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1 The axonal guidance factor Netrin-1 as a potential
2 modulator of swine follicular function

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9
10 ABSTRACT

11 This study was aimed to improve knowledge about swine ovarian follicular
12 function, paying attention to angiogenesis, since new vessel growth is a
13 fundamental event in ovarian function. In particular, we investigated a potential
14 involvement of netrin-1, a protein known as a guidance axon factor.

15 Firstly, we studied the expression and immunolocalization of netrin-1 in swine
16 ovarian follicle and its effect on cultured swine granulosa cell viability and
17 steroidogenesis. Furthermore, aortic endothelial cells were employed to verify a
18 possible netrin-1 effect on angiogenesis.

19 Our data demonstrate the expression and the presence of netrin-1 in swine
20 follicular fluid; in addition, it was shown that netrin-1 inhibits granulosa cell
21 viability and estradiol 17 β levels while it stimulates progesterone production.
22 Netrin-1 also inhibits aortic endothelial cell growth in the angiogenesis bioassay.

1 This effect appears to be mediated by inhibiting Vascular Endothelial Growth
2 Factor and stimulating Nitric Oxide.

3 Therefore, we hypothesize that netrin-1 could be important for follicular
4 function in the swine.

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1 1. Introduction

2 Netrins are a family of highly conserved secreted proteins structurally related to
3 laminins. They were named after the Sanskrit word "netr", which means "one
4 who guides", since they possess guidance cues in axon pathfinding. Recent
5 studies report the involvement of netrins in other developmental process,
6 including the formation of vessels (Cirulli and Yebra 2007). Both neuronal and
7 vascular development require guidance to establish a precise branching pattern
8 and it has been shown that several molecules implicated in axonal navigation
9 have a similar role in regulating vessel sprouting (Larrivéé et al., 2009). In
10 particular, netrin-1 has been related to the fine tuning of the angiogenic
11 process and published data demonstrate pro- and anti-angiogenic effects (Park
12 et al., 2004; Larrivéé et al., 2007; Bouvrée K et al., 2008; Castets M et al.,
13 2009). Maeda et al. (2008) recently demonstrated that netrin-1 is expressed in
14 swine granulosa cells suggesting that the protein could be involved in the
15 modulation of ovarian follicle function. In particular, netrin-1 could play a role in
16 the regulation of the marked cyclical angiogenesis which takes place in the
17 developing follicle and represents an unvaluable physiological model for the
18 investigation of the molecular events responsible for new vessel growth.

19 On these bases, the present study was undertaken to explore the hypothesis
20 that netrin-1 could act as a physiological modulator of swine follicular function.

21 The expression and the immunolocalization of netrin-1 in swine ovarian follicle
22 were explored and the effect of different protein concentrations on the main
23 parameters of swine granulosa cell function were examined. Moreover, an

1 angiogenesis bioassay set up in our laboratory (Basini et al., 2008a) was
2 employed to evaluate the effect of netrin-1 on new vessel growth.

3

4 2. Materials and Methods

5

6 All reagents used in this study were obtained from Sigma (St. Louis, MO, USA)
7 unless otherwise specified.

8

9 *2.1 Collection of ovaries*

10 Swine ovaries were collected at a local slaughterhouse from Large White cross-
11 bred gilts, placed into cold PBS (4°C) supplemented with penicillin (500 IU/ml),
12 streptomycin (500 µg/ml) and amphotericin B (3.75 µg/ml), maintained in a
13 freezer bag and transported to the laboratory within 1 h.

14

15 *2.2 Netrin-1 in swine ovarian follicle*

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17 *2.2.1 Immunohistochemistry*

18 Ovaries were placed in 10% buffered formalin and paraffin embedded.
19 Following routine processing, 6 µm-thick sections were placed on polylysine-
20 treated slides and stained with a polyclonal antiserum raised against a peptide
21 corresponding to 501-604 amino acid sequence of human netrin-1 (Santa Cruz
22 Biotechnology, Santa Cruz, California). Briefly, sections were blocked in 10%
23 normal swine serum for 20 min (200 µl) and then the primary antibody was

1 applied (250 μ l of a solution diluted 1:100 in PBS) and incubated overnight at 4
2 $^{\circ}$ C. After rinsing in PBS, the slides were incubated for 15 minutes with 200 μ l of
3 a biotinilated secondary antibody. After three 5-min washes in PBS, 200 μ l of a
4 solution of streptavidin-peroxidase complex (LSAB[®]+SYSTEM-HRP, Dako,
5 Glostrup, Denmark) was applied for 15 min. Positive reactions were revealed by
6 aminoethylcarbazole (AEC) (Dako, Glostrup, Denmark). Sections were washed
7 three times for 5 min with PBS and counterstained with Gill's ematoxylin for 2
8 min. Control sections were subjected to the same procedure, except that
9 diluted swine serum replaced the primary antibody.

10

11 *2.2.2 Western Blotting of follicular fluid protein content*

12 Follicular fluid was collected with a 26-gauge from follicles >5mm (Basini et al.,
13 2008b), centrifuged (300 \times g; 10 min) to separate rapidly the fluid from the cell
14 fraction and refrigerated at -80 $^{\circ}$ C until assayed. As a positive control, a sample
15 of soluble protein extract from porcine spinal marrow was also prepared. To
16 this aim few grams of tissue were collected from a freshly slaughtered pig and
17 homogenized by Ultra-Turrax (one stroke of 30 seconds at maximum speed) in
18 20 mM Tris/HCl pH 7.8 (1 ml of buffer/gram of tissue). The suspension was
19 centrifuged (18,000Xg) for 5 minutes and the supernatant was stored -80 $^{\circ}$ C.

20 The protein pattern of follicular fluid and spinal marrow samples were resolved
21 by 10% sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-
22 PAGE) according to Laemmli (1970). After electrophoresis, the protein bands
23 were transferred onto a ProBlott membrane (Applied Biosystem, Carlsbad,

1 California) (Towbin et al., 1979) that was blocked with 5% skimmed dry milk
2 dissolved in 20 mm Tris / HCl buffer, pH 7.8 containing 150 mm NaCl, and
3 0.01% (w / v) Tween 20 (TTBS). After washing with TTBS the membrane was
4 incubated overnight with an anti-netrin-1 polyclonal antibody (Santa Cruz
5 Biotechnology, Santa Cruz, California) raised in rabbit diluted (1:50) in TTBS
6 containing 1% (w / v) skimmed dry milk. After three washes in TTBS the
7 membrane was incubated 45 min. with a solution of a biotinilated anti-rabbit
8 IgG antibody (LSAB®+SYSTEM-HRP, Dako, Glostrup, Denmark) diluted 1:5 in
9 TTBS. A solution of complex of streptavidin-peroxidase was then applied for 15
10 min. and positive reactions were revealed by AEC. A replicate SDS-PAGE gel
11 was stained with Comassie blue.

12

13 *2.2.3 RNA extraction and RT-PCR*

14 Follicles >5 mm were dissected from the ovaries according to Basini et al.
15 (2010). Each follicle was cut open, granulosa cells were separated by means of
16 using a siliconized Pasteur pipette and centrifuged at $300 \times g$ for 10 min. Theca
17 tissue was peeled from each follicle using a forceps, deep frozen in liquid
18 nitrogen and powdered in a mortar.

19 Total RNA was extracted using Nucleospin ® RNA II (Macherey-Nagel GmbH,
20 Duren, Germany) according to the manufacturer's instructions. Total RNA was
21 quantified by absorbance at 260 nm (Gen Quant Pro, Amersham Biosciences,
22 Freiburg, Germany). Total RNA (2 µg) was reverse transcribed with Ready-to-
23 Go You Prime First-Strands Beads (Amersham Biosciences, Freiburg, Germany).

1 Netrin-1 cDNA was PCR amplified in parallel with β actin cDNA as an internal
2 positive control. For Netrin-1, the sense primer (5'-GAG ACC TTC AAC ACG
3 CCG-3') and antisense primer (5'-GGA AGG TGG ACA GCG AGG-3') (MWG
4 Biotec, Ebersberg, Germany) were derived from Gene Bank sequence of porcine
5 Netrin-1 (accession number DQ368597). Swine actin was amplified using the
6 primers pACTIN sense (5'- GAG ACC TTC AAC ACG CCG-3') and pACTIN
7 antisense (5'-GGA AGG TGG ACA GCG AGG-3') (MWG Biotec, Ebersberg,
8 Germany). An aliquot (5 μ l) of the cDNA template was amplified by PCR using
9 0.5 μ l (500 mU) Taq polymerase (Fermentas, Hannover, MD, USA) in 50 μ l PCR
10 buffer containing 10 mM dNTP mix (Fermentas), 25 μ M for Netrin-1 primers
11 and 5 μ M for Actin primers . Amplification was carried out using the thermal
12 cycler PTC-100 Peltier (MJ Research, San Francisco, CA, USA). After an initial
13 denaturation step for 5 min at 95°C, target cDNA was amplified for 3 cycles
14 with a denaturation step at 95 °C for 30 sec, annealing at 60 °C for 30 sec (-
15 1°C in the second and third cycle), elongation at 72 °C for 30 sec, and for 35
16 cycles with a denaturation step at 95 °C for 30 sec, annealing at 57 °C for 30
17 sec, elongation at 72 °C for 30 sec. Reactions were terminated with a final
18 elongation at 72 °C for 10 min. The PCR products were separated on 1.5%
19 agarose gel stained with ethidium bromide (0.5 μ g/ml) and visualized under UV
20 light. Digital images were captured by Power Shot A610 photo camera (Canon,
21 Tokyo, Japan). Quantification of band intensity was performed with NIH Image
22 software (Scion Corporation, MA, USA, <http://rsb.info.nih.gov/nih-image/>).

23

1 *2.3 Netrin-1 effects on swine granulosa cell functions*

2

3 *2.3.1 Granulosa cell culture*

4 Granulosa cells were aseptically harvested by aspiration of follicles with a 26-
5 gauge needle, released in medium containing heparin (50 IU/ml), centrifuged
6 for pelleting and then treated with 0.9% prewarmed ammonium chloride at
7 37°C for 1 min to remove red blood cells. Cell number and viability were
8 estimated using a haemocytometer under a phase contrast microscope after
9 vital staining with trypan blue (0.4%) of an aliquot of the cell suspension. Cells
10 were seeded in DMEM/Ham's F12 supplemented with sodium bicarbonate (2.2
11 mg/ml), bovine serum albumin (BSA 0.1%), penicillin (100 IU/ml), streptomycin
12 (100 µg/ml), amphotericin B (2.5 µg/ml), selenium (5 ng/ml) and transferrin (5
13 µg/ml). Once seeded, cells were incubated for 48 h at 37 °C under humidified
14 atmosphere (5% CO₂) in the presence or absence of Netrin-1 at the
15 concentration of 5, 30 and 100 ng/ml. The protein was produced as reported by
16 Serafini et al., 1994. Briefly, cDNAs encoding chick Netrin-1 tagged with a c-
17 myc epitope at its C-terminus and fused to the constant (Fc) region of the
18 human IgG1, were subcloned into the expression vector pCEP4 (Invitrogen,
19 Carlsbad, California) and used to transfect 293-EBNA cells (Invitrogen,
20 Carlsbad, California). Protein was finally purified from conditioned media by
21 heparin affinity chromatography to 85-90% homogeneity, as assessed by silver
22 staining (Keini-Masu et al., 1996).

23

1 *2.3.2 Granulosa cell viability*

2 2×10^5 cells were seeded in 96-well plates in 200 μ l CM. Cell proliferation was
3 assayed using a bioluminescent assay (ATP-lites; Packard Bioscience,
4 Groningen, Netherlands) which measured intracellular ATP levels. ATP, being
5 present in all metabolically active cells, is a viability marker whose
6 concentration declines very rapidly when the cells undergo necrosis or
7 apoptosis. The ATP lite-M assay system is based on the production of light
8 caused by the reaction of ATP with added luciferase and D-luciferin. The
9 emitted light is proportional to the ATP concentration. Briefly, 50 μ l of
10 mammalian cell lysis solution were added to 100 μ l of cell suspension and the
11 plate was shaken for 5 minutes in an orbital shaker at 700 rpm in order to lyse
12 the cells and stabilize ATP. Then 50 μ l of substrate solution were added to the
13 wells and the microplate was shaken for 5 minutes in an orbital shaker at 700
14 rpm. The plate was placed in the dark for 10 minutes and the luminescence
15 was measured in a luminometer (Victor, Packard Bioscience, Groningen,
16 Netherlands).

17

18 *2.3.3 Granulosa cell steroid production.*

19 10^4 cells/well were seeded in 96-well plates in 200 μ l of CM supplemented with
20 androstenedione (28 ng/ml). Culture media were then collected, frozen and
21 stored at -20 °C until progesterone (P4) and estradiol 17β (E2) were
22 determined by a validated Radio Immuno Assays (Grasselli et al., 1993). P4
23 assay sensitivity and ED_{50} were 0.24 and 1 nmol/l, respectively; E2 assay

1 sensitivity and ED_{50} were 0.05 and 0.2 nmol/l. The intra- and inter-assay
2 coefficients of variation were less than 12% for both assays.

3

4 *2.4 Netrin-1 effects on swine aortic endothelial cell functions*

5

6 *2.4.1 Aortic endothelial cell culture*

7 An immortalized porcine aortic endothelial cell line (AOC) (Carrillo et al. 2002)
8 was generously provided by José Yelamos (Hospital Universitario Virgen de la
9 Arrixaca, El Palmar, 30120 Murcia, Spain). Cells were cultured in M199 medium
10 supplemented with sodium bicarbonate (2.2 mg/ml), FCS 20%, penicillin (100
11 IU/ml), streptomycin (100 μ g/ml), amphotericin B (2.5 μ g/ml). In all
12 experiments, AOC at 19th passage were used.

13

14 *2.4.2 Angiogenesis bioassay*

15 The microcarrier-based fibrin gel angiogenesis assay was performed as
16 described by Basini et al. (2008) with some modifications. Briefly, 12.5 mg
17 gelatin-coated cytodex-3 microcarriers in 1 ml PBS were incubated for 3 h to
18 hydrate. After two washings in PBS and one in CM, the microcarriers were put
19 in flasks containing 5 ml CM; AOC (5×10^5) were added and cultured for 24 h in
20 order to let the endothelial cells coat the microcarriers. For the fibrin gel
21 preparation, 40 μ l microcarriers covered by AOC were pipetted into 6 well
22 plates containing a solution of fibrinogen (1mg/ml PBS, pH 7.6), added with
23 1250 IU thrombin (250 μ l). Fibrin gels were allowed to polymerize for 30 min

1 at 37°C, and then were equilibrated for 60 min with 2 ml M199. After a change
2 of the medium, AOC were incubated in the presence or absence of 5, 30 or 100
3 ng/ml of Netrin-1. Plates were incubated at 37°C under humidified atmosphere
4 (5% CO₂). AOC were cultured for 96 h, renewing totally the treatment after 48h
5 as described above.

6

7 *2.4.3 Quantification of AOC growth on fibrin gel matrix*

8 Endothelial cell proliferation in the fibrin gel matrix was evaluated by means of
9 the public domain NIH Program Scion Image Beta 4.02. Ten pictures were
10 taken for each gel at 48 and 96 h; images were converted into gray scale,
11 resized to 50% (Paintbrush Software, MS Office) and saved as Bitmap 24bit
12 format compatible with Scion. The modified images were then imported into the
13 program and measurements were made drawing the perimeter of the area
14 occupied by AOC expressed as number of pixel. In order to validate the
15 measurement of the area covered by AOC in fibrin gels as a reliable method to
16 evaluate cell proliferation, fibrin gels were stained by the nuclear dye bis-
17 benzimide (Hoechst 33258, 20 µg/ml in PBS for 60 min) and examined by the
18 fluorescence microscope. This procedure was performed 20 times; for each
19 experiment the number of nuclei was counted under fluorescence and pictures
20 of the area covered by AOC were taken in order to measure the surface
21 covered in the fibrin gel. A strong correlation was observed between the area
22 covered by AOC and the number of nuclei found in the same area ($r= 0.96$).

23

1 *2.4.5 Aortic endothelial cell VEGF production*

2 2×10^5 cells were seeded in 96-well plates in 200 μ l M199, in the presence or
3 absence of 5, 30 or 100 ng/ml of Netrin-1 and incubated for 48 h. VEGF content
4 in culture media was quantified by an ELISA (Quantikine, R&D System,
5 Minneapolis, MI, USA); this assay, developed for human VEGF detection, has
6 been validated for pig VEGF (Barboni et al. 2000). The assay sensitivity was
7 8.74 pg/ml, the inter- and intra-assay CVs were always less than 7%. Victor
8 Reader set to read at a wavelength of 450 nm emission was used to quantify
9 the reaction product.

10

11 *2.4.6 Aortic endothelial cell NO production*

12 2×10^5 cells were seeded in 96-well plates in 200 μ l M199, in the presence or
13 absence of 5, 30 or 100 ng/ml of Netrin-1 and incubated for 48 h. Nitric oxide
14 (NO) was assessed by measuring nitrite levels in culture media by microplate
15 method based on the formation of a chromophore after reaction with Greiss
16 reagent, which was prepared fresh daily by mixing equal volumes of stock A
17 (1% sulfanilamide, 5% phosphoric acid) and stock B (0.1% N-[naphthyl]
18 ethylenediamine dhydrochloride). After incubation with Greiss reagent the
19 absorbance was determined with the Victor Reader using a 540 nm against 620
20 nm filter. A calibration curve ranging from 25 to 0.39 μ M was prepared by
21 diluting sodium nitrite in culture medium.

22

23 *2.5 Statistical analysis.*

1 Data are presented as means \pm SEM. Statistical analysis was performed by
2 means of ANOVA using Statgraphics package (STSC Inc., Rockville, MD, USA).
3 When significant differences were found, means were compared by Scheffè's F
4 test. P values < 0.05 were considered to be statistically significant.

5

6 3. Results

7 *3.1 Netrin-1 in swine ovarian follicle*

8

9 *3.1.1 Immunohistochemistry*

10 Immunostaining of Netrin-1 in swine ovarian follicle wall was undetectable. On
11 the contrary, immunoreaction was localized in the ovarian vessels (Figure 1 b).

12

13 *3.1.2 Follicular fluid Western Blotting*

14 The immunoblotting showed the presence of Netrin-1 in swine follicular fluid
15 (Figure 2). The band corresponding to Netrin-1, in fact, was resolved at the
16 expected molecular weight of about 70 kDa both in follicular fluid and in a
17 positive control consisting of a soluble protein extract from porcine spinal
18 marrow.

19

20 *3.1.3 Netrin-1 gene expression*

21 Netrin-1 expression was evidenced both in granulosa and theca layer from
22 swine follicles (Figure 3).

23

1 *3.2 Netrin-1 effects on swine granulosa cell functions*

2

3 *3.2.1 Granulosa cell proliferation*

4 Granulosa cell viability, evaluated as ATP content, was slightly but significantly
5 ($p < 0.05$) inhibited by the treatment with 30 and 100 ng/ml of Netrin-1 for 48 h.

6 The lowest concentration of Netrin-1 tested, 5 ng/ml, was ineffective (Figure 4).

7

8 *3.2.2 Granulosa cell steroid production.*

9 Granulosa cell steroid production, evaluated by RIA assays, was affected by
10 Netrin-1 treatment for 48 h. E2 production was significantly inhibited ($p < 0.01$)
11 by 30 and 100 ng/ml of Netrin-1 (Figure 5); the same concentrations, on the
12 contrary, stimulated ($p < 0.001$) P4 production (Figure 6). The lowest
13 concentration tested, 5 ng/ml, was ineffective on both steroid secretions
14 (Figure 5 and 6).

15

16 *3.3 Netrin-1 effects on swine aortic endothelial cell functions*

17

18 *3.3.1 Angiogenesis bioassay*

19 Netrin-1 significantly inhibited ($p < 0.05$) new vessel growth both after 48 and 96
20 h of incubation. All the concentrations tested (5, 30 and 100 ng/ml) exerted the
21 same effect. (Figure 7 and 8).

22

23 *3.3.2 Aortic endothelial cell VEGF production*

1 VEGF secretion by AOC was significantly inhibited ($p < 0.01$) by the 48 h
2 treatment with all the examined concentrations of Netrin-1 (5, 30 and 100
3 ng/ml) (Figure 9).

4

5 *3.3.3 Aortic endothelial cell NO production*

6 All Netrin-1 concentrations (5, 30 and 100 ng/ml) significantly stimulated
7 ($p < 0.01$) NO generation after 48 h of incubation (Figure 10).

8

9 4. Discussion

10 Ovarian physiology is connoted by follicular growth, a fundamental event which
11 is strictly dependent on cell proliferation, steroid production and new vessel
12 growth. The molecular machinery which regulates ovarian angiogenesis, though
13 extensively studied, is far to be completely clarified. In a recent review, Larrivé
14 et al. (2009) observe that anatomical and structural similarities between blood
15 vessels and nerves have been highlighted by several studies. Among the
16 molecules with attractive and repulsive properties which have been found to
17 modulate the proper guidance of both nerves and blood vessels, netrin-1
18 displays both pro- and antiangiogenic effects (Park et al., 2004; Castets et al
19 2009). Interestingly, netrin-1 has been found to be expressed in swine
20 granulosa cells from 3-5 mm ovarian follicles, thus suggesting its potential
21 function in follicular function regulation (Maeda et al., 2008).

22 In the present study netrin-1 was evidenced in ovarian vessels but was
23 undetectable in follicular wall. In addition, we document its expression both in

1 granulosa and theca layer from swine ovarian follicles >5 mm as well as in
2 swine follicular fluid. These data lead us to hypothesize that netrin-1 could play
3 a regulatory role in the function of the ovarian follicle, a structure which
4 represents a typical example of events of proliferation and differentiation.
5 Similarly, Strizzi et al. (2008) demonstrated that mammary gland, a structure
6 subjected to different stages of development and differentiation, expresses
7 netrin-1, which appears to be involved in the fine-tuning of these complex
8 events. In particular, our data show that the protein inhibits granulosa cell
9 viability and E2 synthesis, while it increases P4 production, thus suggesting its
10 involvement in the induction of granulosa cell terminal differentiation. The
11 relationship between growth and differentiation has been a matter of great
12 interest in cellular biology. In granulosa cells, the differentiative function and
13 growth-related process appear inversely controlled (Basini et al., 1998): netrin-
14 1 could play a pivotal role in the paracrine/autocrine regulation of these events.
15 Since follicular growth is strictly dependent on adequate vascular development,
16 this structure has been suggested as an invaluable physiological model of
17 angiogenesis, a process which represents an important aspect of growth and
18 metastasis of solid tumors (Grimm et al 2009). The similarities between nerve
19 and blood vessel guidance have attracted considerable interest towards the
20 study of a potential involvement of neurotropic factors in angiogenesis control
21 (Strizzi et al., 2009).
22 Among these, netrin-1 deserves particular attention even if its exact role in
23 angiogenesis is still doubtful since numerous studies report both pro- or

1 antiangiogenic activities (Bradford et al., 2009). The results obtained in the
2 fibrin bioassay demonstrate an inhibition of angiogenesis by netrin-1. This is in
3 agreement with Lu et al. (2004), but contrary to Park et al. (2004) and Wilson
4 et al. (2006) who suggested that netrin-1 stimulates new vessel growth.
5 Therefore, it appears that netrin-1 function, as already demonstrated in the
6 nervous system (Masuda et al., 2009), could be dependent on receptor type as
7 well as on the intracellular signal transduction pathways activated by each
8 receptor. Further studies will be needed in order to investigate this fundamental
9 topic in our experimental model. Our data also suggest, for the first time, that
10 this effect most likely appears to be mediated by an inhibition of VEGF
11 production by endothelial cells. Moreover, our data are in agreement with
12 Nguyen et al. (2006) demonstrate that netrin-1 appears to increase NO output
13 by endothelial cells. It should be noted that the role of NO in angiogenesis
14 regulation is far from being clarified. Several studies suggest a stimulatory
15 effect (Kimura et al., 2000; Dulak et al., 2000; Sandau et al., 2000) while
16 others point out inhibitory effects (Powell et al., 2000; Santini et al., 2009). The
17 reasons for these discrepancies are still unknown, even if NO levels could play
18 an important role (Kimura et al. 2003).

19 Taken together our data demonstrate that netrin-1 is physiologically present in
20 swine ovary and it is likely involved in modulating follicular function. In
21 particular, data obtained in granulosa cells let us to hypothesize that netrin-1
22 could be important for granulosa cell terminal differentiation; moreover, the
23 observations in endothelial cells seem to suggest its involvement in the control

1 of new vessel growth. Further studies are strongly advisable to address netrin-1
2 function since the molecular machinery responsible for angiogenesis inhibition is
3 far to be clarified.

4

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11

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- 11

Figure captions

1

2

3

4 Figure 1. Immunohistochemical localization of Netrin 1 in swine ovary. A: antral
5 follicles. B: vessel. C: follicle walls. D: corpus luteum. A positive signal has been
6 detected only in vessels (B, see arrows).

7

8 Figure 2. Netrin 1 protein in swine follicular fluid and spinal marrow revealed by
9 immunoblotting. Protein bands on SDS-PAGE gel were stained with Coomassie
10 blue.

11

12 Figure 3. Netrin-1 expression in swine granulosa and theca cells collected from
13 swine follicles. The representative ethidium bromide-stained gel shows ACT and
14 STC 1 amplicons from at least three follicles.

15

16 Figure 4. Effect of the 48 h treatment with or without (C) Netrin-1 at the
17 concentrations of 5, 30 or 100 ng/ml on granulosa cell viability evaluated as
18 ATP content. Data, expressed as % vs respective C, represent the mean \pm SEM
19 of six replicates/treatment repeated in four different experiments. Different
20 letters indicate a significant difference ($p < 0.05$).

21

22 Figure 5. Effect of the 48 h treatment with or without (C) Netrin-1 at the
23 concentrations of 5, 30 or 100 ng/ml on E2 production in swine granulosa cell
24 culture evaluated by RIA. Data, expressed as ng/ml, represent the mean \pm SEM

1 of six replicates/treatment repeated in four different experiments. The absence
2 of common letters indicate a significant difference ($p < 0.01$).

3

4 Figure 6. Effect of the 48 h treatment with or without (C) Netrin-1 at the
5 concentrations of 5, 30 or 100 ng/ml on P4 production in swine granulosa cell
6 culture evaluated by RIA. Data, expressed as ng/ml, represent the mean \pm SEM
7 of six replicates/treatment repeated in four different experiments. Different
8 letters indicate a significant difference ($p < 0.001$).

9

10 Figure 7. Effect of the 48 h treatment with or without (C) Netrin-1 at the
11 concentrations of 5, 30 or 100 ng/ml on AOC growth in fibrin gel. Panel A:
12 phase contrast micrographs showing AOC growth at 48 h in fibrin gel matrix.
13 Panel B: area covered by AOC. Data, expressed as % vs respective C, represent
14 the mean \pm SEM of six replicates/treatment repeated in four different
15 experiments. Different letters indicate a significant difference ($p < 0.05$).

16

17 Figure 8. Effect of the 96 h treatment with or without (C) Netrin-1 at the
18 concentrations of 5, 30 or 100 ng/ml on AOC growth in fibrin gel. Panel A:
19 phase contrast micrographs showing AOC growth at 96 h in fibrin gel matrix.
20 Panel B: area covered by AOC. Data, expressed as % vs respective C, represent
21 the mean \pm SEM of six replicates/treatment repeated in four different
22 experiments. Different letters indicate a significant difference ($p < 0.05$).

23

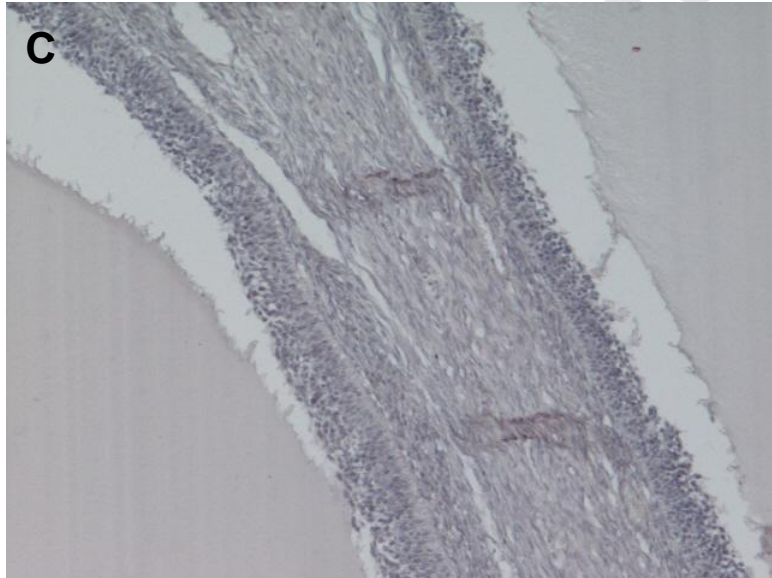
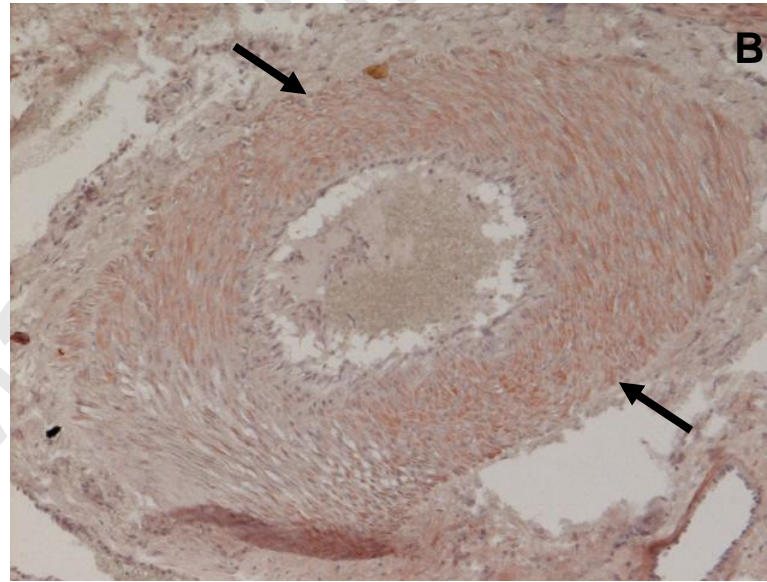
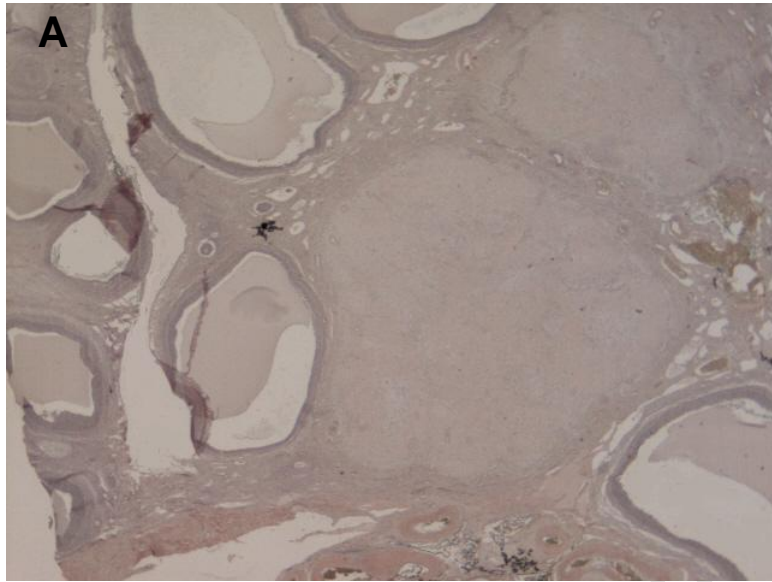
1 Figure 9. Effect of the 48 h treatment with or without (C) Netrin-1 at the
2 concentrations of 5, 30 or 100 ng/ml on VEGF production, evaluated by ELISA,
3 by AOC culture. Data, expressed as pg/ml, represent the mean \pm SEM of six
4 replicates/treatment repeated in four different experiments. Different letters
5 indicate a significant difference ($p < 0.01$).

6

7 Figure 10. Effect of the 48 h treatment with or without (C) Netrin-1 at the
8 concentrations of 5, 30 or 100 ng/ml on NO production, evaluated by Greiss
9 method, by AOC culture. Data, expressed as μM , represent the mean \pm SEM of
10 six replicates/treatment repeated in four different experiments. The absence of
11 common letters indicate a significant difference ($p < 0.01$).

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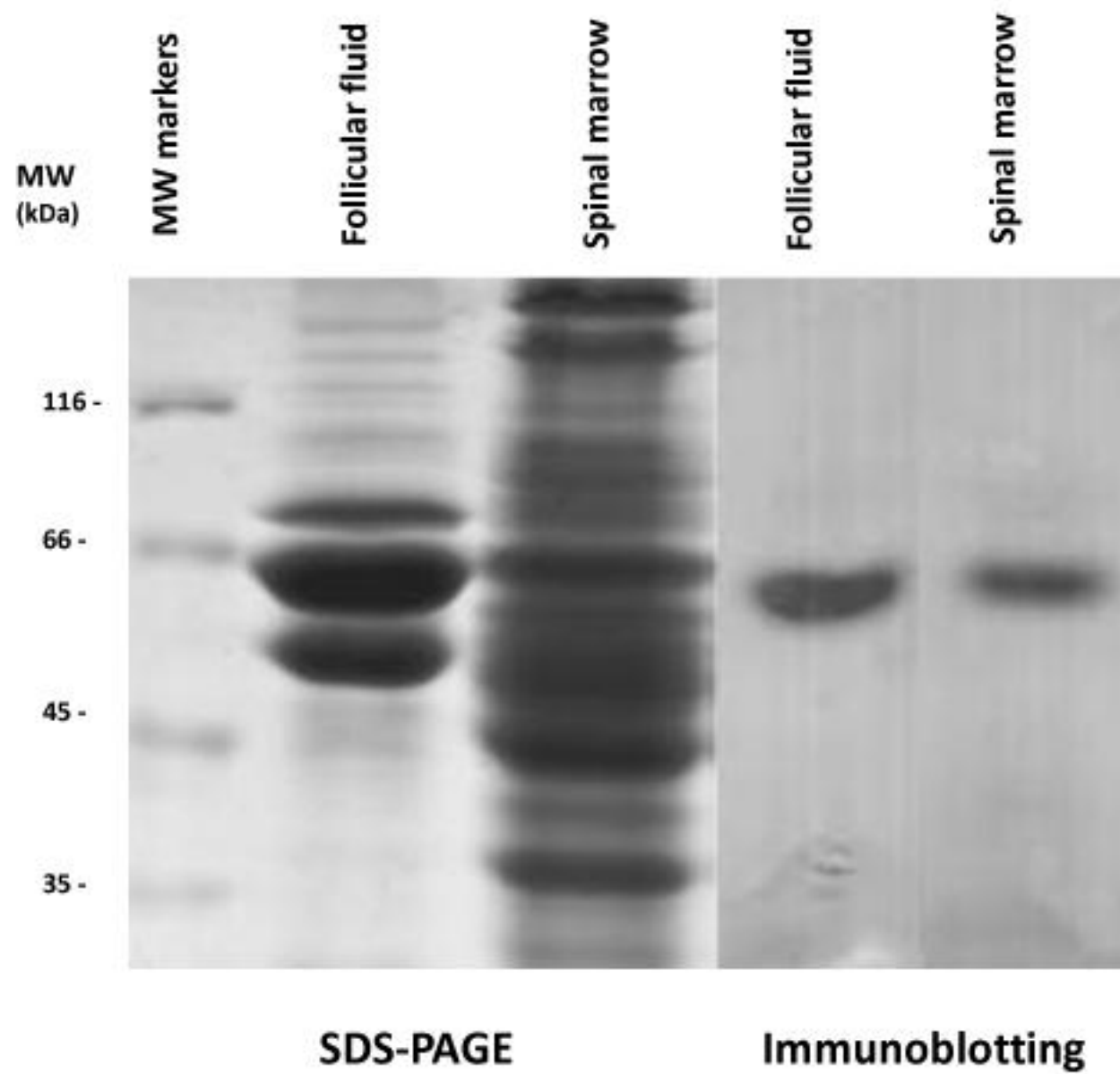
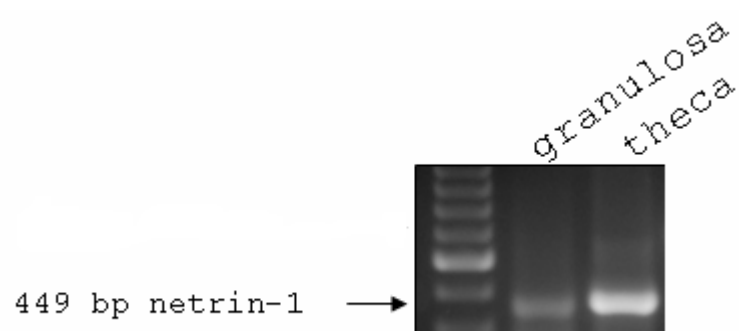


Figure 3



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Figure 4

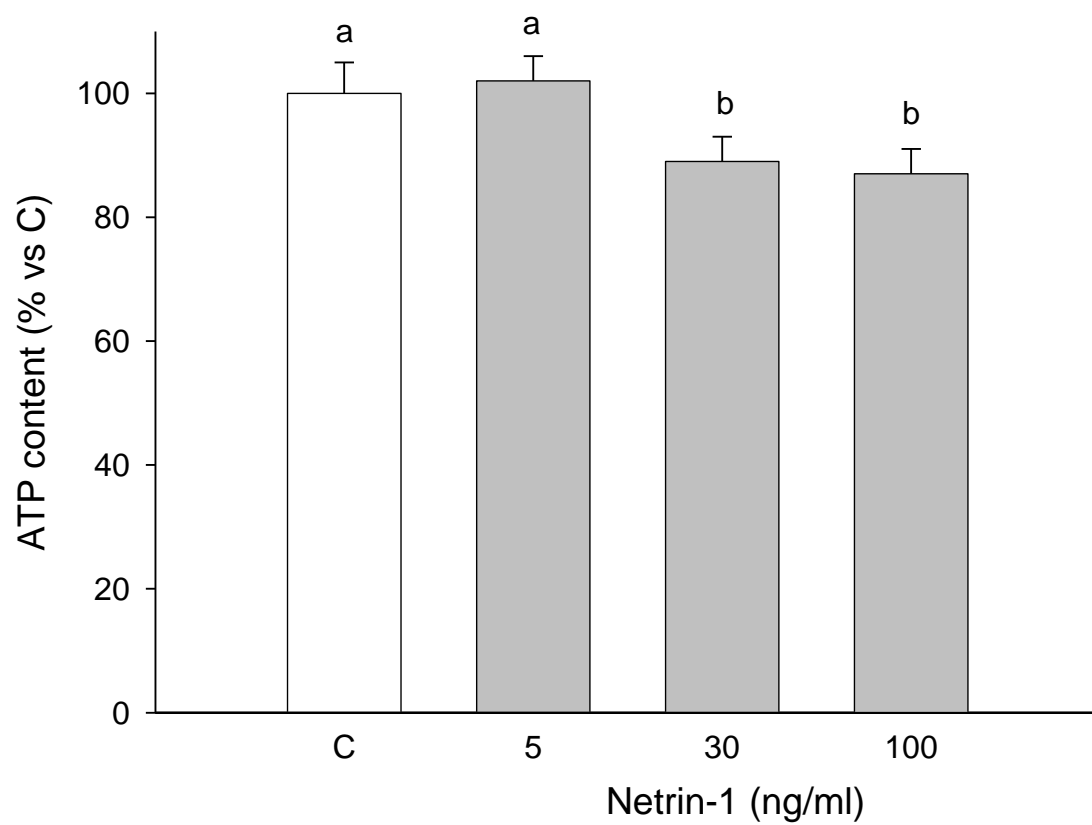


Figure 5

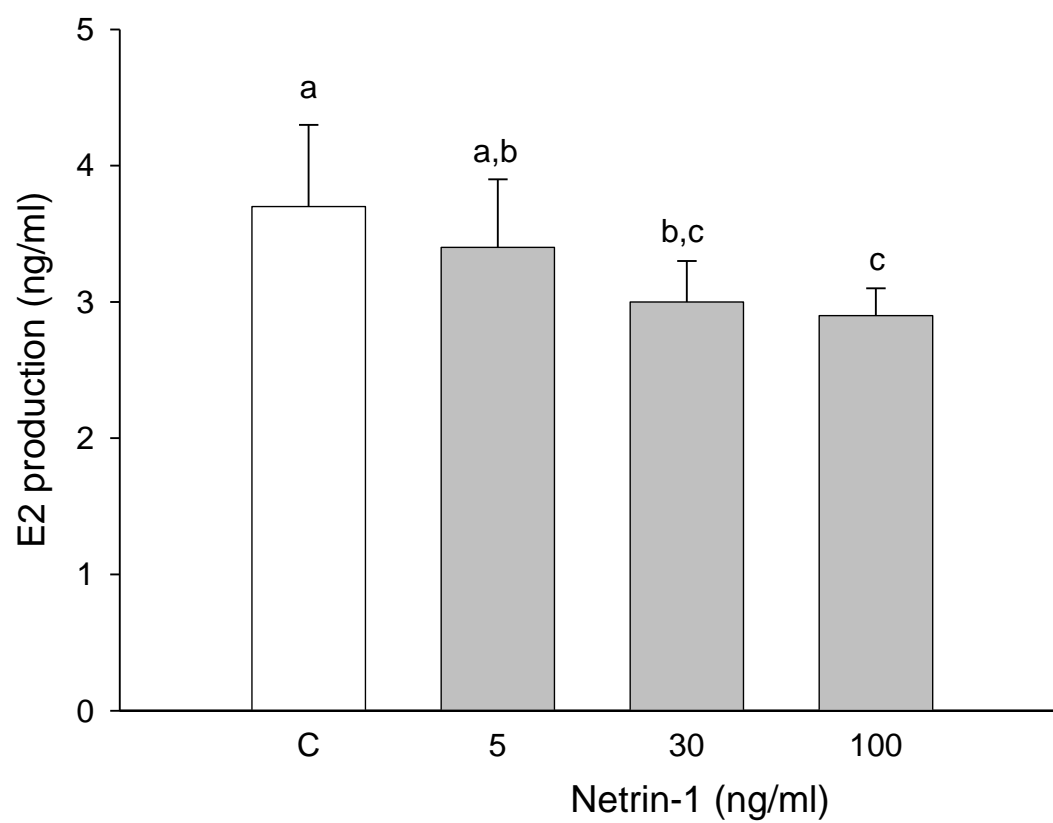


Figure 6

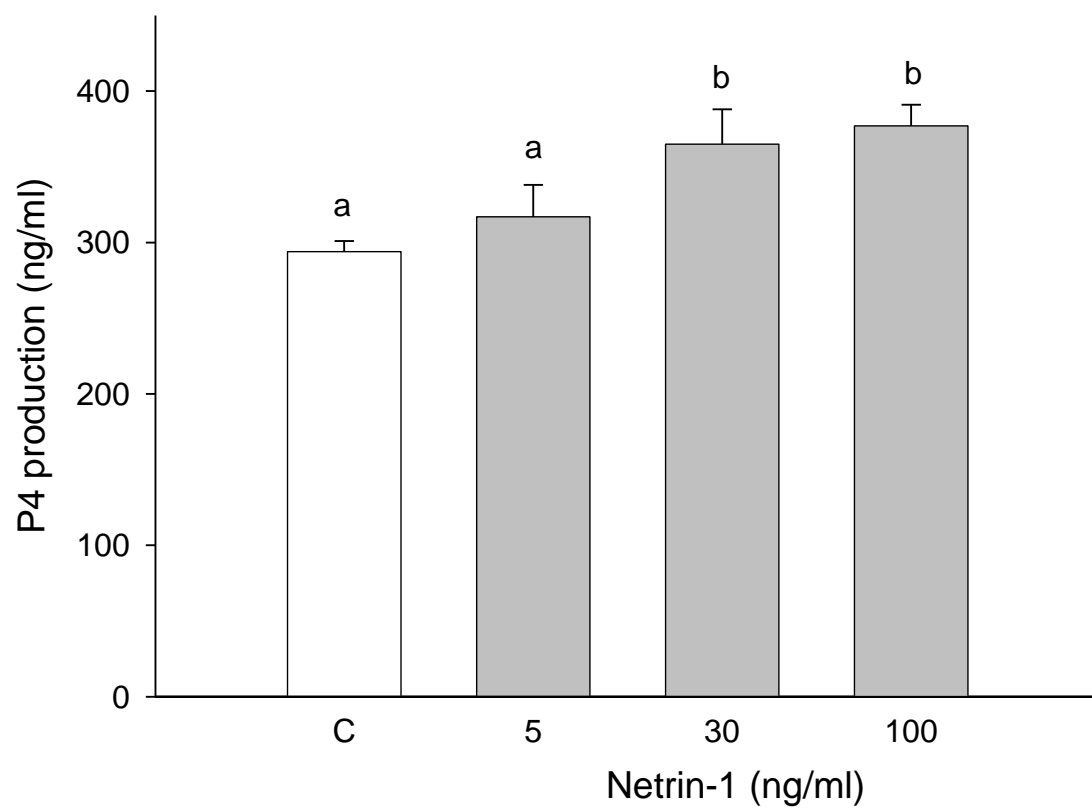


Figure 7

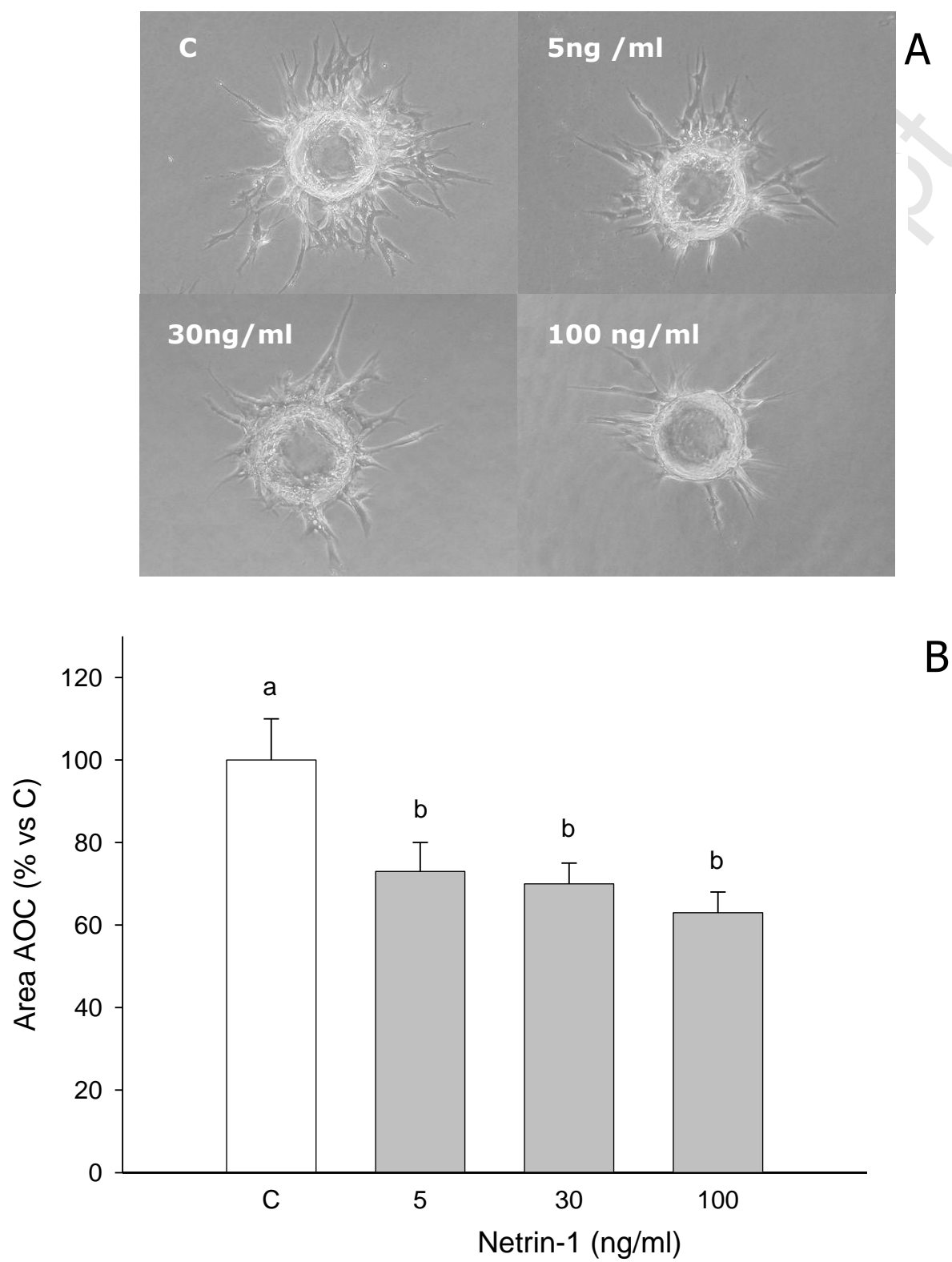


Figure 8

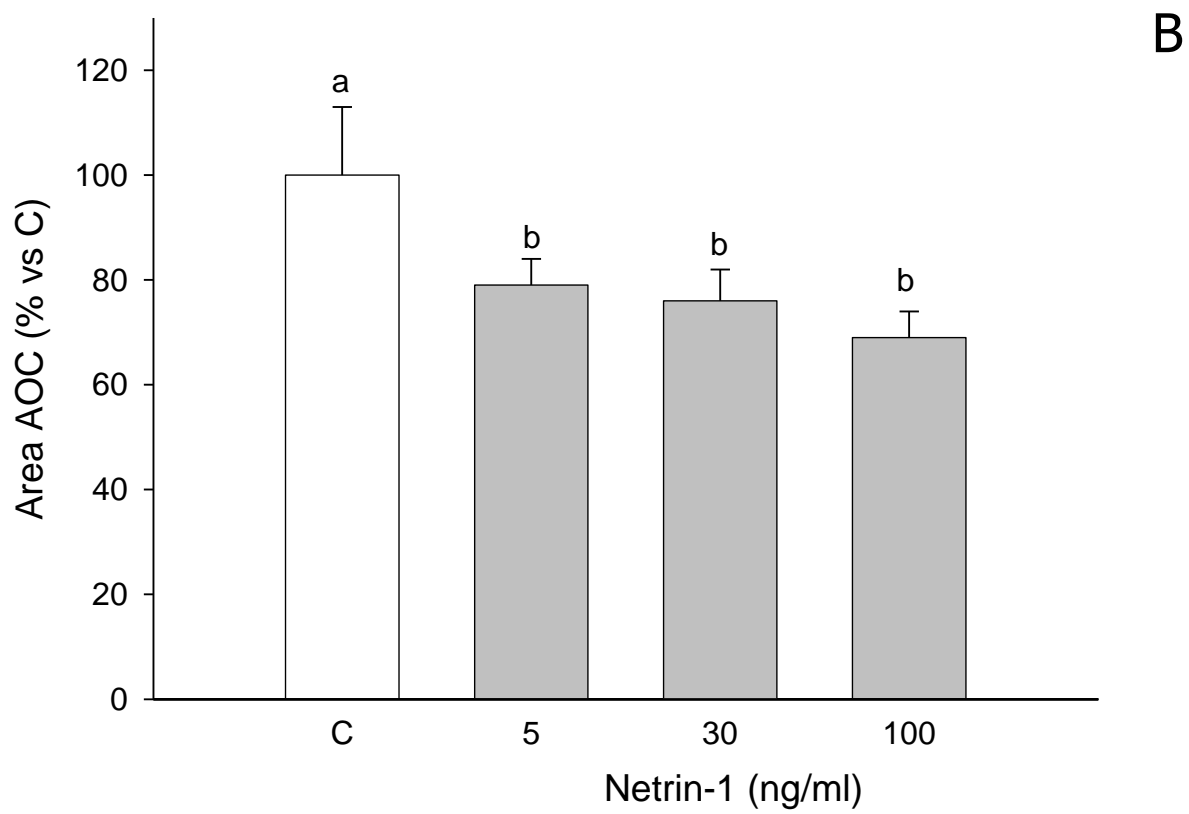
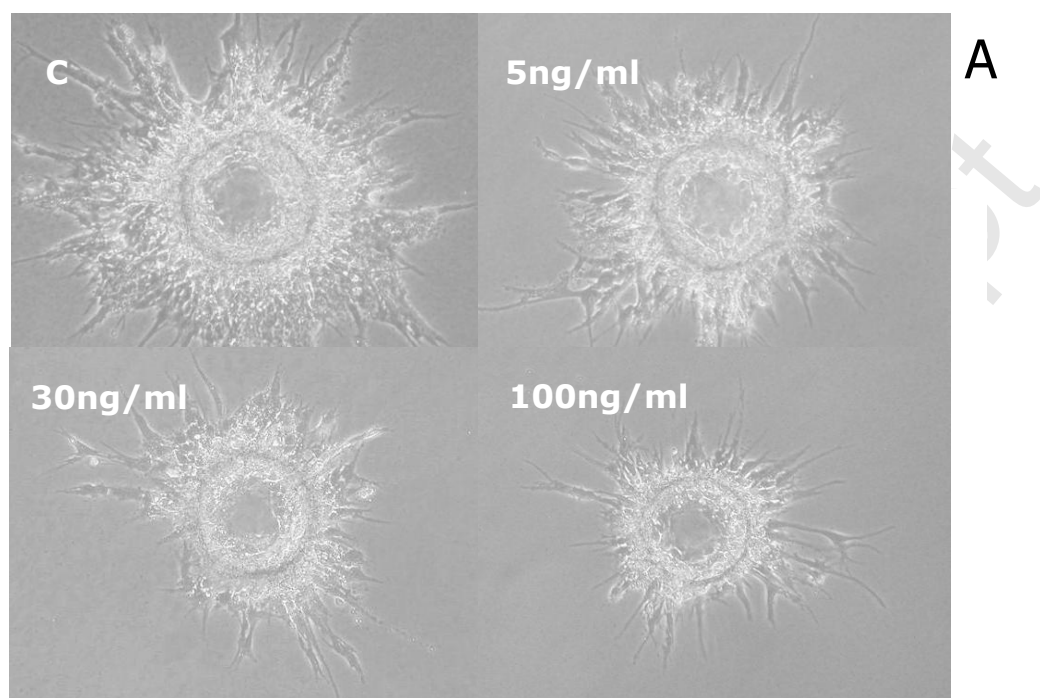


Figure 9

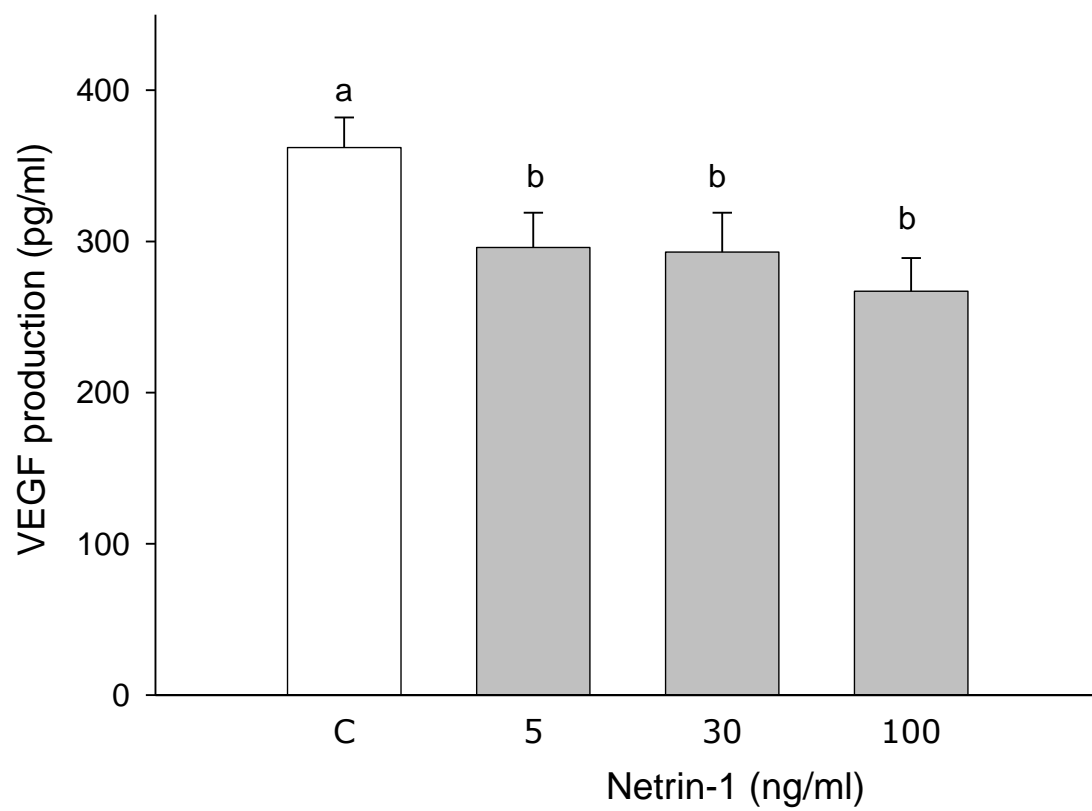


Figure 10

