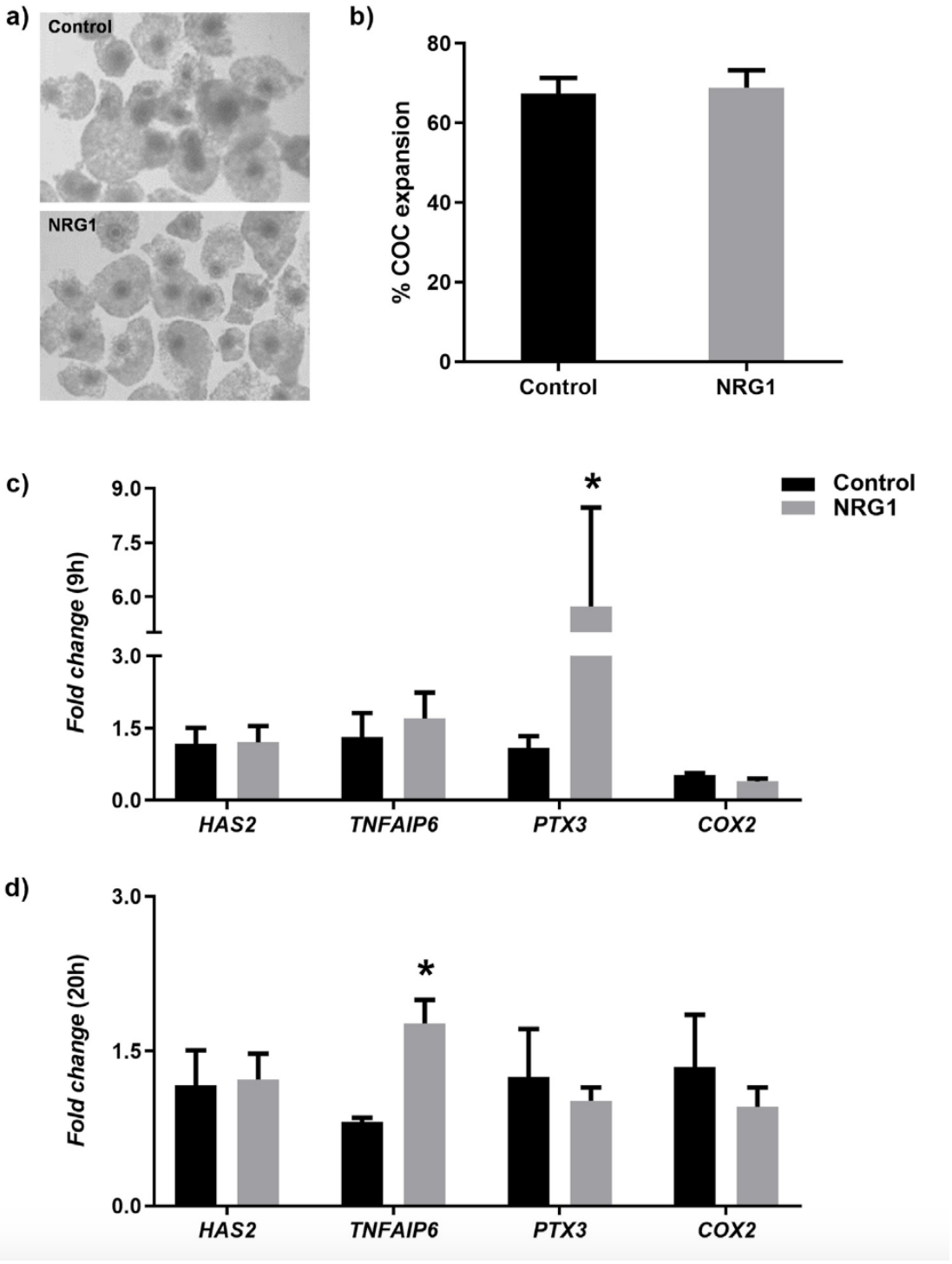

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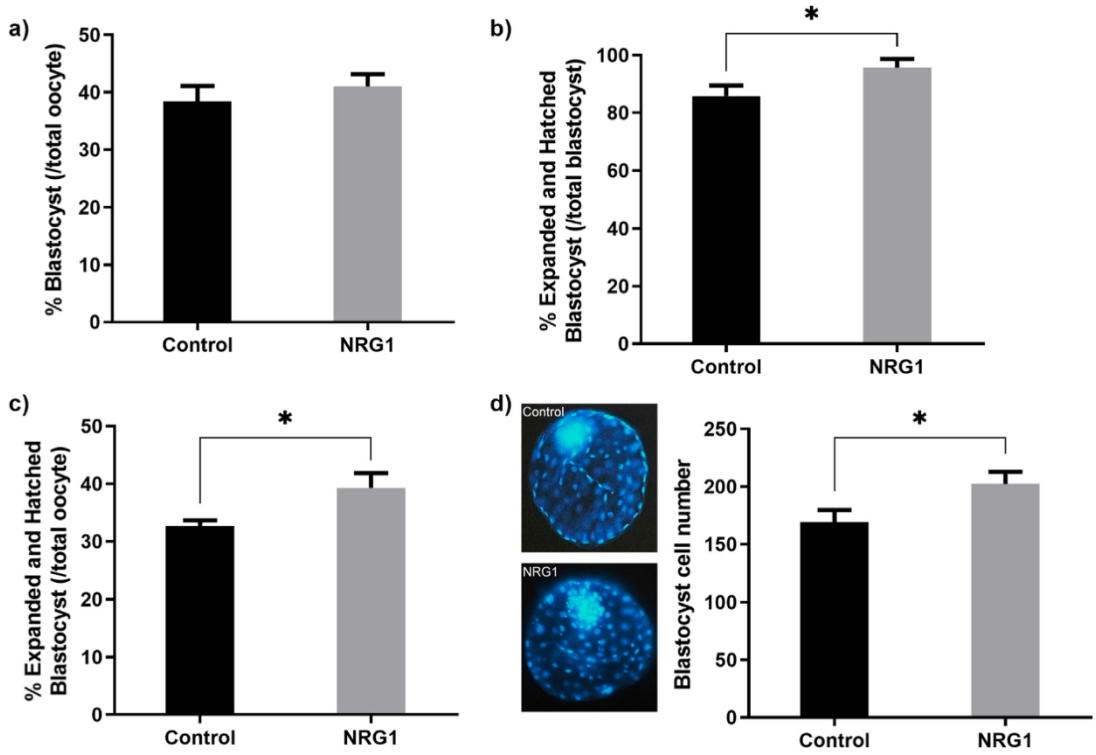
564 FIGURE 4



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