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Targeted suppression of *Has2* mRNA in mouse cumulus cell-oocyte complexes by adenovirus-mediated short-hairpin RNA expression

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Abstract

RNA interference (RNAi) is an effective tool for studying gene function in oocytes, but no studies have targeted somatic cells of primary cultured cumulus cell-oocyte complexes (COCs). This is probably due to difficulty in introducing RNAi-inducing molecules, such as a short-hairpin RNA (shRNA) gene, into COCs by commonly used transfection reagents. We therefore tested whether a developmental process of intact COCs could be suppressed by adenovirus-mediated shRNA expression. *Has2*, encoding hyaluronan synthase 2, was selected as the target transcript, because the process of cumulus expansion depends upon expression of *Has2* mRNA and this process is easily evaluated in vitro. Intact COCs were infected with replication-incompetent adenoviruses containing an expression sequence of shRNA targeting either *Has2* (*Has2* shRNA) or a control transcript not expressed in cumulus cells, and the effects on epidermal growth factor (EGF)-stimulated cumulus expansion were determined. *Has2* shRNA expression suppressed *Has2* mRNA levels in COCs by more than 70%, without affecting expression levels of *Ptgs2*, *Ptx3*, *Tnfrsf6* mRNAs, which are also required for cumulus expansion, or other transcripts not related to expansion. Interestingly, levels of *Areg* and *Ereg* mRNAs were decreased in COCs expressing *Has2* shRNA when compared with those in controls, while *Btc* mRNA levels remained unaffected. Furthermore, the degree of cumulus expansion by *Has2* shRNA-expressing COCs was significantly less than that of controls. Thus adenovirus-mediated introduction of shRNA produces specific gene silencing and a phenotype in intact COCs, providing proof of principle that this method will be a helpful tool for understanding mechanisms of COC development.

Keywords

RNA interference; cumulus expansion; hyaluronan synthase 2; shRNA

INTRODUCTION

RNA interference (RNAi), initially discovered in *Caenorhabditis elegans* (Fire et al. 1998), is a conserved mechanism by which small interfering RNAs (siRNAs) derived from double-stranded RNA bring about sequence-specific gene silencing via transcript degradation. Several studies have used the RNAi technology to examine gene function in primary cultured granulosa cells of pig (Hirano et al. 2004), rat (Jo and Curry 2006; Kwintkiewicz et al. 2007; Parakh et al. 2006; Tamura et al. 2007), cattle (Kobayashi et al. 2007) and mouse (Shimada et al. 2007). Since these studies were conducted using granulosa cells cultured as mono-layers, the protocols used are not necessarily applicable to studying cumulus cell

processes that require communication between oocytes and companion cumulus cells in a three dimensional cumulus cell-oocyte complex (COC). One of the reasons for the absence of studies using intact COCs has been the difficulty of commonly used transfection reagents in introducing RNAi producing molecules, such as a vector containing an expression sequence of short hairpin RNA (shRNA), into primary cultured COCs.

shRNA is an artificially designed RNA molecule that contains an intramolecular stem-loop structure. Once expressed in cells, specific shRNA is processed into siRNA by the endogenous DICER1 enzyme, and triggers subsequent cleavage and degradation of target transcripts, thus “silencing” specific gene expression (Brummelkamp et al. 2002; Paddison et al. 2002; Paul et al. 2002; Sui et al. 2002; Yu et al. 2002). shRNA can be introduced into cells by viral vectors, such as adenoviruses. The relative ease in preparing high-titer viral stocks and the high efficiency in delivering shRNA into both actively dividing and non-dividing cells are major advantages of using adenoviral vectors (Hommel et al. 2003; Xia et al. 2002).

Oocytes and companion cumulus cells communicate via paracrine regulatory factors and gap junctions (Eppig 2001). Removal of oocytes from COCs impairs some cumulus cell functions such as cumulus expansion requiring oocyte-derived paracrine factors (Buccione et al. 1990; Eppig et al. 1993; Vanderhyden et al. 1990). The production of hyaluronan, a non-sulfated glycosaminoglycan, is necessary for cumulus expansion (Richards 2005). Hyaluronan synthase 2 (HAS2) is one of the enzymes required for hyaluronan synthesis (Weigel et al. 1997). Since *Has2* mRNA expression in cumulus cells is well correlated with the cumulus expansion process (Fulop et al. 1997), it is generally accepted that HAS2 is one of the key enzymes required for this process (Richards 2005).

The objective of this study was to determine whether a maturational process of intact oocyte-cumulus cell complexes, cumulus expansion, could be suppressed by adenoviral vector-mediated expression of shRNA, without disrupting the three dimensional COC structure, thus with maintaining the cumulus cell-oocyte communication. *Has2* was therefore selected as the specific shRNA target transcript because the functional consequence of *Has2* silencing could be easily evaluated by assessing the degree of cumulus expansion. Recombinant adenoviruses containing an expression sequence of shRNA targeting *Has2* (*Has2* shRNA) or human lamin A/C (*LMNA* shRNA, as control) were produced. Then, the effects of infecting intact COCs with these viruses on the mRNA levels of *Has2* and other cumulus expansion-related transcripts, *Ptgs2*, *Ptx3* and *Tnfaip6*, encoding prostaglandin synthase 2, pentraxin 3 and tumor necrosis factor alpha induced protein 6, respectively (Fulop et al. 2003; Ochsner et al. 2003a; Ochsner et al. 2003b; Varani et al. 2002), as well as the process of cumulus expansion itself, were examined.

RESULTS

Effect of *Has2* shRNA expression on steady-state *Has2* mRNA levels during cumulus expansion process

As a preliminary experiment, three shRNA sequences targeting *Has2* mRNA were designed and tested for the efficiency of gene silencing. The *Has2* shRNA sequence (see Materials and Methods) that exhibited the greatest efficiency in targeting *Has2* mRNA expression was selected for use in the present study (data not shown).

Effects of *Has2* shRNA expression on *Has2* mRNA levels in COCs were determined. As shown in Fig. 1A, infecting with adenovirus containing expression sequence of *Has2* shRNA decreased *Has2* mRNA levels in COCs in a dose dependent manner, whereas control virus infection had no effect on *Has2* mRNA levels. The degree of *Has2* mRNA

knockdown was more than 70% when COCs were infected at a viral concentration of 30×10^6 ifu/COC. Infecting at a higher viral concentration (90×10^6 ifu/COC) did not improve knockdown efficiency (data not shown).

To examine specificity of *Has2* mRNA silencing, levels of other transcripts encoding proteins known to play important roles in cumulus cell functions were examined. The transcripts examined were: *Bmpr2*, encoding BMP type 2 receptor (Shimasaki et al. 2004); *Inha*, encoding inhibin alpha subunit (Matzuk et al. 1992); *Ldha*, encoding a glycolytic enzyme (Sugiura et al. 2005); *Mvd*, encoding an enzyme involved in cholesterol biosynthesis pathway (Su et al. 2008); and *Smad4*, encoding MAD homolog 4 (Diaz et al. 2007). As shown in Fig. 1B, infecting with either the *Has2* targeting virus or the control virus had no effect on the mRNA levels of these transcripts, suggesting that the silencing induced by *Has2* shRNA expression was specific to *Has2* mRNA.

Effect of *Has2* shRNA expression on cumulus expansion

To assess functional significance of silencing *Has2* expression, the effect of *Has2* shRNA expression on the cumulus expansion was determined. As shown in Fig. 2 and Fig. 3, *Has2* shRNA expression significantly reduced the cumulus expansion index (about 70%) when compared with the *LMNA* shRNA expression or the not infected control. Control shRNA-expressing COCs exhibited comparable levels of cumulus expansion to COCs not infected with any viruses (Fig. 2 and Fig. 3).

To test whether viral infection caused any deleterious effects on the viability of oocytes, oocytes in the COCs not infected or infected with viruses were fertilized in vitro and assessed for their ability to develop to blastocyst stage (Fig. 4). As shown in Fig. 4, the percentages of the oocytes resumed meiosis (GVB), emitted the first polarbody (Pb1), developed to 2-cell (2-cell) and blastocyst (Blast) embryos were comparable between viral infected and non-infected COCs. This suggests that viral infection has no obvious effect on viability and developmental ability of the oocytes, therefore, the reduction of the degree of the cumulus expansion was not attributed to the lower viability of the oocytes. These results strongly suggest that *Has2* mRNA silencing with *Has2* shRNA expression was functionally significant.

Effect of *Has2* shRNA expression on levels of cumulus expansion-related transcripts

Expression levels of *Ptgs2*, *Ptx3* and *Tnfaip6* transcripts were not significantly different between COCs expressing *Has2* shRNA and *LMNA* shRNA; however, these mRNA levels increased when compared with those in COCs not infected with viruses (Fig. 5). The levels of *Tnfaip6* mRNA in COCs, infected with either viruses at concentrations of 9×10^6 and 30×10^6 ifu/COC, were significantly higher than those in COCs not exposed to the viruses.

To assess whether the increases in mRNA levels were restricted to *Ptgs2*, *Ptx3* and *Tnfaip6* transcripts, mRNA levels of *Areg*, *Btc* and *Ereg*, encoding EGF-like growth factors, amphiregulin, betacellulin and epiregulin, involved in the cumulus expansion process (Park et al. 2004) were examined. As was the case in other cumulus expansion-related transcripts, the mRNA levels of *Areg*, *Btc* and *Ereg* increased as viral concentration increased (Fig. 6). Therefore, it appears that viral infection causes up-regulation of many transcripts involved in cumulus expansion process, except for *Has2*, in COCs during cumulus expansion.

Levels of *Areg* and *Ereg* transcripts in COCs expressing *Has2* shRNA were significantly lower than those in control shRNA-expressing cells (Fig. 6). This suggests the possibility that typical formation of extracellular matrix may be required to allow the normal levels of *Areg* and *Ereg* transcripts during cumulus expansion process. To assess this hypothesis, effect of serum removal from culture medium on expression levels of cumulus expansion-

related transcripts were examined (Fig. 7). Since serum derived inter alpha trypsin inhibitor is required for the formation of extracellular matrix during cumulus expansion, normal expansion was inhibited when serum was removed from medium (Chen et al. 1992). As shown in Fig. 7, removing serum from medium significantly decreased transcript levels of *Areg* and *Ereg*, but not *Btc* nor the other transcripts related to cumulus expansion. Taking together, these results suggest that normal formation of extracellular matrix may be required for greater expression levels of *Areg* and *Ereg* mRNA during cumulus expansion process. Furthermore, these results support the hypothesis that steady-state levels of *Areg* and *Ereg* mRNAs decreased when *Has2* mRNA levels were silenced as a consequence of silencing this transcript on matrix formation.

Effect of *Has2* shRNA expression on the kinetics of cumulus expansion and mRNA levels

Since the levels of *Has2*, *Ptgs2*, *Ptx3* and *Tnfaip6* mRNA are dynamically regulated during cumulus expansion, it was possible that the decreased level of *Has2* mRNA caused by *Has2* shRNA expression observed at the 6 hr time point could be explained by differences in the kinetics of expression rather than RNAi-mediated silencing. Therefore, to examine the kinetics of mRNA expression in COCs expressing *Has2* shRNA in more detail, mRNA levels of *Has2*, *Ptgs2*, *Ptx3* and *Tnfaip6* at various time points throughout the cumulus expansion process were determined (Fig. 8). As shown in Fig. 8B, expression levels of these transcripts were low before EGF-treatment (0 hr), and EGF-treatment induced dramatic increase of these transcripts in all groups, as expected. Notably, *Has2* shRNA expression prevented *Has2* mRNA levels from ever reaching levels reached in the *LMNA* shRNA expressing group. Moreover, expression levels of *Ptgs2*, *Ptx3* and *Tnfaip6* after EGF treatment were not significantly different between *Has2* shRNA and *LMNA* shRNA expressing COCs.

DISCUSSION

The effects of infecting with adenoviruses containing expression sequence of *Has2* shRNA on expression levels of cumulus expansion-related and non-related transcripts as well as cumulus expansion by intact COCs were examined. *Has2* shRNA expression suppressed *Has2* mRNA expression in intact COCs, without affecting expression levels of *Ptgs2*, *Ptx3*, *Tnfaip6* and other transcripts not related to cumulus expansion process. Interestingly, levels of *Areg* and *Ereg* mRNAs were decreased in *Has2* mRNA-targeted COCs when compared with those in control shRNA-expressing COCs, while *Btc* mRNA levels remained unaffected. Furthermore, the degree of cumulus expansion by *Has2* shRNA-expressing COCs was lower than those by control shRNA-expressing COCs, indicating that *Has2* transcript silencing was functionally significant. The reduction of *Has2* mRNA levels and degree of cumulus expansion were not attributable to potential deleterious effects of viral infection on the COC viability; since (1) the control virus did not cause significant effects on the *Has2* mRNA or cumulus expansion; (2) *Has2* shRNA-expressing COCs responded to EGF by elevating mRNA levels of the cumulus expansion-related transcripts; (3) expression levels of the transcripts not involved in cumulus expansion process were not affected by the viral infection; and (4) comparable percentages of the oocytes in COCs infected with the viruses were capable in completing meiotic maturation and developing to blastocyst stage after in vitro fertilization compared with oocytes in COCs not infected with viruses.

Compared with other studies using adenoviral vectors, relatively higher viral concentration was required to achieve the effect observed in this study. This is probably because of the difference in the condition of culture (e.g. two-dimensional mono-layers vs. three-dimensional COCs). Since cells cultured as mono-layer are likely to have more opportunity for contact with viral particles than those cultured as COCs, the efficiency of viral infection into cells cultured as intact COCs appears to be significantly lower than that into mono-layer

cells. In addition, a recent study has shown that a rodent specific gene, *Clsp* (also known as *Gm1123*) encoding Car-like soluble protein that might play a role in the anti-viral defense, is expressed in mouse brain and ovary (Kawabata et al. 2007). Although the identity of the cell types expressing CLSP in the ovary is currently not determined, it is possible that mouse granulosa cells are more protected from viral infection by this protein.

Levels of cumulus expansion-related transcripts in COCs infected with viruses tended to be up-regulated compared with those in non-infected COCs. Interestingly, the *PTGS2*, *PTX3*, and *TNFAIP6*, which are necessary for cumulus expansion, also participate in immune/inflammatory response (Richards et al. 2002), and several studies have shown that infecting with recombinant virus itself induced immune-like responses (Culver and Laster 2007; Russell 2000). However, it is important to note that levels of *Ptgs2*, *Ptx3*, *Tnfaip6* mRNAs in adenovirus infected COCs were considerably lower before EGF treatment, and the robust increase of these transcripts occurred only after EGF treatment. Therefore, expression of these transcripts after viral infection still requires EGF stimulation. Clearly, further studies are required for assessing the reasons for the elevated transcript levels. However, one explanation could be that viral infection causes greater sensitivity of cumulus cells to EGF stimulation resulting in higher levels of expression of these immune/inflammatory-related and cumulus expansion-related transcripts. Importantly, levels of the other transcripts not related to cumulus expansion that were assessed in this study were not stimulated by viral infection.

The mRNA levels of *Areg* and *Ereg*, but not *Btc* in *Has2* shRNA-expressing COCs were significantly lower than those in COCs expressing control shRNA. Although the mechanisms underlying gene regulation during cumulus expansion are not fully understood, a reasonable model was proposed by Shimada et al. (2006). According to their model, expression of the EGF-like growth factors in cumulus cells is promoted by prostaglandin E2 secreted from cumulus and mural granulosa cells by autocrine and paracrine mechanisms. Supporting this model, they showed that mRNA levels of *Areg* and *Ereg*, but not *Btc*, in COCs in *Ptgs2* null mice were decreased compared with those in wild-type mice. Coincidentally, in the present study, mRNA levels of *Areg* and *Ereg*, but not *Btc*, were decreased by specific suppression of *Has2* expression, whereas *Ptgs2* mRNA levels were not affected. One possibility is that proper formation of extracellular matrix during cumulus expansion is required for retaining prostaglandin E2 produced by cumulus cells within the cumulus oophorus; thus, without sufficient production of hyaluronan, prostaglandin E2 diffuses into medium, impairing efficient transduction of its signal cascade. Supporting this hypothesis, *Areg* and *Ereg* mRNA levels, but not *Btc* mRNA, were significantly reduced when absence serum from medium did not allow proper formation of extracellular matrix. Alternatively, it is possible that hyaluronan/hyaluronan receptor signaling plays a critical role in *Areg* and *Ereg* expression, since several studies have shown that mRNA encoding receptors for hyaluronan, *Cd44* and *Hmmr*, are expressed in porcine and bovine cumulus cells, and these transcripts were also detectable in mouse COCs (Hernandez-Gonzalez et al. 2006; Kimura et al. 2002; Schoenfelder and Einspanier 2003).

Although it is generally accepted that hyaluronan synthase is a critical enzyme required for cumulus expansion, no studies have demonstrated participation of the HAS2 isoform in the cumulus expansion process. Homozygous mutation of *Has2* gene results in embryonic lethality during midgestation (Camenisch et al. 2000), thus HAS2 requirement in cumulus expansion could not be assessed. Historically, based on the observations that the expanded cumulus oophorus contains hyaluronan and is readily lysed to single cells by specific hyaluronidase treatment (Ball et al. 1982; Eppig 1979; Leonard and Kurzrok 1945; McClean and Rowlands 1942; Talbot 1984), and a potent inhibitor of glucosamine synthesis completely inhibits cumulus expansion in vitro (Buchanan 1973; Chen et al. 1993), it has

been accepted that activity of hyaluronan synthase is required for cumulus expansion. Subsequently, in 1997, *Has2* was cloned as a novel isoform of hyaluronan synthase family, of which expression increases during cumulus expansion process (Fulop et al. 1997). Therefore, it is likely that HAS2 is one of the key enzymes required for hyaluronan synthesis during cumulus expansion. However, recent studies have shown that other hyaluronan synthase isoforms are also expressed in bovine COCs and porcine oocytes (Kimura et al. 2002; Schoenfelder and Einspanier 2003). It was possible that these hyaluronan synthase isoforms and/or other unidentified homologues of the hyaluronan synthase related proteins could compensate for the reduction of HAS2 activity, and specific inhibition of the HAS2 isoform could have little effect on the cumulus expansion. The shRNA sequence targeting *Has2* mRNA used in the present study has no homology to other known hyaluronan synthase genes and therefore do not target them. That specific silencing of *Has2* mRNA levels significantly attenuated the cumulus expansion, strongly suggests that HAS2 is the primary hyaluronan synthase isoform required for cumulus expansion and no other hyaluronan synthase isoforms can compensate for the absence of HAS2 activity.

In summary, we have demonstrated that expression of a transcript, *Has2*, in cumulus cells cultured as intact COCs can be specifically suppressed by adenovirus-mediated shRNA expression. The efficiency of the *Has2* transcript knockdown in the present study was more than 70% and this reduction was functionally significant. This study shows that adenovirus-mediated introduction of shRNA produces specific gene silencing in intact oocyte-cumulus cell complexes, and provides proof of principle that this method will be a helpful tool for understanding mechanisms and processes involved in development of the oocyte-granulosa cell complex. However, when implementing this approach, especially when studying protein functions during cumulus expansion/ovulation process, the response of cumulus cells to viral infection, independently of the function of delivered silencing constructs, must be properly controlled.

MATERIALS AND METHODS

Adenovirus preparation

Replication incompetent recombinant adenoviruses expressing shRNAs were constructed using BLOCK-iT™ Adenoviral RNAi Expression System (Invitrogen, Carlsbad CA) that consists of BLOCK-iT™ U6 RNAi Entry Vector Kit, Gateway® LR Clonase™ II Enzyme Mix, 293A cell line and pAd/BLOCK-iT™-DEST Gateway® Vector Kit, according to the manufacturer's instructions. The adenoviral vectors produced with this system are based on the second-generation vectors developed by Bett et al. (1994) and expression of the shRNA is controlled by the human U6 promoter (Paddison et al. 2002; Paul et al. 2002).

Sequences of shRNA targeting mouse *Has2* (accession number NM_008216) were designed using Invitrogen's RNAi Designer (www.invitrogen.com/rnai). Two single-stranded DNA oligonucleotides, one encoding a shRNA sequence targeting *Has2* and an additional sequence CACC at 5' end (5'-

CACCGCATTGTGAGAGGTTTCTATGCGAACATAGAAACCTCTCACAATGC-3')

and the other encoding complementary sequences and an additional sequence AAAA at 5' end (5'-

AAAAGCATTGTGAGAGGTTTCTATGTTTCGCATAGAAACCTCTCACAATGC-3')

were purchased (Integrated DNA Technologies, Inc, Coralville, IA). The CACC and AAAA sequences are complementary to the overhang sequences in the pENTR™/U6 vector. These two single-stranded DNA oligonucleotides were annealed and then the generated double-stranded DNA oligonucleotide was cloned into the pENTR™/U6 vector to produce pENTR™/U6-*Has2* shRNA vector using BLOCK-iT™ U6 RNAi Entry Vector Kit

(Invitrogen). The ligation reaction product was introduced into competent *E. coli*, and individual clones were isolated and sequenced to confirm the shRNA coding sequence.

The U6 RNAi cassette in the pENTR™/U6-*Has2* shRNA vector (consisting of the human U6 promoter, the sequence encoding *Has2* shRNA and the polymerase III terminator (Bogenhagen and Brown 1981)) was transferred to the adenoviral expression plasmid by LR recombination reaction using Gateway® LR Clonase™ II Enzyme Mix and pAd/BLOCK-iT™-DEST Gateway® Vector Kit (Invitrogen). The LR recombination reaction product was introduced into competent *E. coli* and individual clones were isolated. Then the isolated adenoviral expression and pAd-GW/U6-lamin^{shRNA} plasmids, that was included in the pAd/BLOCK-iT™-DEST Gateway® Vector Kit, were digested with the restriction enzyme, *Pac I* (New England Biolabs, Inc., Ipswich, MA). *Pac I*-digested plasmids were transfected into 293A cells using Lipofectamine™ 2000 (Invitrogen) to produce crude adenoviral stocks. The crude adenoviral stocks were used to infect 293A cells to amplify the adenoviral stocks. These stocks were purified using the Adeno-X™ Virus Purification Kit (Clontech Laboratories, Inc., Palo Alto, CA) and stored at -80°C. The titers of the adenoviral stocks were determined using Adeno-X™ Rapid Titer Kit (Clontech Laboratories) with 293A cells, and adjusted to 3×10^{11} infectious units (ifu)/ml using the Elution buffer provided in the Adeno-X™ Virus Purification Kit.

COC isolation and viral infection

B6SJLF1 mice were bred and raised in the research colony of the authors at The Jackson Laboratory and used for all experiments. Animals were maintained according to the Guide for the Care and Use of Laboratory Animals (Institute for Learning and Animal Research). COCs were isolated from antral follicles of 22 to 24-day-old mice that had been primed with 5 IU equine chorionic gonadotropin 44 to 48 hr earlier as reported previously (Sugiura et al. 2005). The basic culture medium used was bicarbonate-buffered MEM α (Life Technologies, Inc., Grand Island, NY) with Earle's salts, supplemented with 75 μ g/ml penicillin G, 50 μ g/ml streptomycin sulfate, 0.23 mM pyruvate, and 3 mg/ml bovine serum albumin (Sigma-Aldrich Co., St. Louis, MO).

Twenty COCs were infected with the viruses for 24 hr in one well of 4-well (Nalge Nunc International, Rochester, NY) or 24-well plates (Corning Inc., Lowell, MA) in 250 μ l volume of the basic culture medium supplemented with the phosphodiesterase inhibitor, milrinone (10 μ M) (Sigma) (to maintain the oocytes at germinal vesicle stage) and 10^{-8} M 17 β -estradiol (Sigma) with the viral concentrations indicated in the text. After 24 hr of infection, the cells were washed thoroughly and cultured for additional 24 hr in fresh medium. All the cultures were maintained at 37°C in a modular incubation chamber (Billups Rothenberg, Del Mar, CA) infused with 5% O₂, 5% CO₂ and 90% N₂.

Induction and evaluation of cumulus expansion

Twenty four hr after the viral infection period, the COCs were washed thoroughly in the basic culture medium, without milrinone (allowing oocyte maturation to occur) or 17 β -estradiol but supplemented with 5% fetal bovine serum (FBS) and then cultured in 50 μ l of culture medium supplemented with 5% FBS and with or without 10 ng/ml epidermal growth factor (EGF, 10 ng/ml, BD Biosciences, San Jose, CA) under washed mineral oil in 35-mm Petri dishes. In some experiments, freshly isolated COCs were cultured in the basic culture medium supplemented with or without 5% FBS or 10 ng/ml EGF. Samples of COCs were taken before (0 hr) and after (3, 6, 9 and 12 hr) induction of cumulus expansion to assess levels of mRNAs with real-time PCR. The cumulus expansion index was evaluated after 16 hr of culture as reported previously (Eppig et al. 1993).

Fertilization and preimplantation embryonic development in vitro

For in vitro fertilization of oocytes, fetuin (1 mg/ml) was added to the culture medium during viral infection- and subsequent 24 h-culture periods to prevent zona pellucida hardening (Eppig et al. 1992). In vitro maturation and fertilization of oocytes and preimplantation embryonic development were conducted as described previously (Eppig and O'Brien 1996). After maturation culture, prior to in vitro fertilization, oocytes were denuded of cumulus cells by hyaluronidase treatment (Sigma) and pipetting to assess germinal vesicle breakdown (GVB, a marker of resumption of meiosis) and emission of the first polarbody (Pb1) as described previously (Eppig and O'Brien 1996).

Reverse-transcription (RT) real-time polymerase chain reaction (PCR)

RT-real-time PCR analysis was conducted as previously reported (Sugiura et al. 2007). The PCR primers used are presented in Table 1. The other PCR primers used are reported previously (*Has2*, *Ptgs2*, *Ptx3*, *Tnfaip6* (Diaz et al. 2006), *Rpl19*, *Mvd* (Su et al. 2008), and *Ldha* (Sugiura et al. 2007)). The results are presented as the relative expression levels to the transcript amount of a standard sample, as indicated in the text, after normalization to the expression levels of a housekeeping gene, *Rpl19*, by the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). To avoid false positive signals, dissociation-curve analyses were performed at the end of analyses, and the PCR products were applied to agarose gel electrophoresis to confirm the sizes. Moreover, the PCR products were purified and were sequenced to verify sequence identity as preliminary experiments. The reactions were conducted at least in duplicate.

Statistical analysis

All experiments were repeated at least 3 times and statistical analyses were conducted using computer software JMP (SAS Institute, Inc., Cary, NC). The Tukey-Kramer HSD test was used to compare multiple groups, and a student's t-test was used for paired comparison. A *p* value <0.05 was considered statistically significant.

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REFERENCES

- Ball GD, Bellin ME, Ax RL, First NL. Glycosaminoglycans in bovine cumulus-oocyte complexes: morphology and chemistry. *Mol Cell Endocrinol.* 1982; 28(1):113–122. [PubMed: 6290289]
- Bett AJ, Haddara W, Prevec L, Graham FL. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc Natl Acad Sci U S A.* 1994; 91(19):8802–8806. [PubMed: 8090727]
- Bogenhagen DF, Brown DD. Nucleotide sequences in *Xenopus* 5S DNA required for transcription termination. *Cell.* 1981; 24(1):261–270. [PubMed: 6263489]
- Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. *Science.* 2002; 296(5567):550–553. [PubMed: 11910072]
- Buccione R, Vanderhyden BC, Caron PJ, Eppig JJ. FSH-induced expansion of the mouse cumulus oophorus in vitro is dependent upon a specific factor(s) secreted by the oocyte. *Dev Biol.* 1990; 138:16–25. [PubMed: 2155145]
- Buchanan JM. The amidotransferases. *Adv Enzymol Relat Areas Mol Biol.* 1973; 39:91–183. [PubMed: 4355768]
- Camenisch TD, Spicer AP, Brehm-Gibson T, Biesterfeldt J, Augustine ML, Calabro A Jr, Kubalak S, Klewer SE, McDonald JA. Disruption of hyaluronan synthase-2 abrogates normal cardiac

- morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. *J Clin Invest.* 2000; 106(3):349–360. [PubMed: 10930438]
- Chen L, Mao SJ, Larsen WJ. Identification of a factor in fetal bovine serum that stabilizes the cumulus extracellular matrix. A role for a member of the inter-alpha-trypsin inhibitor family. *J Biol Chem.* 1992; 267(17):12380–12386. [PubMed: 1376324]
- Chen L, Russell PT, Larsen WJ. Functional significance of cumulus expansion in the mouse: roles for the preovulatory synthesis of hyaluronic acid within the cumulus mass. *Molecular Reproduction and Development.* 1993; 34(1):87–93. [PubMed: 8418823]
- Culver CA, Laster SM. Adenovirus type 5 exerts multiple effects on the expression and activity of cytosolic phospholipase A2, cyclooxygenase-2, and prostaglandin synthesis. *J Immunol.* 2007; 179(6):4170–4179. [PubMed: 17785856]
- Diaz FJ, O'Brien MJ, Wigglesworth K, Eppig JJ. The preantral granulosa cell to cumulus cell transition in the mouse ovary: development of competence to undergo expansion. *Dev Biol.* 2006; 299(1):91–104. [PubMed: 16908014]
- Diaz FJ, Wigglesworth K, Eppig JJ. Oocytes determine cumulus cell lineage in mouse ovarian follicles. *J Cell Sci.* 2007; 120(Pt 8):1330–1340. [PubMed: 17389684]
- Eppig JJ. FSH stimulates hyaluronic acid synthesis by oocyte-cumulus cell complexes from mouse preovulatory follicles. *Nature.* 1979; 281(5731):483–484. [PubMed: 492307]
- Eppig JJ. Oocyte control of ovarian follicular development and function in mammals. *Reproduction.* 2001; 122(6):829–838. [PubMed: 11732978]
- Eppig JJ, O'Brien MJ. Development in vitro of mouse oocytes from primordial follicles. *Biol Reprod.* 1996; 54(1):197–207. [PubMed: 8838017]
- Eppig JJ, Wigglesworth K, O'Brien MJ. Comparison of embryonic developmental competence of mouse oocytes grown with and without serum. *Mol Reprod Dev.* 1992; 32(1):33–40. [PubMed: 1381197]
- Eppig JJ, Wigglesworth K, Chesnel F. Secretion of cumulus expansion enabling factor by mouse oocytes: relationship to oocyte growth and competence to resume meiosis. *Dev Biol.* 1993; 158(2):400–409. [PubMed: 8344458]
- Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature.* 1998; 391(6669):806–811. [PubMed: 9486653]
- Fulop C, Salustri A, Hascall VC. Coding sequence of a hyaluronan synthase homologue expressed during expansion of the mouse cumulus-oocyte complex. *Arch Biochem Biophys.* 1997; 337(2):261–266. [PubMed: 9016821]
- Fulop C, Szanto S, Mukhopadhyay D, Bardos T, Kamath RV, Rugg MS, Day AJ, Salustri A, Hascall VC, Glant TT, Mikecz K. Impaired cumulus mucification and female sterility in tumor necrosis factor-induced protein-6 deficient mice. *Development.* 2003; 130(10):2253–2261. [PubMed: 12668637]
- Hernandez-Gonzalez I, Gonzalez-Robayna I, Shimada M, Wayne CM, Ochsner SA, White L, Richards JS. Gene expression profiles of cumulus cell oocyte complexes during ovulation reveal cumulus cells express neuronal and immune-related genes: does this expand their role in the ovulation process? *Mol Endocrinol.* 2006; 20(6):1300–1321. [PubMed: 16455817]
- Hirano T, Yamauchi N, Sato F, Soh T, Hattori MA. Evaluation of RNA interference in developing porcine granulosa cells using fluorescence reporter genes. *J Reprod Dev.* 2004; 50(5):599–603. [PubMed: 15514468]
- Hommel JD, Sears RM, Georgescu D, Simmons DL, DiLeone RJ. Local gene knockdown in the brain using viral-mediated RNA interference. *Nat Med.* 2003; 9(12):1539–1544. [PubMed: 14634645]
- Jo M, Curry TE Jr. Luteinizing hormone-induced RUNX1 regulates the expression of genes in granulosa cells of rat periovulatory follicles. *Mol Endocrinol.* 2006; 20(9):2156–2172. [PubMed: 16675540]
- Kawabata K, Tashiro K, Sakurai F, Osada N, Kusuda J, Hayakawa T, Yamanishi K, Mizuguchi H. Positive and negative regulation of adenovirus infection by CAR-like soluble protein, CLSP. *Gene Ther.* 2007; 14(16):1199–1207. [PubMed: 17538635]

- Kimura N, Konno Y, Miyoshi K, Matsumoto H, Sato E. Expression of hyaluronan synthases and CD44 messenger RNAs in porcine cumulus-oocyte complexes during in vitro maturation. *Biol Reprod.* 2002; 66(3):707–717. [PubMed: 11870078]
- Kobayashi SI, Sakatani M, Kobayashi S, Okuda K, Takahashi M. Gene Silencing of Cyclooxygenase-2 mRNA by RNA Interference in Bovine Cumulus-Granulosa cells. *J Reprod Dev.* 2007
- Kwintkiewicz J, Cai Z, Stocco C. Follicle-stimulating hormone-induced activation of Gata4 contributes in the up-regulation of Cyp19 expression in rat granulosa cells. *Mol Endocrinol.* 2007; 21(4):933–947. [PubMed: 17227882]
- Leonard SL, Kurzrok R. A study of the hyaluronidase effect in the collicle cells of ovulated rat ova. *Endocrinology.* 1945; 37:171–176.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (Duluth).* 2001; 25(4):402–408.
- Matzuk MM, Finegold MJ, Su JG, Hsueh AJ, Bradley A. Alpha-inhibin is a tumour-suppressor gene with gonadal specificity in mice. *Nature.* 1992; 360(6402):313–319. [PubMed: 1448148]
- McClellan D, Rowlands IW. Role of hyaluronidase in fertilization. *Nature.* 1942; 150:627–628.
- Ochsner SA, Day AJ, Rugg MS, Breyer RM, Gomer RH, Richards JS. Disrupted function of tumor necrosis factor-alpha-stimulated gene 6 blocks cumulus cell-oocyte complex expansion. *Endocrinology.* 2003a; 144(10):4376–4384. [PubMed: 12959984]
- Ochsner SA, Russell DL, Day AJ, Breyer RM, Richards JS. Decreased expression of tumor necrosis factor-alpha-stimulated gene 6 in cumulus cells of the cyclooxygenase-2 and EP2 null mice. *Endocrinology.* 2003b; 144(3):1008–1019. [PubMed: 12586778]
- Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, Conklin DS. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev.* 2002; 16(8):948–958. [PubMed: 11959843]
- Parakh TN, Hernandez JA, Grammer JC, Weck J, Hunzicker-Dunn M, Zeleznik AJ, Nilson JH. Follicle-stimulating hormone/cAMP regulation of aromatase gene expression requires beta-catenin. *Proc Natl Acad Sci U S A.* 2006; 103(33):12435–12440. [PubMed: 16895991]
- Park JY, Su YQ, Ariga M, Law E, Jin SL, Conti M. EGF-like growth factors as mediators of LH action in the ovulatory follicle. *Science.* 2004; 303(5658):682–684. [PubMed: 14726596]
- Paul CP, Good PD, Winer I, Engelke DR. Effective expression of small interfering RNA in human cells. *Nat Biotechnol.* 2002; 20(5):505–508. [PubMed: 11981566]
- Richards JS. Ovulation: new factors that prepare the oocyte for fertilization. *Mol Cell Endocrinol.* 2005; 234(1–2):75–79. [PubMed: 15836955]
- Richards JS, Russell DL, Ochsner S, Espey LL. Ovulation: new dimensions and new regulators of the inflammatory-like response. *Annu Rev Physiol.* 2002; 64:69–92. [PubMed: 11826264]
- Russell WC. Update on adenovirus and its vectors. *J Gen Virol.* 2000; 81(Pt 11):2573–2604. [PubMed: 11038369]
- Schoenfelder M, Einspanier R. Expression of hyaluronan synthases and corresponding hyaluronan receptors is differentially regulated during oocyte maturation in cattle. *Biol Reprod.* 2003; 69(1):269–277. [PubMed: 12620932]
- Shimada M, Hernandez-Gonzalez I, Gonzalez-Robayna I, Richards JS. Paracrine and autocrine regulation of epidermal growth factor-like factors in cumulus oocyte complexes and granulosa cells: key roles for prostaglandin synthase 2 and progesterone receptor. *Mol Endocrinol.* 2006; 20(6):1352–1365. [PubMed: 16543407]
- Shimada M, Yanai Y, Okazaki T, Yamashita Y, Sriraman V, Wilson MC, Richards JS. Synaptosomal-associated protein 25 gene expression is hormonally regulated during ovulation and is involved in cytokine/chemokine exocytosis from granulosa cells. *Mol Endocrinol.* 2007; 21(10):2487–2502. [PubMed: 17595323]
- Shimasaki S, Moore RK, Otsuka F, Erickson GF. The bone morphogenetic protein system in mammalian reproduction. *Endocr Rev.* 2004; 25(1):72–101. [PubMed: 14769828]
- Su YQ, Sugiura K, Wigglesworth K, O'Brien MJ, Affourtit JP, Pangas SA, Matzuk MM, Eppig JJ. Oocyte regulation of metabolic cooperativity between mouse cumulus cells and oocytes: BMP15

- and GDF9 control cholesterol biosynthesis in cumulus cells. *Development*. 2008; 135(1):111–121. [PubMed: 18045843]
- Sugiura K, Pendola FL, Eppig JJ. Oocyte control of metabolic cooperativity between oocytes and companion granulosa cells: energy metabolism. *Dev Biol*. 2005; 279(1):20–30. [PubMed: 15708555]
- Sugiura K, Su YQ, Diaz FJ, Pangas SA, Sharma S, Wigglesworth K, O'Brien MJ, Matzuk MM, Shimasaki S, Eppig JJ. Oocyte-derived BMP15 and FGFs cooperate to promote glycolysis in cumulus cells. *Development*. 2007; 134(14):2593–2603. [PubMed: 17553902]
- Sui G, Soohoo C, Affar el B, Gay F, Shi Y, Forrester WC, Shi Y. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc Natl Acad Sci U S A*. 2002; 99(8):5515–5520. [PubMed: 11960009]
- Talbot P. Hyaluronidase dissolves a component in the hamster zona pellucida. *J Exp Zool*. 1984; 229(2):309–316. [PubMed: 6736889]
- Tamura K, Matsushita M, Endo A, Kutsukake M, Kogo H. Effect of insulin-like growth factor-binding protein 7 on steroidogenesis in granulosa cells derived from equine chorionic gonadotropin-primed immature rat ovaries. *Biol Reprod*. 2007; 77(3):485–491. [PubMed: 17522074]
- Vanderhyden BC, Caron PJ, Buccione R, Eppig JJ. Developmental pattern of the secretion of cumulus-expansion enabling factor by mouse oocytes and the role of oocytes in promoting granulosa cell differentiation. *Dev Biol*. 1990; 140:307–317. [PubMed: 2115479]
- Varani S, Elvin JA, Yan C, DeMayo J, DeMayo FJ, Horton HF, Byrne MC, Matzuk MM. Knockout of pentraxin 3, a downstream target of growth differentiation factor-9, causes female subfertility. *Mol Endocrinol*. 2002; 16(6):1154–1167. [PubMed: 12040004]
- Weigel PH, Hascall VC, Tammi M. Hyaluronan synthases. *J Biol Chem*. 1997; 272(22):13997–14000. [PubMed: 9206724]
- Xia H, Mao Q, Paulson HL, Davidson BL. siRNA-mediated gene silencing in vitro and in vivo. *Nat Biotechnol*. 2002; 20(10):1006–1010. [PubMed: 12244328]
- Yu JY, DeRuiter SL, Turner DL. RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc Natl Acad Sci U S A*. 2002; 99(9):6047–6052. [PubMed: 11972060]

Figure 1. Effect of *Has2* shRNA expression on levels of transcripts encoding HAS2 or proteins not involved in the cumulus expansion process

COCs infected with adenoviruses containing expression sequences of either *Has2* shRNA (solid bar) or *LMNA* shRNA (hatched bar) or without any viruses (open bar) were sampled after 6 hr of EGF treatment, and expression levels of (A) *Has2* mRNA and (B) mRNAs encoding proteins not involved in the cumulus expansion process, but are known to play important roles in functions of cumulus cells, were examined with real-time PCR. Data are presented as the relative levels to the control non-virus-infected (+EGF) groups (mean $\pm$ SEM). An asterisk denotes a significant difference between the two indicated groups ($p < 0.05$).

Figure 2. Representative photographs of virus-infected COCs after induction of cumulus expansion

COCs infected with adenoviruses containing expression sequences of either *Has2* shRNA (F–H) or *LMNA* shRNA (C–E) or without any viruses (A, B) were treated with (B–H) or without (A) EGF for 16 hr. After 16 hr, oocytes were denuded of cumulus cells and assessed for GVB and the first polarbody emission (insert). Bars, 100 μ m.

Figure 3. Effect of *Has2* shRNA expression on cumulus expansion

COCs infected with adenoviruses containing expression sequences of either *Has2* shRNA (solid bar) or *LMNA* shRNA (hatched bar) or without any viruses (open bar) were treated with EGF, and cumulus expansion index was assessed after 16 hr. Mean $\pm$ SEM. An asterisk denotes a significant difference between the two indicated groups ($p < 0.05$).

Figure 4. Effect of exposure to virus on the developmental competence of the oocytes

Oocytes in COCs infected with adenoviruses containing expression sequences of either *Has2* shRNA (solid bar) or *LMNA* shRNA (hatched bar) or without any viruses (open bar) were matured and fertilized in vitro, and the preimplantation embryonic development were assessed. GVB, oocytes underwent germinal vesicle breakdown. Pb1, oocytes emitted the first polarbody. 2-cell, 2-cell embryo. Blast, blastocyst embryo.

Figure 5. Effect of *Has2* shRNA expression on mRNA levels of *Ptgs2*, *Ptx3* and *Tnfaip6* during cumulus expansion

COCs infected with adenoviruses containing expression sequences of either *Has2* shRNA (solid bar) or *LMNA* shRNA (hatched bar) or without any viruses (open bar) were sampled after 6 hr of EGF treatment, and expression levels of *Ptgs2*, *Ptx3* and *Tnfaip6* mRNA were examined with real-time PCR. Data are presented as the relative levels to the control non-virus-infected (+EGF) groups (mean $\pm$ SEM). The letter “a” denotes a significant difference from the control groups ($p < 0.05$). An asterisk denotes a significant difference between the two indicated groups ($p < 0.05$).

Figure 6. Effect of *Has2* shRNA expression on levels of transcripts encoding EGF-like growth factors during cumulus expansion

COCs infected with adenoviruses containing expression sequences of either *Has2* shRNA (solid bar) or *LMNA* shRNA (hatched bar) or without any viruses (open bar) were sampled after 6 hr of EGF treatment, and expression levels of *Areg*, *Btc* and *Ereg* mRNA were examined with real-time PCR. Data are presented as the relative levels to the control non-virus-infected (+EGF) groups (mean $\pm$ SEM). The letter "a" denotes a significant difference from the control groups ($p < 0.05$). An asterisk denotes a significant difference between the two indicated groups ($p < 0.05$).

Figure 7. Effect of serum removal from culture medium on levels of the cumulus expansion-related transcripts

Freshly isolated COCs were cultured with or without EGF or serum and sampled after 6 hr of EGF treatment, and expression levels of the cumulus expansion-related transcripts were examined with real-time PCR. An asterisk denotes a significant difference between the two indicated groups ($p < 0.05$).

Figure 8. The kinetics of *Has2*, *Ptgs2*, *Ptx3* and *Tnfaip6* mRNA expression in *Has2* shRNA expressing COCs during cumulus expansion
 (A) Representative photographs of COCs at different time points were shown. Bar, 100 μ m.
 (B) COCs infected with adenoviruses containing expression sequences of either *Has2* shRNA (solid bar) or *LMNA* shRNA (hatched bar) or without any viruses (open bar) were sampled at the time points indicated, and expression levels of *Has2*, *Ptgs2*, *Ptx3* and *Tnfaip6* mRNAs were examined with real-time PCR. Data are presented as the relative levels to the control non-virus-infected groups at 6 hr (mean $\pm$ SEM). *, $p < 0.05$.

Table 1

Sequence of PCR primers used for real-time PCR

Gene	Forward	Reverse
<i>Areg</i>	TCCAAGATTGCAGTAGTAGCTGTCA	TATCGTTTCAAAGGTGCACTGT
<i>Btc</i>	AACTGCACAGGTACCACCCCTAGA	ACAGATGCAGGAGGGAGTTTGC
<i>Ereg</i>	CCATCATGCATCCCAGGAGAA	TAGCCGTCCATGTCAGAACTACACT
<i>Bmpr2</i>	CTTTCACCCCCTGACACAA	GCGACTATCAAAACAGCTAACACAGA
<i>Inha</i>	GAAGATGTCTCCCAGGCTATCCTT	TGGCCGGAATACATAAGTGAAGA
<i>Smad4</i>	GCCAGCTTCTCTGTCCAGGTAGTA	TGTCCACAGGACAGAAGCGATT