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Tyrosine kinase A, C and fibroblast growth factor-2 receptors in bovine embryos cultured in vitro

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Abstract

Neurotrophins and basic fibroblast growth factor are ligands of tyrosine kinase receptors, though they bind to different tyrosine kinase receptor classes. Neurotrophins bind to receptor tyrosine kinase class VII, Trk receptor family, while basic fibroblast growth factor binds to receptor tyrosine kinase class IV, FGF receptor family. The mammalian uterine tract immunolocalizes neurotrophins and bFGF; therefore their cognate receptors might exert a role during embryonic development. Using RT-PCR, we found mRNA for p75^{NTR} TrkA, TrkC and FGFr2 throughout the early bovine embryonic development in vitro. Immunofluorescent staining, assessed by confocal microscopy, showed the expression of TrkA and TrkC proteins in oocytes and all embryonic stages analyzed. We have provided a novel description of TrkA and TrkC proteins, and TrkA, TrkC, p75^{NTR} and FGFr2 mRNA expression throughout mammalian embryonic development. This work may help to design future research with neurotrophins in bovine embryo culture and embryonic stem cells.

Keywords: In vitro; Cattle; Embryo; Neurotrophin; Tyrosine kinase receptor

1. Introduction

Tropomyosin-receptor-kinase receptors (Trks) are a family of membrane receptors that promote neural development, survival and division, although they can be found in non-neural tissues as well [1,2]. The common ligands of Trks are neurotrophins (NTs). Subtypes of Trks are neurotrophin-specific, such a way nerve growth factor (NGF) binds to TrkA, brain derived growth factor (BDNF) and neurotrophin-4 (NT4) binds to TrkB and neurotrophin-3 (NT3) binds to TrkC. NTs also bind to the low-affinity p75 neurotrophin receptor (p75^{NTR}). NTs may strengthen their effects by cooperation both among them and with the basic fibroblast growth factor (bFGF) [3,4]. The bFGF belongs to the fibroblast growth factor (FGF) family, whose members are involved in angiogenesis, wound healing, and embryonic development [5]. bFGF binds to the high-affinity FGF receptor-2 (FGFr2), although it also interacts with other members of the FGF receptor family. NTs, bFGF and their receptors might exert a role during embryonic development, provided that the uterine tract immunolocalizes BDNF, NGF and NT-3 in rodents [6–8], NGF in the goat [9] and bFGF in pigs [10] and humans [11]. Furthermore, the goat expresses p75^{NTR} and TrkA proteins in granulosa, thecal and oviductal cells, and, as shown in the hamster, in uterine cells [8,9]. Bovine granulosa cells express FGFr2 and

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Table 1
Details of primers used for PCR on bovine embryos.

Gene	Primer sequence (primer concentration)	Fragment size	Annealing temperature	Accession number	Reference
TrkA	ATGTCAACAACGGCAACTAC (0.2 μM) GTCTCATCCTTCTCCACTGG (0.2 μM)	181 bp	53 °C	XM_613650	Authors' design
TrkC	CGAAGAAACCTGTCAAGTAACC (0.2 μM) ATGTTCATGCGGAAGAGTGG (0.2 μM)	221 bp	60 °C	XM_592957	Authors' design
p75	TGGACAGCGTGACCTTCTC (0.2 μM) TCGTCTCGTCCTGGTAATAGC (0.2 μM)	153 bp	53 °C	DV807392	Authors' design
FGFr2	TCCTCCATGAACTCCAATACC (0.2 μM) GGCTTCTCCTTGTCAATTCC (0.2 μM)	218 bp	60 °C	XM_880233	Authors' design
H2a1	GTCGTGGCAAGCAAGGAG (0.4 μM) GATCTCGGCCGTTAGGTACTC (0.4 μM)	182 bp	58.6 °C	U62674	[16]

TrkA, whilst thecal and cumulus cells express TrkA and TrkB, respectively [12–14]. Moreover, $p75^{NTR}$ mRNA and protein localizes to cumulus cells and oocytes [13]. However, in the bovine early embryo, the expression of protein and mRNA for Trks, $p75^{NTR}$ and FGF2r has been not explored.

In this work we analyze gene expression for TrkA, TrkC, $p75^{NTR}$ and basic FGF receptor-2 during the early bovine embryonic development in vitro. Following mRNA analysis, we described protein expression for TrkA and TrkC.

2. Materials and methods

All reagents were purchased from Sigma (Madrid, Spain) unless otherwise stated.

2.1. In vitro embryo production

Bovine embryos were in vitro produced from slaughterhouse ovaries as previously reported [15]. Presumptive zygotes were cultured in synthetic oviduc fluid (SOF) with 6 g/l BSA at 38.7 °C, 5% CO_2, 5% O_2 and 90% N_2. Embryonic development was evaluated on days 3, 6, 7 and 8.

2.2. RNA extraction

RNA was extracted from immature oocytes, zygotes, morulae, hatched blastocysts and a 1.5-month old bovine fetus (positive control). Isolation of mRNA was performed using the Dynabeads mRNA Direct KIT (Dynal, Oslo, Norway), following the manufacturer's instructions.

2.3. Reverse transcription

For each tissue, reverse transcription was performed twice with the first-strand complementary DNA (cDNA) synthesis kit for RT-PCR (AMV reverse transcriptase; Roche, Barcelona, Spain) with oligo-d(T) and random primers, respectively. Samples were then cooled at 4 °C, mixed and stored at −20 °C until use.

2.4. Real-time PCR

PCR was performed with an i-Cycler iQ Real-Time PCR detection System (Bio-Rad, Hercules, CA, USA) as previously reported [15] Primer sequences, PCR traits for target genes, annealing temperature, sizes of the amplified fragments, and GeneBank accession number are shown in Table 1. Data were normalized to histone H2a1 transcript levels [16].

2.5. Immunocytochemical analysis

TrkA and TrkC receptors were immunocytochemically analyzed in oocytes, 2–4 cell embryos, morulae and hatched blastocysts. Primary rabbit polyclonal antibodies to TrkA and TrkC (SC-118, SC-14025, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used to detect protein expression following previously described procedures [17]. Briefly, samples ($n = 10$) were fixed in 4% paraformaldehyde and permeabilized. After incubation of samples in Image-iT FX Signal Enhacer (Molecular Probes) to reduce background, primary antibodies to TrkA and TrkC (2 μg/ml) were applied overnight at 4 °C. Samples were then incubated with the appropriate secondary antibodies (Alexas 488 goat anti-rabbit secondary antibody; Molecular Probes) for 2 h at room temperature, washed and mounted on glass slides in VectaShield anti-fade medium containing DAPI (Vector Laboratories). Oocytes and embryos were examined on a confocal microscope (Leica Ultra-Espectral Confocal Microscope TCS-SP2-AOBS). Serial z-axis optical sections were acquired in order to accurately characterize the pattern of expression of

TrkA and TrkC in all samples analyzed. Staining controls using secondary antibody alone were included.

2.6. Western blot analysis

Western blot with bovine tissues was performed to confirm primary antibody specificity. Rat brain tissue was used as a positive control, as the available primary antibodies (SC-118, SC-14025, Santa Cruz Biotechnology, Santa Cruz, CA, USA) had been raised to detect TrkA and TrkC from mouse, rat or human origin.

Samples consisted of either 30 blastocysts, or biopsies from bovine corpus luteum or rat brain and were processed for Western blot analysis as described by Sambrook [18]. Briefly thawed samples were recovered in 50 μl buffer (0.125 M Tris–HCl, 20% glycerol, 4% SDS, 0.2% 2-mercaptoethanol, 0.001% bromophenol blue), boiled during 5 min at 100 °C and separated on a 6% polyacrylamide gel for 3 h at 100 mV. Following electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, which were incubated overnight at 4 °C with primary antibodies (SC-118, SC-14025) diluted in blocking solution (2 μg/ml). Following incubation membranes were incubated with the appropriate (HRP)-conjugated secondary antibody (Amersham Biosciences, UK). To induce enzymatic reactions, the membranes were incubated with chemiluminiscence reagents (ECL Pierce Biotechnology Inc., Rockford, IL) for 1 min and immediately exposed to autoradiographic film. Protein bands were referreed to pre-stained bands (Bio-Rad Laboratories, Hercules, CA).

3. Results

3.1. Gene expression

Gene expression was made in 4 replicates that consisted each of 50 immature oocytes, 50 zygotes, 30 morulae and 10 hatched blastocysts, and bovine fetus (positive control). The absence of DNA was confirmed by amplification of a single product from β-actin with the expected size for RNA (299 bp). Dissociation curves performed after each PCR cycle ensured that a single product had been amplified. Electrophoresis confirmed the expected size of the products, while amplicon sequencing confirmed the expected mRNA and no other pseudo- or retro-genes. All stages and replicates analyzed contained mRNA for p75^{NTR}, TrkA, TrkC and FGFr2. The mRNA levels for all genes detected remained low in the oocyte and in the zygote stage, but

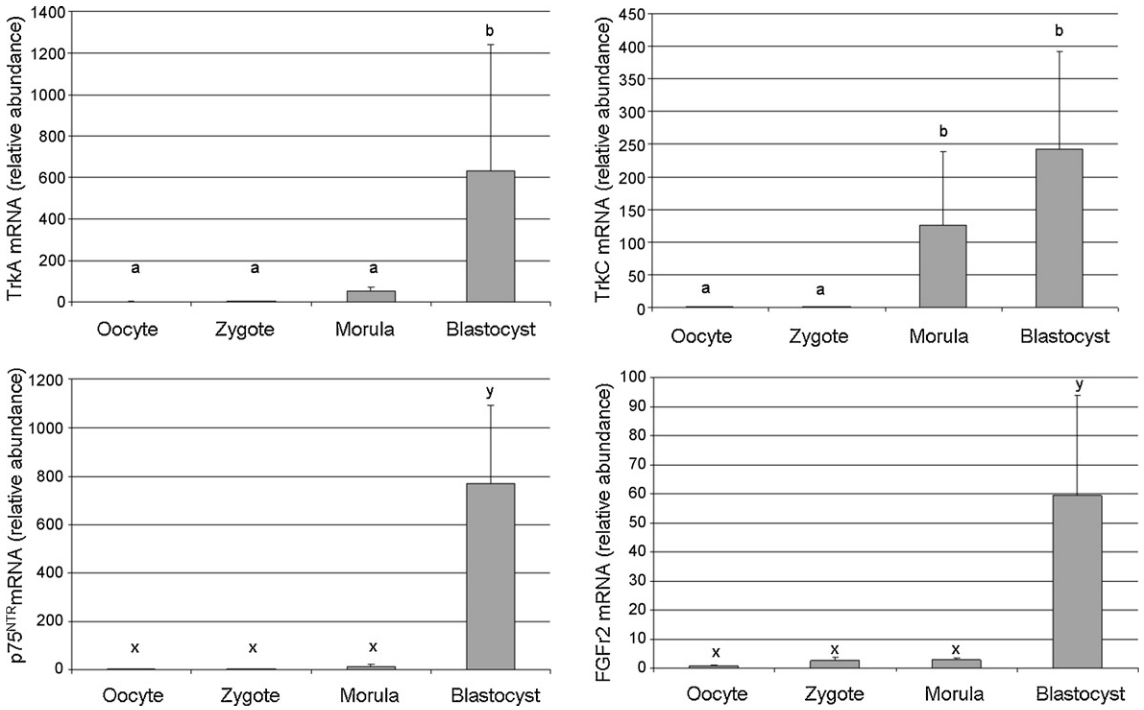

Fig. 1. Relative abundance (arbitrary units) of p75^{NTR} TrkA, TrkC and FGFr2 mRNA in bovine immature oocytes, and embryos cultured in synthetic oviduct fluid (SOF) with 6 g/l BSA up to the hatched blastocyst stage. Data are means ± S.E. Different superscripts express significant differences (a and b) $p < 0.05$; (x and y) $p < 0.005$.

they sharply increased at hatching (Fig. 1, $p7^{NTR}$ and FGFr2: $p < 0.005$; TrkA and TrkC: $p < 0.05$).

3.2. Immunocytochemical analysis and Western blot

TrkA and TrkC were detected in all stages analyzed from the immature oocyte up to the blastocyst stage. Serial z-axis optical sections showed predominant cytoplasm labelling for TrkA and TrkC within all samples analyzed (Fig. 2), however, at the blastocyst stage, TrkA staining was found in the nucleus and in the cytoplasm (Fig. 2C). TrkA and TrkC receptors were localized both to the trophectoderm (TE) and the inner cell mass (ICM) of bovine blastocysts (Fig. 2C and F, respectively). TrkA and TrkC were identified in bovine tissues by Western blot. In blastocysts, TrkA recognized a single band at 70 kDa, which is likely to correspond to a truncated isoform of the receptor. Nevertheless, in corpus luteum, we obtained a 140 kDa band, in full coincidence with the positive control, rat brain. This is in proof that the TrkA antibody used in our study is able to bind to both full length and truncated TrkA isoforms in bovine tissues. In addition, TrkC detected a single 145 kDa band both with bovine blastocysts and rat brain, confirming that the antibody used binds to full length TrkC isoforms in bovine tissues (Figs. 3 and 4).

4. Discussion

Tropomyosin-receptor-kinase receptors, FGFr2 and their ligands are present in the genital tract of several mammals [6–12,14]. Neurotrophins and other cytokines promote oocyte developmental competence, early embryonic development and implantation [13,19–22]. However, to our knowledge, there is no available information on the expression of neurotrophin or bFGF receptors in the bovine preimplantation embryo.

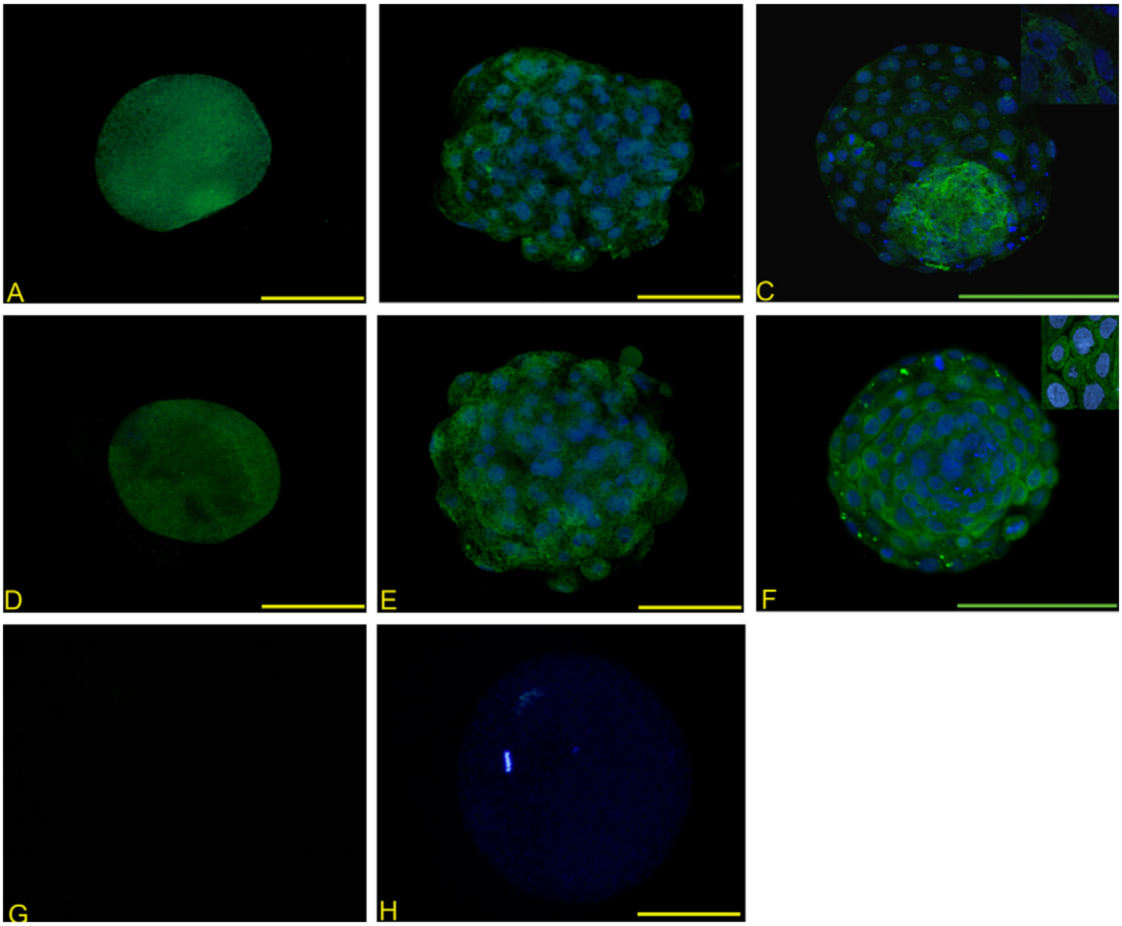

Fig. 2. Localization of TrkA (A–C) and TrkC (D–F) in bovine oocytes (A and D), morulae (B and E) and blastocysts (C and F) Negative controls with secondary antibody alone show no signal. (G) and (H) show representative microphotographs of a negative control oocyte (G) and its corresponding nuclear staining (H). Yellow scale bars 50 μm, blue scale bars 100 μm.

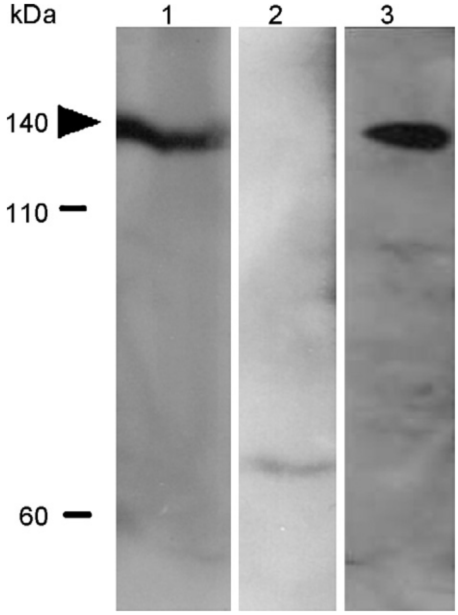

Fig. 3. Chemiluminiscent detection of TrkA receptor in bovine corpus luteum (lane 1), bovine blastocysts (lane 2) and rat brain (lane 3).

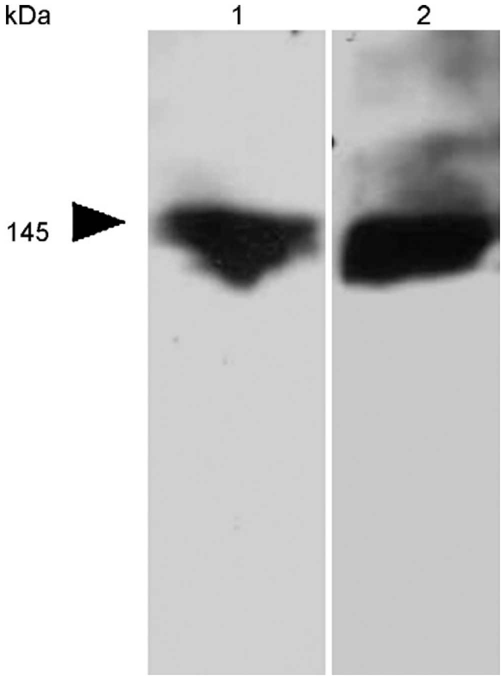

Fig. 4. Chemiluminiscent detection of TrkC receptor in bovine blastocysts (lane 1) and rat brain (lane 2).

In this study, we have described the presence of TrkA and TrkC proteins and TrkA and TrkC, p75^{NTR}, FGFr2 mRNA through the bovine embryo development. The levels of mRNA for all genes analyzed were lower in stages previous to the major genome activation. However, gene expression appeared to increase at the morula stage and reached their highest level in the hatched blastocysts. A similar expression pattern was observed in mice for TrkB [22]. It seems that expression of some Trks might be conserved across species, suggesting a developmentally important role. The increased concentration of FGFr2 mRNA we found in blastocysts is consistent with the presence of its ligand bFGF in bovine blastocysts [23].

Protein expression for TrkA and TrkC were detected in the bovine oocyte and throughout embryonic development. According to the Western blot analysis, the TrkC receptor we detected in bovine embryos corresponds to its full isoform. However, the detected TrkA receptor corresponds to a truncated isoform. Competent truncated forms of TrkA receptor have been previously reported [24]. The pictures obtained show that TrkC is mainly localized in the cytoplasm, although in blastocysts TrkA localizes both to the cytoplasm and nucleus. The spatial distribution found for TrkA in bovine embryos, is in agreement with the transit of transmembrane receptors to the nucleus described for several members of the tyrosine kinase receptor family, including TrkA [25–27] As belonging to the same receptor family, and having no available adverse evidences, we cannot discard that TrkC may also translocate into the cell, which would explain the distribution pattern we found for TrkC.

The expression of Trks in bovine preimplantation embryos suggests that neurotrophins might exert a role in the bovine embryonic development. In fact, in mice, neurotrophins promote embryo development and suppress apoptosis [22]. Moreover, the presence of Trks in the bovine ICM is consistent with their expression in human embryonic stem cells (hESC) [3], suggesting that neurotrophins may contribute to improve bovine stem cells derivation and maintenance. Unlike hESC, it is unknown whether the human ICM expresses Trks. However, in contrast with the bovine species, in the mouse blastocyst TrkB mRNA and protein localizes to the TE and not the ICM [22].

The information provided in our work may help to design future research with neurotrophins in bovine embryo culture and embryonic stem cell derivation and maintenance.

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