Basonuclin: a novel mammalian maternal-effect gene

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Basonuclin is a zinc-finger protein found in abundance in oocytes. It qualifies as a maternal-effect gene because the source of pre-implantation embryonic basonuclin is maternal. Using a transgenic-RNAi approach, we knocked down basonuclin specifically in mouse oocytes, which led to female sub-fertility. Basonuclin deficiency in oocytes perturbed both RNA polymerase I- and II-mediated transcription, and oocyte morphology was affected (as evidenced by cytoplasmic and cell surface abnormalities). Some of the affected oocytes, however, could still mature to and arrest at metaphase II, and be ovulated. Nevertheless, fertilized basonuclin-deficient eggs failed to develop beyond the two-cell stage, and this pre-implantation failure accounted for the sub-fertility phenotype. These results suggest that basonuclin is a new member of the mammalian maternal-effect genes and, interestingly, differs from the previously reported mammalian maternal-effect genes in that it also apparently perturbs oogenesis.

Key WORDS: Female fertility, Oogenesis, Pre-implantation development, Transcription regulation, Transgenic RNAi

INTRODUCTION

Maternal effect, which can refer to the dependence of early embryonic development on maternal products, is common in both animal and plant kingdoms. This dependence is on maternal proteins and mRNAs that are recruited during oocyte maturation or following fertilization. The dependence on maternal mRNA is because the fully grown oocyte and the one-cell embryo are essentially transcriptionally silent but translationally active, and the maternal-to-zygotic transition occurs during the late one-cell/early two-cell stages (Ko et al., 2000; Schultz, 2002; Wang et al., 2004). Mammalian maternal-effect genes have only been identified recently, largely by gene-knockout approaches [e.g. Mater (Nalp5 – Mouse Genome Informatics), Hsf1, Zar1, Npm2 and Stella (Dppa3 – Mouse Genome Informatics)]. Unlike their counterparts in invertebrates and lower vertebrates, the molecular mechanisms of mammalian maternal-effect genes have not been well understood. In the strictest sense, mutations in maternal-effect genes should not affect oocyte development, maturation and ovulation, but should impair embryo development. Indeed, null mutations of the mammalian maternal-effect genes identified so far have little effect on oocyte maturation and fertilization, and interferes only with early embryo development (Tong et al., 2000; Christians et al., 2000; Wu et al., 2003; Burns et al., 2003; Payer et al., 2003). Hence, genes expressed in oocytes can be classified as required for oogenesis (i.e. oocyte development, maturation, ovulation and fertilization) and for embryonic development (i.e. maternal effect) (Dean, 2002).

A hallmark of oocyte development is the synthesis and accumulation of macromolecules and organelles that constitute the maternal capital used to support early development prior to activation of the embryonic genome (Schultz, 1993). In mammals, this maternal store includes proteins and RNAs, which are involved in a wide range of processes, as evidenced by the variety of implicated functions of the currently identified maternal-effect genes (Tong et al., 2000; Christians et al., 2000; Wu et al., 2003; Burns et al., 2003; Payer et al., 2003). Furthermore, it is still not clear how mammalian oocytes monitor and respond to a deficiency in maternal-effect genes, even if malfunction of these genes does not directly affect oocyte maturation itself.

Basonuclin (Bnc1 – Mouse Genome Informatics) is a zinc-finger protein with a very restricted tissue distribution; it is mainly found in keratinocytes of the stratified epithelium and germ cells (i.e. male germ cells and oocytes) (Yang et al., 1997; Mahoney et al., 1998). There is highly suggestive evidence that basonuclin regulates rRNA transcription (Iuchi and Green, 1999; Tseng et al., 1999). For example, the zinc fingers of basonuclin interact with three evolutionarily conserved binding sites in the rDNA promoter (Tian et al., 2001). In mouse oocytes, basonuclin co-localizes with RNA polymerase I (Pol I) activity in the nucleolus, and a dominant-negative mutant of basonuclin interferes with Pol I transcription in oocytes (Tian et al., 2001). Basonuclin, however, differs from the dedicated Pol I transcription factors (e.g. UBF), in that it is also abundant in nucleoplasm and can interact with Pol II promoters (Tseng et al., 1999). Such observations led to the proposal that basonuclin may have target genes other than rDNA (Tian et al., 2001). Together, these results led us to propose that basonuclin is necessary for oocyte rRNA synthesis and possibly other processes, and perturbing basonuclin function in the oocyte will disrupt oogenesis, leading to infertility. To test this hypothesis, we employed a recently developed transgenic-RNAi approach (Svoboda et al., 2000; Stein et al., 2003) to inhibit basonuclin function specifically in mouse oocytes.

We report here that transgenic-RNAi effectively and specifically reduces basonuclin level in mouse oocytes. The transgenic mice are apparently normal, except females are subfertile. Basonuclin-deficient oocytes display a reduced rate of Pol I transcription and perturbation of a large number of Pol II-transcribed genes. Many basonuclin-deficient oocytes appear morphologically and biochemically abnormal, but their maturation and ovulation proceed without apparent problems. Although normal numbers of eggs are ovulated and inseminated, pre-implantation development is severely compromised. Thus, basonuclin joins the short, but growing, list of mammalian maternal-effect genes; among them, it is the first to also perturb oogenesis.
MATERIALS AND METHODS
RNAi transgene construct
Two transgenic constructs differing in their vector backbones and sequence of the basonuculin inverted repeats were employed in this study (see also Results). The Zp3 promoter directs oocyte-specific expression of the basonuculin double-stranded (ds) RNA. To prepare the inverted repeat for generating the dsRNA hairpin, basonuculin-coding sequence was amplified by PCR and then ligated 3’ to 3’. The inverted repeat was transferred to pRNAi-ZP3 cassette (Stein et al., 2003), to yield pB1-rnai-zp3-1 or and pB1rani-zp3-2.

Generating transgenic mice
The transgene construct was used for microinjection at the Transgenic and Chimeric Mouse Facility at University of Pennsylvania and Transgenic Animal Model Core at University of Michigan with the approval of the Institutional Animal Care and Use Committee at the respective universities. The zygotes for microinjection were produced by mating of B6SJLF1/J mice. Transgenic out-crossed (TG-OC) mice were obtained by mating transgenic founder males (B6SJLF1/J) with wild-type females (CF1), and genotyped by PCR using the EGF primer pair (5’-CGACGTA-AACGGGCAAAGT and 5’-CTCGTGGGGCTTTTGCATCA) with 34 cycles at 55°C for 20 seconds, 72°C for 40 seconds and 94°C for 20 seconds.

Fertility
Each founder transgenic female (B6SJLF1/J) was mated with the same three stud males (B6SJLF1/J) in a random sequence. The same males were also used to assess the fertility of TG-OC females. Successful mating was verified by detecting a vaginal plug; the mated females were then housed separately for observation and recording the number of pups delivered per litter, which was averaged to assess fertility. Other than the fertility tests, all further studies were carried out with TG-OC females.

Collection and culture of oocytes and embryos
GV, MII eggs and fertilized eggs were collected as previously described (Schultz et al., 1983). GV oocytes were cultured in CZB medium (Chatot et al., 1989) containing 0.2 mM IBMX (Sigma) to inhibit GV breakdown; MII eggs were cultured in CZB medium and fertilized eggs cultured in KSOM medium (Erbach et al., 1994) and their development was recorded at 36, 60 and 84 hours post-coitum (hpc).

RNA isolation and real-time PCR
Total RNA was isolated from 20 oocytes and reverse transcribed with Superscript II reverse transcriptase (Invitrogen) using oligo dT as primer. The resulting cDNA was quantified by real-time PCR using an ABI Prism 7000 thermocycler (Applied Biosystems). The primers for basonuculin were: 5’-ACTGGACCCTGGAATTACATC and 5’-CCTCGTGGGGCTTTTGCATCA with 34 cycles at 55°C for 20 seconds, 72°C for 40 seconds and 94°C for 20 seconds.

Fertilization
Fertilized eggs were collected at 10 hpc and labeled with 10 μM BrdU for 6 hours. The embryos were then fixed with paraformaldehyde, treated with 2 N HCl at 37°C for 1 hour, and then neutralized in 0.1 M borate buffer (pH 8.5), for 15 minutes. Incorporated BrdU was visualized with 1:30 diluted Alexa fluor 488-conjugated anti-BrdU antibody (Invitrogen). Run-on assays and quantification of the transcription loci
Run-on assays were conducted on meiotically incompetent oocytes obtained from 13- to 14-day-old mice as previously described (Tian et al., 2001). Briefly, oocytes were permeabilized and then incubated at 37°C for 1 hour with 0.2 mM Br-UTP and 100 μg/ml α-amanitin. Oocytes were then fixed in 2% paraformaldehyde and the incorporated BrdU was detected with Alexa Fluor 488-conjugated anti-BrdU monoclonal antibody (Molecular Probes) at 1:20 dilution. The amount of incorporated BrdU was quantitated by measuring the overall fluorescence intensity at the largest cross-section of the nucleus (recorded on an epi-fluorescence digital micrograph) with the ‘measurement’ function of the ImageJ, a software package created by W. Rasband (http://rsb.info.nih.gov/ij/). This value, in arbitrary units, was subtracted by the background fluorescence of an area equal in size in the nucleoplasm, which was not labeled in the presence of α-amanitin. The number of labeled transcription foci was scored using microphotographs, which represented a stack of selected optical planes of the nucleolus. The microphotograph stacks were produced with a Zeiss Axiosvert 200 using a 100× objective under epifluorescence and by manual focusing. The informative optical planes containing unique transcription foci were selected manually and photographed. Scoring transcription foci in a nucleolus required usually 8 to 12 microphotographs. The foci that had been scored in the previous plane were marked on photos to avoid repetition. This method also ensured that no informative optical plane was missed because the out-of-focus foci image of the previous plane could be seen.

RNase protection assay
RNase protection assays were conducted to quantify rRNA in oocytes. [32P]-labeled antisense probe for rRNA was prepared using the Maxiscript T7 kit and pTRI- RNA-28s/pTRI-RNA-18S antisense control templates (Ambion). Fifty oocytes were transferred to the lysis buffer of the Direct Protect RPA kit (Ambion). The samples were then incubated with the probe, treated with nuclease and electrophoresed according to the manufacturer’s protocol. Protected fragments were detected by autoradiography and quantified by density measurement.

RNA isolation and microarray analysis
Total RNA was extracted from oocyte samples, each originated from one of the six TG-OC (T#50) and four wild-type eCG-stimulated mice, and subjected to cRNA preparation (Pan et al., 2005; Zeng et al., 2004). The control 1, 3, 4 and transgenic 4, 5, 6 mice were from one litter, and the rest from another. Each Affymetrix MOE 430 2.0 GeneChip was probed with 15 μg of cRNA and processed according to the Affymetrix instructions. Raw microarray data (*-cel) (available at the Gene Expression Omnibus Repository, Accession Number GSE4029) were processed using the GC-RMA or MAS-5 algorithms to obtain the normalized expression summarization for each probe set. The expression level in our data set ranged between 1 and 66,582. Genes with an expression level of less than 10 were excluded before GeneSpring (Silicon Genetics) K-mean hierarchical clustering and other filter analysis. Genes whose expression was significantly changed in the transgenic oocytes compared with the control were identified by Significance Analysis of Microarrays (v1.21, two-class, unpaired analysis with the permutation parameter set at 500, FDR<1%) or by one-way ANOVA analysis (FDR<5%) and annotated in the GeneSpring. The statistically significant gene lists obtained by ANOVA were imported into EASE v2.0 (Hosack et al., 2003) to test for overrepresentation of biological processes in transgenic versus wild-type samples. An EASE score was calculated for the likelihood of overrepresentation in the Gene Ontology Consortium annotation categories (i.e. biological process, cell component, molecular function), as well as KEGG pathways and SwissProt keywords.

Immunofluorescence staining and immunoblotting
Oocytes/embryos were fixed in 2.5% paraformaldehyde/PBS and immunocytochemistry was conducted as previously described (Yu et al., 2004) using an affinity-purified anti-basonuculin antibody (MBP-1023) diluted 1:30. Affinity-purified pre-immune serum was used as a negative control.

For immunoblotting, 75 oocytes/embryos were lysed in 2× SDS-PAGE buffer, run on a 7.5% SDS-PAGE and transferred to PVDF membrane (Hybond-P, Amersham Biosciences). Immunodetection was performed using horsederadhis peroxidase-conjugated secondary antibodies and ECL Advance reagents (Amersham) according to the manufacturer’s instructions.

DNA replication assay
Fertilized eggs were collected at 10 hpc and labeled with 10 μM BrdU for 6 hours. The embryos were then fixed with paraformaldehyde, treated with 2 N HCl at 37°C for 1 hour, and then neutralized in 0.1 M borate buffer (pH 8.5), for 15 minutes. Incorporated BrdU was visualized with 1:30 diluted Alexa fluor 488-conjugated anti-BrdU antibody (Invitrogen).

Histology
For histological analysis, adult ovaries were fixed in Bouin’s fixative for 16 hours before transfer to 70% ethanol. Samples were then embedded in paraffin, serially sectioned (5 μm) and stained with Hematoxylin and Eosin.

TUNEL labeling assay
TUNEL (TdT-mediated dUTP nick end labeling) assays were carried out with the In Situ Cell Death Detection Kit (Roche Diagnostics) according to the manufacturer’s instructions.
Protein synthesis assay and transcription-requiring complex (TRC) quantification
Oocytes were metabolically radiolabeled with [35S]-methionine and acid-insoluble radioactivity was then determined following TCA precipitation as previously described (Conover et al., 1991). Quantification of the transcription-requiring complex (TRC) was also conducted as previously described (Conover et al., 1991).

RESULTS
Expression profile of basonuclin during oogenesis and pre-implantation development
Immunostaining showed that basonuclin was not detected in eight-cell embryos (Q. J. Tian and H.T., unpublished). Consistent with this finding, a recent microarray study indicated that basonuclin mRNA was present in oocytes and one-cell embryos but its level rapidly decreased by over 30-fold during pre-implantation development; by the eight-cell stage, it was at background levels (Zeng et al., 2004). We first confirmed this basonuclin mRNA expression profile by RT-PCR (Fig. 1, broken line) and then established the expression profile for basonuclin protein in oocytes and pre-implantation embryos by immunoblotting and immunocytochemistry. Immunoblotting revealed an apparent decline in basonuclin level from the immature oocyte (GV-stage) to the mature metaphase II (MII) stages (Fig. 1). Similar amounts of basonuclin were present in MII eggs and one-cell embryos, but no basonuclin was detected in two-cell and morulae/blastocysts, which is consistent with the disappearance of its mRNA during pre-implantation development. Immunocytochemical analysis also revealed a similar and progressive decrease of immunoreactive material from oocytes to pre-implantation embryos (Fig. 1). Thus, maternal basonuclin is not replaced by zygotic basonuclin during pre-implantation development and therefore could have a maternal effect.

Generating basonuclin-RNAi transgenic mice
The presence of basonuclin in oocytes and early embryos suggests it performs: (1) the same function for both oocytes and embryos; (2) unrelated functions in oocyte maturation and/or in early embryos; or (3) an oocyte function, which is required by the embryos (maternal effect). To resolve these possibilities, we employed a recently developed transgenic RNAi approach to inhibit basonuclin function in oocytes (Svoboda et al., 2001). We constructed two types of RNAi transgene plasmids, pB1mai-zp3-1 and pB1rani-zp3-2 (Fig. 2A); the details of generating transgenic mice are described in the Materials and methods. Twenty-four founder mice (13 females and 11 males) were obtained, of which 14 were generated by pB1mai-zp3-1 and 10 by pB1mai-zp3-2.

Transgenic basonuclin-RNAi females are sub-fertile
We analyzed the fertility of both founder transgenic females and female progeny of the transgenic founder males (Fig. 2B). To reduce the influence of male fertility on the outcome, three normal males were mated in a randomized sequence with each female being tested. Eight of the nine transgenic founder females showed a wide range of sub-fertility – from moderate to severe (infertile) – when compared with the average of the seven non-transgenic littersmates in the control group (Fig. 2B). The sub-fertility was unlikely to be due to the vector backbone and/or transgene integration sites because the founder females were derived from multiple injections of the two transgene-constructs. Southern analysis also failed to establish any correlation between the integration sites and transgene copy number with the observed reduced fertility (data not shown).

The fertility of transgenic female progeny of the male founders mirrored that of the transgenic female founders (Fig. 2C). Other than female sub-fertility, the transgenic male and female mice appeared normal. Because transgenic females were sub-fertile, we used transgenic males to transmit the transgene and focused subsequent studies on the female progeny of two transgenic lines, T50 and T8, which had ~10% and 50% normal fertility, respectively. To date, the female sub-fertility phenotype of these lines has been stable after two generations of breeding.

Transgenic oocytes contain reduced amounts of basonuclin mRNA and protein
We examined fully grown transgenic oocytes for basonuclin mRNA and protein to determine the cumulative effect of RNAi during the growth phase. Real-time PCR demonstrated that the amount of basonuclin mRNA in transgenic oocytes from both lines was dramatically reduced. For T8 and T50 oocytes, respectively, an additional 4 and 6 cycles were required to reach threshold, when compared with non-transgenic counterparts (Fig. 2D). This increase in cycle number translates into a 94% and 98% reduction in basonuclin mRNA. Indeed, immunocytochemical (Fig. 2F) and western blot (Fig. 2G) examinations confirmed the dramatic decrease in basonuclin protein content in these transgenic oocytes. These assays, however, failed to distinguish the small difference in the severely reduced basonuclin protein levels of the two transgenic lines. Conceivably, the exponential nature of the RT-PCR method is more sensitive in detecting the small variation between the very low basonuclin levels, which were below the level of detection of immunocytochemistry and western blot. Notably, the small
difference in mRNA levels appeared to produce an effect on the fertility in T8 and T50 lines, which were 50% and 90%, respectively (Fig. 2E) and correlate with the 94% and 98% reduction of basonuclin mRNA content.

Our analysis also showed high targeting specificity of the transgenic RNAi approach in oocytes, consistent with previous reports (Stein et al., 2003; Fedoriw et al., 2004; Yu et al., 2004). In transgenic oocytes, no detectable change was observed in the amount of mRNA for UBF, a ubiquitous Pol I-specific transcription factor (Fig. 2D). Furthermore, we observed no difference in the abundance of basonuclin 2 mRNA, which is a closely related homolog and shares more than 57% of the nucleotide sequence with basonuclin (Romano et al., 2004; Vanhoutteghem and Djian, 2004). This high targeting specificity is consistent with a previous report that transgenic RNAi targeting oocyte Msy2 mRNA has no effect on the abundance of the closely related Msy4 transcript (Yu et al., 2004). These results demonstrate the efficacy and specificity of the transgenic RNAi approach. Thus, as predicted, perturbing basonuclin function in oocytes reduces fertility (Tian et al., 2001).

**Increased oocyte developmental failure in basonuclin-deficient oocytes**

To characterize the cause of the sub-fertility phenotype, we first examined the histology of transgenic ovaries. Compared with the control (Fig. 3A,B), histological sections revealed clear changes in follicular structures in adult transgenic ovaries (Fig. 3C,D), which appeared normal in size and weight. Although the number of follicles appeared unaffected, some follicles exhibited abnormal morphologies, which could be classified into two discrete categories. In the first category, the follicles were invariably large and the
oocytes contained cytoplasmic vesicles or cavities (Fig. 3C,D, arrows), which was not observed in non-transgenic ovaries (Fig. 3A,B). In the second category, the follicles were small and irregular in shape, and the oocytes were apparently being degraded and absorbed (Fig. 3C,D arrowheads). Moreover, cumulus cells in contact with the degenerating oocyte had enlarged nucleoli, which were intensely stained by hematoxylin (Fig. 3C,D). Transgenic ovaries contained corpora lutea, suggesting ovulation had occurred. However, when compared with non-transgenic ovary (Fig. 3A,B), the transgenic corpora lutea contained more cells, which made them appear darker because of hematoxylin nuclear staining (Fig. 3C,D). The ovarian morphology suggested heterogeneity in the transgenic oocyte population, some oocytes failed, whereas others succeeded in reaching maturity.

Maturation of basonuclin-deficient oocytes

To characterize the defect of basonuclin-deficient oocytes, GV-intact oocytes were isolated and observed in culture. The isolated oocytes most probably belonged to the subpopulation that survived basonuclin-deficiency during the growth phase. A striking difference between the non-transgenic and transgenic GV-intact oocytes was that ~60% of the latter had dark, granular and even opaque cytoplasm (Fig. 3F, arrowhead shows an example). This cytoplasmic change was accompanied by a roughing of the oocyte surface, as revealed by differential interference contrast (DIC) microscopy (Fig. 3G,H, arrowhead). To ascertain if oocytes with altered morphology differed in their ability to mature, GV-intact transgenic oocytes were isolated and divided into two groups according to their cytoplasm transparency (transparent and light; opaque and dark) and cultured in vitro. Surprisingly, both the transgenic GV-intact oocytes with light or dark cytoplasm reached MII, as evidenced by emission of the first polar body, at incidence similar to the non-transgenic controls (Table 1). These observations strongly suggested that the cytoplasmic and cell surface changes did not interfered with the maturation of this subpopulation. This conclusion was confirmed when the numbers of MII eggs isolated from PMSG/hCG-primed transgenic mice were found to be the same when compared with controls (Table 1).

Pol I-mediated transcription is impaired in basonuclin-deficient oocytes

Because of the suspected role of basonuclin in rRNA transcription, we used a run-on assay with BrUTP to assess Pol I transcription in basonuclin-deficient growing oocytes, which were transcriptionally active (Fig. 4) as shown by incorporation of BrdU into both nucleoplasm and nucleolus (Fig. 4A,B). In the presence of a high concentration of α-amanitin, incorporation of BrU into nucleoplasm was abolished, suggesting Pol II transcription was inhibited (Fig. 4C,D). Under this condition, fluorescence intensity in the basonuclin-deficient oocytes was reduced by ~38% (Fig. 4E). Also reduced were the numbers of transcription foci, where fluorescence is brighter than other regions in the nucleolus, suggesting intensive incorporation of BrUTP, i.e. a site of Pol I transcription. Because rDNA is a multi-copy gene, these foci probably represent the rDNA copies that are actively transcribed during the run-on labeling. Scoring the number of transcription loci by optically sectioning the nucleolus (Fig. 4F,G) indicated that the number of foci was reduced by ~25% in T50 transgenic oocytes when compared with controls (P<0.001) (Fig. 4H). Nevertheless, we detected neither a significant change in mature 28S and 18S rRNA level by RNase-protection assay (data not shown), nor an apparent change in the rate of protein synthesis in both growing and fully grown transgenic oocytes (Table 1).

Perturbed Pol II transcription in basonuclin deficient-oocytes

Because of the potential of basonuclin as a Pol II transcription regulator, we investigated the effect of basonuclin deficiency on Pol II transcription by analyzing the global gene expression pattern in

![Fig. 3. Basonuclin-RNAi transgene affected the morphology of ovary and oocytes.](image)

Histology of non-transgenic (A,B) and T50 transgenic (C,D) ovaries are shown at two magnifications: the entire ovary (A,C) and an enlarged region (B,D). Arrows indicate follicles that contain abnormal oocytes; arrowheads indicate remnants of degenerating follicles. Follicles and oocytes are apparently normal in the transgenic ovary (*). cl, corpus luteum. GV-stage oocytes were isolated and observed under the phase-contrast (E,F) and differential interference contrast (DIC) microscopy. Non-transgenic oocytes are shown in E,G, and transgenic ones in F,H. Arrowheads indicate examples of dark granules in the transgenic cytoplasm (F) and the bumps on transgenic cell surface (H). The transgenic population is heterogenous: both morphologically normal and abnormal oocytes are present. Scale bars: 50 μm in E; 10 μm in H.
fully grown, GV-intact oocytes by microarray analysis. After an active period of transcription during the first two-thirds of oocyte growth, transcription decreases such that the fully grown oocyte is essentially transcriptionally quiescent, which stays so during oocyte maturation until zygotic gene expression initiates in the late one-cell embryo (Hamatani et al., 2004; Zeng and Schultz, 2005). Therefore, the mRNA composition at the GV-intact stage reflects not only the cumulative effect of Pol II transcription during oocyte growth, but also is the source of the mRNA at the beginning of oocyte maturation and thus may have important bearings on early embryonic development.

RNAs of oocytes of four non-transgenic and six transgenic mice were analyzed individually on Affymetrix MOE 430 2.0 gene chips, which contains more than 34,000 transcripts. The resulting microarray data had a strong tendency to cluster within control and transgenic groups (Fig. 5A), when compared with an unbiased gene list. There were 772 and 253 genes, the mRNA levels of which were up- or downregulated more than twofold, respectively. As expected, basonuclin mRNA level was reduced by 210-fold, the largest magnitude of change among the up- and downregulated genes; the mRNA level of the related basonuclin 2 was not affected. An EASE analysis (Hosack et al., 2003) (Fig. 5B) showed that the genes whose
expression was upregulated more than twofold belonged to four processes: transcription and DNA binding (39.1%), development (17.5%), intercellular junction/extracellular space (11.3%) and metal ion binding (6.5%), whereas the genes downregulated more than twofold were mostly related to cell motility/adhesion (37.8%), intracellular transport (30.0%) and protein binding (17.8%). Although it remains to be seen how these perturbations in gene expression contributed to the phenotype, it is clear that: (1) our RNAi approach was effective and highly specific; (2) Pol II transcripts were evidently affected by basonuclin-deficiency; and (3) some of the transcript upregulation was probably a secondary response to basonuclin deficiency (see also Discussion). Moreover, these results support the proposal that basonuclin regulates Pol II transcription, in addition to its function as a Pol I transcription regulator.

Basonuclin-deficient oocytes lead to early embryonic failure

Our analysis of isolated oocytes suggested that sub-fertility occurred following oocyte maturation and ovulation. Accordingly, we examined the ability of transgenic oocytes to be fertilized and support pre-implantation development. Fertilized transgenic and control eggs were collected at ~10 hpc, cultured and scored for developmental stages at 36, 60 and 84 hpc. Developmental progression of transgenic embryos was clearly delayed or arrested (Fig. 6A). At 36 hpc, virtually all non-transgenic embryos reached the two-cell stage, whereas only 75% and 40% of T8 and T50 embryos, respectively, did (Fig. 6B). At this point, 60% of the T50 embryos were at the one-cell stage, and during the next 48 hours, few of them cleaved and progressed beyond the two-cell stage. Overall, very few T50 embryos could develop beyond the two-cell stage (Fig. 6A,B). Although development of T8 embryos was better, ~60% of them reached the blastocyst stage, the rate of development was nonetheless slower than controls. Once the T8 transgenic embryos reached the eight-cell stage, when an increasing number of zygotic genes are activated (Hamatani et al., 2004; Wang et al., 2004; Zeng et al., 2004), they readily developed to the blastocyst stage without further loss (Fig. 6B). We noted that, overall, at the end of this culture period (84 hpc or 3.5 days), 63±11% and 10±3% of the T8 and T30 embryos, respectively, developed beyond the four- to eight-cell stages, which could account for the fertility of these two transgenic lines (53% and 10% respectively). Thus, early embryonic failure caused the sub-fertility of the transgenic females.

Because most of the embryo loss occurred during the one- and two-cell stages, we focused on development during this time. We first assessed if genome activation, which is required for development beyond the two-cell stage, occurred in basonuclin-deficient two-cell embryos (Schultz, 2002). Genome activation, as assayed by expression of the transcription-requiring complex (TRC), a set of structurally related proteins that are accepted markers of genome activation (Conover et al., 1991), did occur in transgenic embryos to the same extent as in controls (Fig. 7M). Thus, it is unlikely that failure to activate the embryonic genome was the basis for developmental failure and hence we looked at earlier times. We noted a variety of abnormalities in the one-cell transgenic embryos. First, and reminiscent of transgenic oocytes (Fig. 4G), transgenic one-cell embryos appeared ‘rough’ under Normaski optics (Fig. 7A-D). Nuclear morphology of the transgenic embryos was also altered; most notably, the male pronuclei were 18.7% smaller in diameter than that of the control (Fig. 7E-H, Table 1), suggesting a chromatin de-condensation failure. The distribution of chromatin/DNA surrounding nucleolus in the transgenic pronuclei also differed clearly from that of controls (Fig. 7F arrow). The most striking defect, however, was chromatin fragmentation, seen as additional
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pieces of DAPI-positive materials, along with the diminutive pronuclei (Fig. 7G,H,J). Polyspermy could be a source of additional chromatin, because the frequency of poly-pronuclei observed in transgenic one-cell embryos (12 hpc) increased by more than tenfold (Table 1). Another cause of DNA fragmentation was probably due to stress on segregating chromatin during mitotic division, which led to unequal distribution of DNA between daughter cells (Fig. 7K,L). Cytoplasm fragmentation was just as frequent as nuclear fragmentation (Fig. 7L). There was, however, no single common defect observed in the transgenic embryos. Despite these failures, the apoptotic pathway was not activated when assayed by TUNEL (not shown). It is very likely that this range of abnormalities underlies the reduced developmental competence of embryos derived from basonuclin-deficient oocytes and, in particular, cell cycle regulation. Consistent with this, in transgenic one-cell embryos, our result indicated a reduction or block in DNA replication (Fig. 7M), which, in non-transgenic embryos, was observed between 8 and 16 hours post-fertilization as previously reported (Moore et al., 1996).

**DISCUSSION**

The results presented here imply a novel and crucial role for basonuclin in female fertility, because developmental competence is markedly compromised in basonuclin-deficient oocytes. This phenotype is unlikely to be due to the transgenic RNAi approach used to inhibit basonuclin specifically in oocytes, because several transgenic lines, generated with different vector backbones, exhibit similar phenotypes. Our microarray study of the transgenic oocytes agrees with this result and showed that expression of known maternal-effect genes (Zar1, Mater, Hsf1, Npm2, Pms2, Stella, Dnmt1 and Ctcf) is not affected (not shown). These observations are consistent with mounting evidence that the transgenic RNAi approach in oocytes does not elicit a classical interferon response to the expressed dsRNA and is very specific, i.e. off-targeting by the generated siRNAs is minimal (Stein et al., 2005).

Basonuclin appears to be another member of a growing family of mammalian maternal-effect genes. The source of embryonic basonuclin is maternal and targeted depletion of basonuclin in oocytes leads to sub-fertility. The observed embryonic failure at the one- to two-cell stages, which accounts for the reduced fertility of basonuclin-RNAi transgenic females, is similar to the phenotypes of several recently described mouse maternal-effect genes [e.g. Mater, Hsf1, Zar1, Npm2 and Stella (Tong et al., 2000; Christians et al., 2000; Wu et al., 2003; Burns et al., 2003; Payer et al., 2003)]. Like basonuclin-deficient embryos, these embryos show perturbed fertilization (Zar1–/–), fragmentation or abnormal cleavage at the first mitosis (Mater–/–, Stella–/– and Npm2–/–), and delayed cell cycle progression leading to cleavage arrest during one- to two-cell transition (Zar1–/–). Although we suspect that genome activation in the early embryo was compromised by the failure of chromatin reorganization, the apparent normal expression of the TRC suggests that at least some aspect of genome activation occurs in basonuclin-deficient transgenic embryos.

Our observations suggest an interesting difference between basonuclin and other maternal-effect genes reported so far. Most of the known homozygous-null maternal-effect mutants have little effect on the histology of the ovary, follicular structure, oocyte morphology and ovulation (Tong et al., 2000; Christians et al., 2000; Howell et al., 2001; Wu et al., 2003; Burns et al., 2003; Payer et al., 2003), i.e. there appears to be little, if any, overt problem with oocyte development. By contrast, basonuclin-deficient oocytes appear abnormal and are often present in degenerating follicular structures, which may reflect improper communication between the oocyte and follicle cells that is required for proper and coordinated follicle development (Matzuk et al., 2002). The transgenic oocyte population is apparently heterogeneous, suggesting either a variable expression of the transgene or a variable resilience to basonuclin-deficiency. The observation that normal numbers of eggs are ovulated implies that mouse ovary can compensate for the loss of oocytes during their pre-implantation development of basonuclin-RNAi transgenic embryos. Eggs fertilized in vivo were collected and cultured in vitro for 84 hours (3.5 days). (A) Development of the transgenic (T50) embryos was arrested at the two-cell stage. (B) At 36, 60 and 84 hours post-coitum, the developmental stages of the embryos were scored and expressed as a percentage (±s.d.) of the population. The data are the average of three experiments, in which a total of 40-60 embryos for each transgenic line were scored. The dead category includes collapsed, degenerated or fragmented embryos; 1C, one cell, 2C, two cell (etc.); M/B, morula/blastocyst.
development and maintain the overall output. However, very little is known about how ovary determines and regulates its output. Another level of compensation might take place within basonuclin-deficient oocytes. Consistent with this proposal is that although we detect reduced rates of Pol I transcription using the run-on assay, the level of mature rRNA and protein synthesis remain unchanged in fully grown basonuclin-deficient oocytes. Another sign that a compensatory mechanism exists is that microarray analysis of basonuclin-deficient oocytes reveals a large number of upregulated genes, and the most notable among them is a ~16-fold increase in abundance of Gli2 mRNA, which encodes a transcriptional effector (GLI2) that can upregulate basonuclin transcription (Cui et al., 2004). Upregulation of Gli2 in basonuclin-deficient oocytes suggests an apparently futile attempt of the transgenic oocytes to compensate for the reduced level of basonuclin mRNA by increasing its transcription. Another likely source of compensation comes from basonuclin 2, the transcript of which is also abundantly present in mouse oocytes and not affected by RNAi targeting basonuclin. It is clear, however, that the putative compensation of basonuclin 2 can be only partial, because of the observed embryonic developmental failure of embryos derived from basonuclin-deficient oocytes. Thus, the detrimental effect of basonuclin deficiency, be it reduction of Pol I and Pol II-mediated transcription or other impairments, may be alleviated by the ability of the oocyte to compensate for these perturbations.

In summary, our data demonstrate that basonuclin is a maternal-effect gene, and both Pol I- and Pol II-mediated transcriptions are perturbed in basonuclin-deficient oocytes; however, our data do not indicate in molecular terms how impairment of basonuclin function leads to this phenotype. We can propose, nevertheless, that basonuclin is likely to be required for both oogenesis and early embryogenesis. Whether these functions are related needs to be resolved. Although our microarray analysis did not detect any significant change in the mRNA level of the known maternal-effect genes in basonuclin-RNAi transgenic oocytes, it remains a formal possibility that basonuclin is required for the expression of one or more unidentified maternal-effect genes in the oocyte, i.e. the maternal effect of basonuclin may be secondary. Our results also suggest that in the absence of basonuclin, a compensatory mechanism is activated to ensure that normal numbers of eggs are ovulated. This mechanism may reflect a more general response that would enhance reproductive fitness.

Fig. 7. Abnormalities of basonuclin-RNAi transgenic embryos. Fertilized eggs were examined under DIC (Nomaski) optics (A-D) or fluorescence (DAPI) (E-G). (H-L) Merged images. Compared with non-transgenic one-cell embryos (A), the transgenic T50 embryos had a much rougher cell surface (B-D) caused by numerous ‘bumps’ (B-D,L, black arrowheads). At the same one-cell stage, the transgenic pronuclei, as revealed by DAPI staining, were smaller (F-H) and contained irregular DNA distribution (white arrow in F) when compared with the control (E). In some transgenic embryos, additional nucleus/chromatin aggregates were seen (white arrowhead in G,H), some of which were probably derived from polyspermy (arrow indicates the head of an attached sperm). At the two-cell stage and beyond, additional abnormalities were seen in transgenic embryos but not in control (I); some examples were collapsed embryos with disorganized chromosomes (J), incomplete chromosome segregation during mitosis (K, arrowhead indicates the two chromatin mass was still linked by a thread of DNA) and cytoplasmic fragmentation (L, white arrow). (M) A summary of the defects of transgenic T50 embryos during the early pre-implantation development in culture. The observed defects were indicated above the time line (thick black line) of one-cell to two-cell embryonic development (Moore et al., 1996). The cell-cycle stages (G1, S, G2 and M) were shown above the time line and the hours post copulation (hpc) below. The black dots indicate that the timing of the observation could be determined to a particular stage of the cell cycle; the short thick lines imply that the measurements were made on pooled one- or two-cell embryos at varying stages of the cell cycle. The autoradiogram of a PAGE gel shows the presence of TRC in both the control and transgenic embryos. Scale bars: 50 μm in C; 10 μm in E, I.
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References