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Impact of oocyte-secreted factors on its developmental competence in buffalo

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Summary

Oocyte-secreted factors (OSFs) play an important role in the acquisition of oocyte developmental competence through bidirectional cross-talk between oocyte and cumulus cells via gap junctions. Thus, the present study was designed to investigate the effect of two OSFs, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), on the developmental competence of buffalo oocytes derived from two different follicle sizes. Cumulus–oocyte complexes (COCs) from large follicles (LF, >6 mm) or small follicles (SF, <6 mm) were collected and matured *in vitro* either in the presence of GDF9 or BMP15, or both, or with the denuded oocytes (DOs) as a source of native OSFs. Cleavage and blastocyst rates were significantly ($P < 0.05$) higher in LF-derived than SF-derived oocytes. Cleavage and blastocyst rates were significantly higher ($P < 0.05$) in the DOs and the combination groups compared with the control, GDF9 alone and BMP15 alone groups, both in LF-derived and SF-derived oocytes, although the cleavage and blastocyst rates did not differ significantly ($P > 0.05$) between DOs and combination groups. Relative mRNA analysis revealed significantly higher ($P > 0.05$) expression of the cumulus cell marker genes *EGFR*, *HAS2*, and *CD44* in LF-derived than SF-derived oocyte; the expression of these markers was significantly higher ($P > 0.05$) in DOs and combination groups, irrespective of the follicle size. These results suggested that LF-derived oocytes have a higher developmental competence than SF-derived oocytes and that supplementation of GDF9 and BMP15 modulates the developmental competence of buffalo oocytes by increasing the relative abundance of cumulus-enabling factors and thereby increasing cleavage and the quality of blastocyst production.

Keywords: BMP15, Buffalo, Developmental competence, GDF9, Oocyte-secreted factors

Introduction

The success of an *in vitro* embryo production (IVEP) programme depends on the developmental competence of oocytes. Oocyte developmental competence itself is regulated by several factors such as origin of oocytes (Lonergan *et al*., 1994), follicular health (Hagemann, 1999), culture milieu (Lee *et al*., 2009) and communication between oocytes and their surrounding cumulus cells (CCs) (Krisher, 2004). This bidirectional communication in cumulus–oocyte complexes (COCs) is mediated through gap junctions (Conti *et al*., 2006; Gilchrist *et al*., 2008) and is crucial for the promotion of cell growth (Li *et al*., 2000), prevention of cell death (Hussein *et al*., 2005), suppression of luteinization (Sutton-McDowall *et al*., 2012) and maintenance of CC metabolism (Gilchrist *et al*., 2008; Sutton-McDowall *et al*., 2015). An understanding of the role of oocyte secretary factors (OSFs) that regulate oocyte developmental competence has helped to improve IVEP protocols.

OSFs are amongst the important mediators of bidirectional communication, which regulates gene expression in CCs that are associated with oocyte maturation and subsequent embryo development. Major OSFs that regulate oocyte developmental competence

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are growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) (Galloway *et al*., 2000; Hanrahan *et al*., 2004). The function and potential of both these proteins differ markedly between species (Crawford & McNatty, 2012). In mono-ovulatory animals, the ratio of GDF9 and BMP15 is nearly equivalent, suggesting that both factors are important for follicular development. However, in the oocytes of multi-ovulatory animals, GDF9 expression is higher than BMP15 expression, suggesting a minor role for BMP15 (Crawford & McNatty, 2012). In humans, rare mutations of GDF9 and BMP15 are closely linked with polycystic ovarian syndrome (Wang *et al*., 2010) and ovulation failure (Dixit *et al*., 2005). Hussein *et al*. (2006) reported the positive effect of OSFs on oocyte developmental competence. They found enhanced bovine oocyte developmental competence after the addition of 175 ng/ml GDF9 and 10% v/v BMP15. As buffalo and bovine are related species, similar doses were chosen for this study. Rossi *et al*. (2015) evaluated the effect of different concentrations of BMP4, as well as the interaction between BMP4 and FSH on growth, ultrastructural integrity, and mRNA expression for GDF-9, BMP15, maternal antigen that the embryo requires (MATER) and nucleoplasmin-2 in bovine secondary follicles cultured *in vitro*. To date, limited information has been available regarding the role of GDF9 and BMP15 during the maturation process in buffalo oocytes and no reliable molecular markers of oocyte developmental competence have been identified.

Therefore, the present study was designed with the hypothesis that OSFs (GDF9 or BMP15 alone, or in combination) may enhance oocyte developmental competence by upregulating the expression of oocyte quality predictor genes in CCs. Large follicle (LF)-derived and small follicle (SF)-derived oocytes were cultured independently to analyze the effect of OSF supplementation on oocytes from differently sized follicles. We investigated the role of DOs as a source of endogenous OSFs in COC co-culture during *in vitro* maturation (IVM) and compared the control with the exogenous recombinant OSF-supplemented oocytes. We also evaluated the effect of OSFs on expression of epidermal growth factor receptor (*EGFR*), hyaluronan synthase 2 (*HAS2*) and cluster differentiation 44 (*CD44*) genes as potential oocyte competence markers.

Material and Methods

Chemicals

All chemicals used in this study were procured from Sigma Chemicals Co. (St. Louis, MO, USA), except when specifically mentioned in the text. All these chemicals were culture grade and endotoxin free. Plastic dishes and tubes were procured from Nunc (Thermo Scientific, Denmark). All chemicals for reverse transcription were purchased from Thermo Scientific, Denmark.

Oocyte recovery and *in vitro* maturation

Oocyte recovery

Buffalo oocyte collection and *in vitro* maturation was performed as per the established laboratory protocol (Bhardwaj *et al*., 2016b). In brief, buffalo ovaries were collected from the local abattoir and transported to the laboratory in pre-warmed saline (35–37°C). COCs were aspirated separately from the LFs (≥6 mm) and SFs (<6 mm) using a 18G needle fitted with a 5-ml syringe. Culture-grade oocytes (COCs with more than three layers of compact cumulus and evenly granular ooplasm) were selected under a stereozoom microscope for further experimental study. Different experiments were performed in five replicates.

Generation of DOs

DOs were generated by removing CCs from COCs by washing them with 0.01% hyaluronidase for 1–2 min. Any remaining leftover CCs were removed by pipetting followed by four to six times serial washing with TCM-199 medium (M2154, Sigma).

In vitro *maturation*

Culture-grade oocytes were washed four to five times in maturation medium containing TCM-199 (HEPES modified) supplemented with 5 μg/ml LH, 0.5 μg/ml FSH, 1 μg/ml estradiol-17β, 0.25 mM sodium pyruvate, 0.68 mM L-glutamine, 10 μg/ml gentamicin, 3 mg/ml bovine serum albumin (BSA) and 10% FBS. This medium was used as a basal medium for *in vitro* maturation. COCs of both follicle sizes were matured *in vitro* in basal medium supplemented either with GDF-9 (175 ng/ml; human recombinant; SRP4872-20UG, Sigma) or BMP15 (100 ng/ml; human recombinant; 5096-BM-005, Sigma) alone or in combination. To determine the role of DOs as a source of endogenous OSFs, COCs were co-cultured with DOs at a ratio of 2:1. COCs harvested from the LFs and SFs that were cultured in basal medium were used as the control. In total, about 10–15 COCs were kept for maturation in 50 μl droplets of maturation medium overlaid with mineral oil for 24 h at 38.5°C and 5% CO_2 in air with maximum relative humidity. Cumulus expansion was graded on a scale of +1 to +5.

In vitro fertilization and embryo production

Buffalo embryos were produced *in vitro* as per the established laboratory protocol (Bhardwaj *et al*., 2016b).

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Table 1 Gene-specific oligonucleotide primers pairs with annealing temperature, amplicon sizes and references

Gene	Primer pairs	Annealing temp (°C)	Predicted size (bp)	References/GenBank accession number
GAPDH	F-CGACCACTTTGTCAAGCTCA R-GGACCTTACTCCTTGGAGGC	60	82	NM001034034
β-Actin	F-AGTTCGCCATGGATGATGA R-TGCCGGAGCCGTTGT	60	154	Dangi *et al*. (2014)
EGFR	F-CCAGGAGGTTGCCGGCTATGT R-GCAGCTCCCTCAGTCCGGTTTT	60	161	HM749883.1
HAS2	F-ATAAATGTGGCAGGCGGAAGAAGG R-GTCTTTGTTCAAGTCCCAGCAGCA	60	183	Assidi *et al*. (2008)
CD44	F-TGAAGAACATGGGGCAAACACAAC R-TTCTGCCCACACCTTCTCCTACTG	60	147	XM006062370.1

In brief, *in vitro* matured COCs of all groups were fertilized with frozen–thawed buffalo bull semen using FerTALP medium supplemented with 0.2 mM sodium pyruvate, 6 mg/ml BSA, 20 µg/ml heparin and 50 µg/ml gentamycin. After 18 h co-incubation of sperms and oocytes, presumptive zygotes were washed in modified synthetic oviductal fluid (mSOF) containing BSA (3 mg/ml fraction V), 10% FBS, sodium pyruvate (0.25 mM), L-glutamine (0.68 mM) with essential and non-essential amino acids. Presumptive zygotes were then cultured (10–15 zygotes/50 µl droplet) in mSOF at 38.5°C, 5% CO_2 and maximum relative humidity. Medium was changed on alternate days until the blastocyst stage or day 8 post insemination.

RNA isolation and reverse transcription

For the isolation of RNA, CCs of all the groups were separated from the oocytes ($n = 20$ for each sample) after 24 h of *in vitro* maturation by vortexing for 1 min. All the denuded oocytes were removed under a stereozoom microscope and CCs were suspended in phosphate-buffered saline (PBS)-PVP and then were transferred with a pipette in an autoclaved Eppendorf tube (0.5 ml). Cells were centrifuged at 1500 rpm for 10 min, followed by washing in PBS. The pellet formed was used for RNA isolation. Total RNA was isolated from CCs using TRIzol® reagent (Ambion cat. no. 15596-026). In total, 1 µg of total RNA was reverse transcribed using a single-stranded cDNA synthesis kit (Thermo Scientific, cat. no. AB1453/B) as per the manufacturer's instructions. The quality of cDNA was assessed by amplification reaction for housekeeping genes (*GAPDH* and β-*actin*). The PCR product was analyzed using 1.8% agarose gel electrophoresis.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was carried out in duplicate for relative quantification of mRNA expression of competence markers (*EGFR*, *HAS2*, and *CD44*) and reference genes (*GAPDH* and β-*actin*).The primer sequences and amplicon sizes are summarized in Table 1. qPCR was performed using the DyNAmo Flash SYBR green qPCR kit (Thermo Scientific, cat#415L) and Stratagene Mx3000P (Agilent Technology, Santa Clara, CA USA) as per the manufacturer's instructions. In brief, a master mix contained 10 µl of SYBR green, 0.5 µl of forward and reverse primers each (20 pM), 8 µl nuclease free water and 1 µl of cDNA. The reaction was performed for 40 cycles with the following cycling conditions: denaturation at 95°C for 30 s, annealing at 60°C for 15 s and extension at 72°C for 30 s. A non-template control was run to check for any contamination and to minimize errors. The geometric mean of C_t values of the β-actin and GAPDH were used as the C_t of reference genes for the calculation of relative gene expression by the Pfaffl method (Pfaffl, 2001). For each group, three samples were taken and used for statistical analysis. LF-derived oocytes from the control group were used as the endogenous control for relative expression analysis.

Statistical analysis

For the analysis of cleavage and blastocysts data, all replicated proportional development data were arc-sin transformed prior to analysis. Statistical analysis within groups was carried out by independent *t*-tests and one-way analysis of variance (ANOVA) to make comparison between the groups using SAS 9.2 software (SAS Institute Inc., Cary, NC, USA) using Tukey's test and differences with a P-value < 0.05 were considered as statistically significant. All data were presented as mean ± standard error (SE). qPCR data were analyzed statistically by independent *t*-test for within group analysis followed by one-way ANOVA for comparison between the groups

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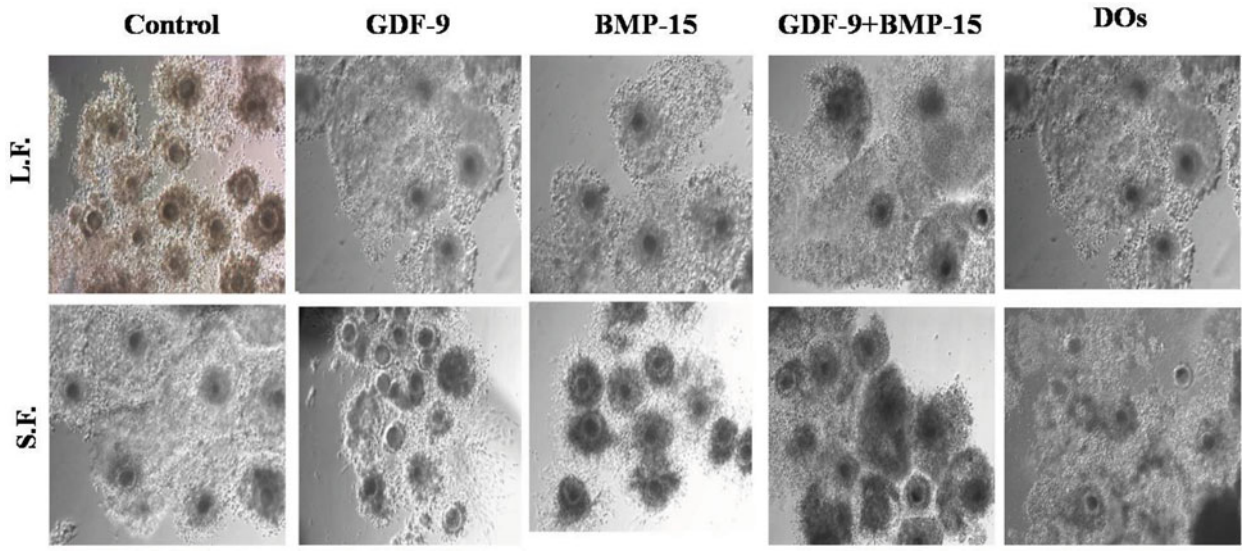

Figure 1 Cumulus expansion in different experimental groups supplemented with OSFs: cumulus expansion was observed at 24 h of maturation. Expansion of cumulus cells is an index of measurement for the developmental competence of oocytes.

using SAS 9.2 software (SAS Institute Inc., Cary, NC, USA) and Graph Pad In Stat Software (Peter Russell, Royal Veterinary College) and a P-value < 0.05 was considered to be statistically significant.

Results

Effect of OSFs on *in vitro* embryo production

Cumulus expansion was improved in the GDF9–BMP15 combination group followed by the DO, GDF9, BMP15 and control groups and was better in the LF groups than in the SF groups for all respective treatments (Fig. 1 and Table 2). The LF-derived oocytes depicted significantly ($P < 0.05$) higher cleavage and blastocyst rates as compared with the SF-derived oocyte for the control groups compared with other groups; no significant ($P > 0.05$) differences were noticed among other groups. Cleavage and blastocyst rates were significantly ($P < 0.05$) higher in the GDF9–BMP15 combination group and the DO group than in the other groups, irrespective of follicle sizes (Table 2). No significant difference ($P > 0.05$) was observed between the GDF9–BMP15 combination group and the DO group. Similarly, there were no significant differences ($P > 0.05$) between control, GDF9 and BMP15 groups.

Genes expression profile of oocyte competence markers

All the oocyte competence markers, *EGFR*, *HAS2*, and *CD44*, were expressed in CCs of *in vitro* matured oocytes, irrespective of follicle sizes. Although, mRNA expression analysis revealed no specific pattern when compared between LF-derived and SF-derived oocytes, overall *EGFR*, *HAS2*, and *CD44* expression was higher in LF oocytes than SF oocytes (Fig. 2). The relative expression of *EGFR* was higher in CCs from LF-derived oocytes compared with the SF-derived oocytes in all the treated groups. Supplementation of GDF9 in culture medium significantly ($P < 0.05$) upregulated *EGFR* expression as compared with BMP15 supplementation; while, the combination of GDF9–BMP15 exhibited significantly ($P < 0.05$) higher expression as compared with the control. A significant ($P < 0.05$) upregulation in *EGFR* expression in oocytes matured in the presence of DOs as compared with the other groups was noted. Similarly, *HAS2* was upregulated significantly ($P < 0.05$) by GDF9 supplementation, but was further upregulated by BMP15 supplementation and further upregulated significantly ($P < 0.05$) in DOs, as well in the GDF9–BMP15 combination group (Fig. 2). *CD44* expression was relatively not influenced ($P > 0.05$) by supplementation of GDF9 and BMP15 independently, irrespective of the follicle size. However, it was upregulated significantly ($P < 0.05$) in the GDF9–BMP15 combination group in comparison with all other groups. A non-significant ($P > 0.05$) increase in *CD44* expression was registered in SF-derived oocytes.

Discussion

The present study investigated the effect of OSF supplementation during IVM on buffalo oocyte developmental competence and the effect of OSFs

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Table 2 Effect of OSFs on LF-derived and SF-derived oocytes in different experimental groups

Experimental groups	No. of oocytes cultured		Cumulus expansion in *in vitro* matured oocytes		Cleavage rate *n* (%)		Blastocyst rate *n* (%)	
	LF	SF	LF	SF	LF	SF	LF	SF
Control	126	245	++++	++	81 (64.4 ± 2.29^{A})	133 (54.65 ± 1.41^{a*})	17 (21.0 ± 1.0^{A})	22 (17.08 ± 0 .79^{a*})
GDF9	122	243	+++++	+++	71 (58.5 ± 2.0^{A})	127 (52.5 ± 2.5^{a})	17 (23.25 ± 0.25^{A})	27 (20.5 ± 0.28^{a})
BMP15	128	246	+++++	+++	81 (63.8 ± 1.90^{A})	144 (58.6 ± 2.03^{a})	18 (22.8 ± 0.37^{a})	32 (20.4 ± 0.50^{a})
COCs + DOs	124	242	+++++	++++	89 (72.20 ± 2.35^{B})	160 (66.2 ± 1.4^{b})	27 (30.0 ± 0.63^{B})	45 (28.4 ± 0.67^{b})
GDF9 + BMP15	125	248	+++++	++++	91 (72.86 ± 0.92^{B})	169 (68.4 ± 2.03^{b})	25 (28.3 ± 0.21^{B})	46 (27.5 ± 0.29^{b})
Total	625	1224			413	733	104	172

^{A,B,a,b}Values (mean ± SE) along column represented by different superscript letters differ significantly ($P < 0.05$).
Values (mean ± SE) within group represented by asterisk mark differ significantly ($P < 0.05$).
Five replicates were taken for each group.
Cumulus expansion was graded on a scale of +1 to +5.
Cleavage rate: proportion of total oocytes cultured resulting in two-cell embryos.
Blastocyst rate: proportion of cleaved oocytes resulting in blastocyst development.

on the expression profile of competence markers in CCs. Fatehi *et al*. (2005) reported that BMP2, BMP4, and the types I and II receptor systems play an important role in development and functioning of follicles rather than in final oocyte maturation and cumulus expansion. During the study, the effect of OSF supplementation during IVM on blastocyst rate varied among the groups. Co-culture of COCs (derived from LFs and SFs) with denuded oocytes during IVM significantly improved the cleavage and blastocyst rates. This result indicated that the denuded oocytes secrete some mitogenic factors that stimulated further cytoplasmic maturation of oocytes. Improved developmental competence has been documented following DO–COC co-culture in mouse (Sudiman *et al*., 2014), pig (Gomez *et al*., 2012), cattle (Hussein *et al*., 2011; Yeo *et al*., 2008) and goat (Romaguera *et al*., 2010; Dey *et al*., 2012).

No significant difference was noticed for the cleavage and blastocyst rates following supplementation of GDF9 and BMP15 independently in LF-derived and SF-derived oocytes in comparison with their respective controls. This finding is in agreement with the study conducted by Sudiman *et al*. (2014) in cattle, in which no significant difference was observed on supplementation of GDF9 and BMP15 independently in comparison with the control. However, in contrast with these results, increase in blastocyst rate has been reported by other groups on administration of OSFs independently in cattle (Hussein *et al*., 2006) and mice (Sudiman *et al*., 2014). Cleavage and blastocyst rates were significantly improved in the GDF9 and BMP15 combination and DO groups in comparison with other groups, although there was no significant difference between the GDF9 and BMP15 combination and DO groups. GDF9 and BMP15, therefore, act synergistically and thus improve developmental competence. This simple process, of addition of OSFs, helped overcome the deficiency in endogenous OSFs during IVM. It is possible that BMP15 and GDF9, in combination, activate SMAD1/5/8 and SMAD2/3 pathways, respectively, that are involved in the regulation of oocyte developmental competence. Activation of these pathways is important for oocyte development in mono-ovulatory large animals (Hanrahan *et al*., 2004). BMP15 supplementation during IVM is likely to promote the uniform distribution of active mitochondria, thereby improving functional competence (Sutton-McDowall *et al*., 2012). Hussein *et al*. (2011) depicted qualitative temporal changes in oocyte paracrine factor production during maturation and Li *et al*. (2014) found that *GDF9* and *BMP15* mRNA expression levels were closely associated with oocyte maturation, fertilization, embryo quality, and pregnancy outcome. OSFs exert synergistic beneficial effects on nuclear and cytoplasmic maturation, rapid

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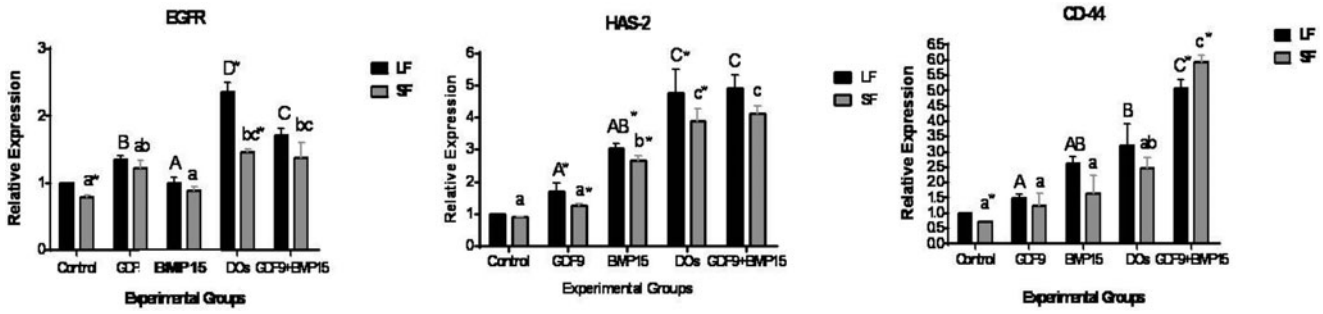

Figure 2 Relative gene expression of oocyte competence markers (*EGFR*, *HAS-2* and *CD44*). Data are presented as mean ± standard error (SE) and groups with different superscripts differ significantly ($P < 0.05$). Capital and small letters represents LF-derived and SF-derived oocytes respectively. Subgroups with different superscripts vary significantly. *EGFR*: epidermal growth factors receptor, *HAS2*: hyaluronan synthase, *CD44*: cluster of differentiation 44.

utilization of energy and management of oxidative stress (Romaguera *et al*., 2010). In contrast with our report, Hussein *et al*. (2006) observed no increase in blastocyst yield when supplementing the combination of GDF9 and BMP15 in cattle COCs. In this study, cleavage and blastocysts rates were significantly higher in LF-derived than SF-derived oocytes in control groups and by supplementation of GDF9 or BMP15 or DOs. There was no significant difference between LF-derived and SF-derived oocytes, this result indicated that such supplementation is more effective in SF-derived oocytes than in LF-derived oocytes. This result is in corollary with the fact that large follicle-derived oocytes are more competent than small follicle-derived oocytes (Lonergan *et al*., 1994).

Competent oocytes express higher levels of CCs transcripts, such as *HAS2*, *GREM1*, *EGFR* and *TNFAIP6*, which are responsible for cumulus expansion (Bhardwaj *et al*., 2016a). The effect of OSF supplementation during *in vitro* maturation on relative gene expression of competence markers in CCs was also studied. A significant increase in gene expression was observed in both the DO co-cultured and in the GDF9–BMP15 combination group in LF oocytes.

CD44 and *HAS2* expression in the BMP15 supplemented group was significantly higher than in the control and was related to hyaluronic acid production in CCs (Schoenfelder & Einspanier, 2003) as *HAS2* mRNA expression in CCs correlates with hyaluronic acid (HA) production. *HAS2* was differentially expressed in human CCs, as the quality of human oocytes varies with *HAS2* expression (Cillo *et al*., 2007). Hyaluronic acid was found to delay death and prevent fragmentation of porcine oocytes (Tunjung *et al*., 2009). In SF-derived oocytes, the DO group and the GDF9–BMP15 combination group had a significantly higher transcript level of oocyte competence markers, whereas no significant differences were noticed in the other two groups compared with the control. *CD44* expression was higher in the DO-supplemented group than in the GDF9 and control groups, with no significance for the BMP15-supplemented experimental group. *CD44* is a cell adhesion receptor with HA as its main ligand. It is mainly involved in cell adhesion, migration, inflammatory response and apoptosis inhibition (Lesley *et al*., 2004; Eshkar *et al*., 2007). *CD44* has only been reported in mature oocytes, while it was absent in humans (Campbell *et al*., 1995), cattle (Furnus *et al*., 2003), pigs (Toyokawa *et al*., 2005) and mice (Wheatley *et al*., 1993). These findings suggest the positive effect of BMP15 on the expression of *CD44* and *HAS2* transcripts which in turn further improved oocyte developmental competence. *CD44* is absent in immature oocytes, as matrix formation during this stage would affect nutrient exchange and paracrine signaling (Yokoo *et al*., 2010).

A significant difference was observed in *EGFR* expression of both LF-derived and SF-derived oocytes, thus *EGFR* can be considered as an important determinant of oocyte competence. *EGFR* expression was significantly higher in the DO group than in the control and GDF9 supplemented groups, with no significant difference compared with the GDF9–BMP15 combination group. OSFs facilitate cross-talk between CCs and oocytes via upregulation of *EGFR*, *HAS2*, and *CD44* expression, therefore the developmental competence of oocytes is increased.

It can be concluded from this study that LF-derived oocytes have higher developmental competence than SF-derived oocytes. Supplementation of OSFs modulates the developmental competence of buffalo oocytes, irrespective of follicular size, by increasing the relative abundance of cumulus-enabling factors and thereby increasing cleavage and the quality of blastocyst production. Furthermore, *EGFR*, *HAS2*, and *CD44* expression could be used as markers in CCs for the selection of competent buffalo oocytes for *in vitro* embryo production.

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