Somatic cells regulate maternal mRNA translation and developmental competence of mouse oocytes

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Germ cells divide and differentiate in a unique local microenvironment under the control of somatic cells. Signals released in this niche instruct oocyte reentry into the meiotic cell cycle. Once initiated, the progression through meiosis and the associated programme of maternal messenger RNA translation are thought to be cell autonomous. Here we show that translation of a subset of maternal mRNAs critical for embryo development is under the control of somatic cell inputs. Translation of specific maternal transcripts increases in oocytes cultured in association with somatic cells and is sensitive to EGF-like growth factors that act only on the somatic compartment. In mice deficient in amphiregulin, decreased fecundity and oocyte developmental competence is associated with defective translation of a subset of maternal mRNAs. These somatic cell signals that affect translation require activation of the PI(3)K–AKT–mTOR pathway. Thus, mRNA translation depends on somatic cell cues that are essential to reprogramme the oocyte for embryo development.

Germ cell differentiation requires a unique microenvironment created by surrounding somatic cells. In gonads of adult Drosophila and Caenorhabditis elegans, this environment provides the ‘niche’ that is key to the maintenance of the germ stem cell pool. A similar niche is critical for spermatogonial stem cell replication and differentiation to maintain spermatogenesis in mammals. In adult mammalian ovaries, the follicle microenvironment in which the oocyte develops is the conduit for bidirectional exchange of signals with surrounding somatic cells. With the possible exception of transcription in model organisms, the molecular basis for somatic/germ cell interactions is still poorly understood.

On signals from the soma in the ovarian follicle, fully grown mammalian oocytes reenter the meiotic cell cycle, complete the first meiotic division, and progress to metaphase II of the second division. Although these transitions still occur if oocytes are freed from surrounding somatic cells and cultured in vitro, it is commonly accepted that, in the absence of somatic cell contacts, oocyte fertilization and embryo development are compromised. These defects probably arise from disruptions in the poorly defined molecular process by which the oocyte acquires developmental competence, termed cytoplasmic maturation. Nuclear transfer experiments indeed show that the defects associated with oocyte denudation and in vitro culture reside in the cytoplasm. Because cytoplasmic maturation of the oocyte and early embryo development proceed in the absence of transcription, competence to develop as an embryo must rely on a genome-wide programme of maternal mRNA translation and degradation.

Oocyte maturation and ovulation induced by the gonadotropin LH (luteinizing hormone) requires activation of paracrine/autocrine signals within the follicle. In addition to the release of prostaglandins and steroids, LH induces large increases in amphiregulin (Areg), epiregulin (Ereg) and betacellulin (Btc) mRNAs within 1–3 h of stimulation in mural granulosa cells, followed by an increase in cumulus cells. These growth factors bind to the epidermal growth factor receptor (EGFR) on granulosa cells, and their release mediates the LH-dependent transactivation of EGFR. Genetic and pharmacological data demonstrate that activation of this EGF network is essential to transmit the gonadotropin signal from the mural granulosa cells to the cumulus cells and the oocyte, to induce oocyte maturation, cumulus expansion and ovulation.

Translation of maternal mRNAs during oocyte maturation requires polyadenylation directed by RNA-binding proteins, the prototype being the cytoplasmic-polyadenylation-element-binding (CPEB) protein. In Xenopus oocytes, CPEB-mediated translation is under the control of cell cycle regulators that function in a cell-autonomous...
fashion. Limited information is available on whether translation during the meiotic cell cycle is affected by somatic cell signals. Here we have tested the hypothesis that the environment in which oocytes complete meiosis and signals from somatic cells control translation in the oocytes. This regulation is critical for mammalian oocyte competence to develop as an embryo.

RESULTS
The accumulation of the spindle component TPX2 is dependent on the environment in which the oocyte matures
TPX2 (targeting protein for the *Xenopus* kinesin xklp2) is a protein essential for spindle assembly and chromosome interaction with microtubules. It binds and activates Aurora A by promoting its autophosphorylation. The level of TPX2 expression is critical for spindle function, and altered expression is associated with aneuploidy and cancer. In agreement with a previous report, we show that TPX2 is undetectable in oocytes in prophase and accumulates during maturation to MII (Fig. 1). It has been proposed that the absence of TPX2 accumulation in prophase is due to protein degradation through APC/Cdh1 (ref. 21). Indeed, little change in TPX2 mRNA translation occurs during the early phases of oocyte maturation, but the late TPX2 accumulation is associated with an increased translation. Surprisingly, we found that TPX2 protein accumulation is not only dependent on the stage of the meiotic cell cycle. Significant differences in TPX2 protein levels were observed when comparing MII oocytes matured in vivo with those matured in vitro in association with somatic cells, or those matured in vitro after being denuded. This initial finding suggests that TPX2 accumulation is sensitive to the environment in which the oocyte matures.

Translation of *TPX2* and other mRNAs in oocytes is sensitive to somatic cell cues
To investigate whether cumulus cells, the somatic cells surrounding the oocyte, play a role in the translation of maternal mRNAs and protein synthesis, we developed an *in vitro* model that preserves the somatic environment in which the oocyte matures. Translational reporters were constructed and injected into oocytes still surrounded by cumulus cells (cumulus cell-enclosed oocyte, CEO; Fig. 2a,b). This model enables monitoring of translation of selected maternal mRNA in oocytes that maintain contact with cumulus cells. Furthermore, translation rates in CEOs can be compared with those measured in denuded oocytes (DOs), which are no longer exposed to somatic signals.

Reporter constructs with luciferase open reading frames under the control of 3' untranslated regions (3' UTRs) of *Tp2* or *Dazl*, an RNA-binding protein essential for gametogenesis, were injected into CEOs. Translation rates of these reporters increased as the oocytes progressed from germinal vesicle (GV) to MII (Fig. 2c), consistent with our report of recruitment of the corresponding endogenous transcripts to the polysomes. However, translation in CEO is further increased by supplementing the incubation medium with amphiregulin (AREG), an EGF-like growth factor that accumulates physiologically in the follicle during ovulation, or EGF itself (Fig. 2c). Both ligands signal through EGFR on cumulus cells and are not expressed by oocytes in culture. Growth-factor-induced effects were not detected when meiotic reentry was prevented with the phosphodiesterase inhibitor milrinone (Supplementary Fig. 1), when a Tpx2 reporter with truncated 3' UTR was injected (Supplementary Fig. 2), or when oocytes were denuded before stimulation (Fig. 2c). These findings demonstrate that the 3' UTR, progression through meiosis and somatic cells are all required for the growth-factor-dependent stimulation. Exposure to AREG did not increase the stability of the reporter, confirming an effect on translation rate (Supplementary Fig. 1b).

Consistent with reporter translation, endogenous TPX2 and DAZL protein levels increased as oocytes matured (Fig. 2d,e). AREG further enhanced these protein levels, confirming that increased translation of the reporter reflects translation of the endogenous mRNA and accumulation of the encoded protein. When incubated with AREG and in contact with somatic cells and AREG, a rise in oocyte protein synthesis was independently confirmed by monitoring accumulation of IL-7, an oocyte-secreted chemokine, in the medium (Fig. 2f).

To conclusively demonstrate that AREG does not directly stimulate the oocyte and that the signals are indirect and mediated by somatic cells, we used a genetic mouse model in which EGFR is downregulated only in somatic cells. Mice carrying a null *Egfr* allele and a floxed allele (*Egfr*fl/fl), and expressing a Cre recombinase under the control of the granulosa-cell-specific CYP19A1 promoter, show a 90% decrease in EGFR expression in granulosa cells. Although LH-dependent *in vivo* oocyte maturation is impaired, *in vitro* spontaneous maturation...
Figure 2  EGF-like growth factor stimulation of cumulus–oocyte complexes in vitro increases translation in oocytes. (a) Schematic illustration describing the experimental conditions to measure translation rates in CEOs. Oocytes still in complex with cumulus cells were injected with two reporters, one coding for the Renilla luciferase (RL) under the control of a maternal mRNA (Tpx2 or Dazl) 3' UTR and one coding for the firefly luciferase (FL) with a polyadenylated 3' UTR. After three hours of incubation in the presence of milrinone to maintain the meiotic arrest, a group of injected CEOs was washed free of inhibitor and incubated with or without EGF-like growth factors. In some experiments, a further group of CEOs was mechanically denuded and oocytes incubated with growth factors as above. At the end of the incubation, oocytes were dissected free of cumulus cells and luciferase activity was measured in oocyte extracts. (b) Bars represent the mean ± s.e.m. of four experiments where IL-7 accumulation was measured in spent media of CEOs cultured as in c with or without AREG. t-test: *P < 0.01 versus MII; (g) CEOs were prepared from pregnant mares serum gonadotrophin (PMSG)-treated Egfrfl/fl, Egfrfl/fl or EgfrΔ/fl; Cyp19 CRE (Cre recombinase) mice. They were then injected with a Tpx2 luciferase reporter and incubated as detailed in a in the absence or presence of AREG. At the end of the incubation, oocytes were dissected free of cumulus cells and luciferase activity was measured in oocyte extracts. The data from Egfrfl/fl and EgfrΔ/fl groups gave similar results and were combined. Data are the mean ± s.e.m. of four independent experiments. Paired t-test: **P = 0.018 MII + AREG versus MII of Egfrfl/fl; EgfrΔ/fl; Cyp19 CRE; **P = 0.033 MII + AREG of Egfrfl/fl versus MII + AREG of EgfrΔ/fl; Cyp19 CRE. See Supplementary Table 3 for statistics source data. Uncropped images of blots and gels are shown in Supplementary Fig. 7.

Defective AREG production in vivo is associated with altered translation and compromised oocyte developmental competence

To determine whether this somatic effect on oocyte translation also occurs in vivo and whether global translation is affected, genetic mouse models perturbing the EGF network were investigated. We have reported that the EGF-like growth factors AREG, EREG and BTC

progresses normally. We used CEOs derived from these mice to test whether Egfr gene inactivation in somatic cells ablates the AREG effects on oocyte translation in vitro. The experiment reported in Fig. 2g demonstrates that AREG does not significantly increase the translation rate of Tpx2 reporter when CEOs from EgfrΔ/fl; CYP19A1–Cre mice are microinjected.
Defective spindle morphology was more frequently detected in MII oocytes derived from Areg−/− mice (Fig. 3e,f). Thus, inactivation of Areg in the somatic cells yields oocytes that mature but with signs of reduced developmental competence. Our model was then used to test whether the translational programme executed during oocyte maturation is affected by the absence of this somatic signal. To capture actively translating mRNAs, polysomes were isolated by sucrose density gradient from WT and Areg−/− oocytes. Maternal transcripts associated with the polysomes were isolated and analysed by microarray hybridization (Fig. 4). Consistent with our previous report, 3,208 transcripts were recruited or released from the polysomes during oocyte maturation in WT oocytes (Supplementary Information 1 and 2). A similar number of transcripts (3,440) also moved in and out of the polysome pool in oocytes from Areg−/− mice, confirming that the translation programme is qualitatively intact. However, quantitative analysis

Figure 3 Compromised developmental competence of oocytes from Areg−/− mice. (a) Summary of offspring from mating of WT, Areg+/− and Areg−/− mice. The number of litters is reported below the scatterplot. *t-test: ****P < 0.0001 versus WT. (b) Bars represent the mean ± s.e.m. of the two-cell embryo yield after in vitro fertilization (IVF) using CEOs from superovulated WT and Areg−/− mice. t-test: ***P < 0.001 versus WT. The number of IVF experiments carried out is reported below the bars. (c) After superovulation, a group of MII CEOs was stripped of cumulus cells and used for IVF. Bars represent the mean ± s.e.m. of two-cell embryo yield. t-test:

are induced by LH at the time of ovulation and that transactivation of EGFR is indispensable for oocyte maturation and ovulation11,13. Whereas inactivation of EGFR causes a block in oocyte maturation in vivo and defective ovulation11, Areg−/− mice ovulate and are fertile11 but litter size is significantly decreased (Fig. 3a). When fertilization rates were assessed in vitro, CEOs or DOs derived from Areg−/− mice fertilize at a rate significantly lower than wild-type (WT) littermates, suggesting a defect in developmental competence (Fig. 3b,c). Identical to the Areg−/− follicle activated in vivo, isolated CEOs from WT mice cultured in vitro are not exposed to AREG. Therefore, the role for AREG in promoting cytoplasmic maturation was further tested by adding exogenous AREG during WT CEO in vitro maturation. This treatment improved the fertilization rate (Fig. 3d), a finding consistent with other studies demonstrating positive effects of the EGF network on developmental competence26–28.
Altered mRNA translation of a subgroup of maternal mRNAs in Areg−/− oocytes. (a,b) Analysis of polysome-associated transcripts in WT and Areg−/− MII oocytes. Three distinct pools of 500–750 oocytes from WT and Areg−/− mice were used for the analysis. The actual data are reported in Supplementary Tables 1 and 2. (c) Comparison of hybridization data and qPCR of total and polysome-bound distribution of selected transcripts; t-test P < 0.05 from three distinct mRNA preparations. (d) Comparison of recruitment to the polysome of most downregulated transcripts in Areg−/− oocytes. The ratio MII/GV was calculated for the top 31 transcripts whose levels were most significantly different in polysomes from MII Areg−/− oocytes when compared with WT MII. Data are reported as mean ± s.e.m. of at least 1.5 were included in the analysis. Enriched gene ontology terms were discovered using DAVID (see Methods for details). (e) In vitro translation of cyclin B1 3′ UTR reporter in CEOs cultured with or without AREG. Bars represent the mean ± s.e.m. of three distinct experiments with different pools of CEOs. (f,h) Comparison of TPX2 and Dazl levels in WT and Areg−/− superovulated MII oocytes. The bars are the mean ± s.e.m. of densitometric analysis of five western blots for TPX2 and four western blots for Dazl proteins done on different groups of oocytes. *P < 0.05 versus WT. (g) Bars represent the mean ± s.e.m. of polysome-associated cyclin B1 mRNA in WT and Areg−/− oocytes. (i) In vitro translation of cyclin B1 3′ UTR reporter in CEOs cultured with or without AREG. Bars represent the mean ± s.e.m. of three distinct experiments with different pools of CEOs. (j) In vitro translation of the Tex19.1 3′ UTR reporter in CEOs cultured with or without AREG. Bars are the mean ± s.e.m. of three separate experiments. In experiments reported in h–k, no statistical difference could be observed between the WT and Areg−/− groups. See Supplementary Table 3 for statistics source data. Uncropped images of blots and gels are shown in Supplementary Fig. 7.
revealed significant changes in the level of transcripts associated with polysomes in mutant oocytes. When using a cutoff of $P < 0.05$ (Fig. 4a), polysome association of approximately 200–300 transcripts was altered in the Areg$^{-/-}$ oocytes when compared with WT (Fig. 4b). Transcripts normally recruited to the polysome during maturation were decreased in the Areg$^{-/-}$ oocytes (Fig. 4c). We confirmed the decrease of selected transcripts in the polysome pool by quantitative PCR (qPCR; Fig. 4d).

The affected transcripts included the main functional categories of metabolism, embryonic development, cell cycle and RNA regulators (Fig. 4e). Analysis of the 3’ UTRs affected in the Areg$^{-/-}$ oocytes did not reveal the presence of a common signature, with the possible exception of two consensus elements enriched in a subset of transcripts (Supplementary Fig. 3). Protein levels of DAZL and TPX2 in MII oocytes of the Areg$^{-/-}$ mice were decreased (Fig. 4f,g), which is consistent with the

Figure 5 PI(3)K–AKT–mTOR signalling is involved in the somatic regulation of oocyte mRNA translation. (a) Representative western blot of the time course of AKT Ser 473 phosphorylation in CEOs incubated with AREG for different times. Extracts from total CEOs were used for the western blot. (b) Densitometric analysis of AKT Ser 473 phosphorylation at different times after AREG exposure. Total CEO extracts were used for these measurements. Each point is the mean ± s.e.m. of three different experiments. t-test: *$P < 0.05$ and **$P < 0.01$ versus zero time point. (c) Representative western blot of AKT Ser 473 phosphorylation measured in oocyte extracts after stimulation when still in complex with cumulus cells (CEO) or after denudation (DO). (d) Densitometric analysis of AKT Ser 473 phosphorylation measured in oocytes incubated with or without AREG when still in complex with cumulus cells (CEO) or after denudation (DO). Each point is the mean ± s.e.m. of three different experiments. t-test: *$P < 0.05$ and **$P < 0.01$ versus DO groups. Of note, phosphorylation of AKT at 0 and 30 min was highly variable. Although not significant, this trend to an increase in Ser 473 phosphorylation at 30 min is probably due to mechanical stress from manipulation of the oocytes during denudation. This increase is observed whether oocytes are derived from CEO or are incubated as DOs in the absence or presence of AREG. (e,f) The AREG-dependent activation of translation of the TPX2 reporter is prevented by PI(3)K inhibitors (LY294002 and wortmannin), which block AKT phosphorylation in the oocyte. After microinjection of the TPX2 reporter, CEOs were preincubated with inhibitors for 30 min and then incubated with or without AREG for two more hours. At the end of the incubation, oocytes were freed of cumulus cells and used for western blot analysis (e). For luciferase assays (f), CEOs were incubated overnight. All oocytes used in the luciferase assays had reached the MII stage as indicated by the presence of polar bodies. Data are the mean ± s.e.m. of four independent experiments. t-test: ****$P < 0.0001$ and **$P < 0.01$. (g) Effect of the mTOR inhibitor rapamycin (Rapa) on the AREG-dependent increase in TPX2 reporter translation. Data are the mean ± s.e.m. of four independent experiments. t-test: *$P < 0.05$ versus AREG. (h) Intact PI(3)K signalling in the oocyte is necessary for the AREG-dependent increase in Tpx2 reporter translation. CEOs were derived from Pten$^{fl/fl}$,Zp3-CRE mice, where the Pten gene is deleted only from the oocytes, and Pten$^{fl/fl}$ littermates, which behave as WT mice. The incubation and the luciferase assay were conducted as detailed in Fig. 2. Each point is the mean ± s.e.m. of three experiments conducted on different days. t-test: **$P = 0.009$ Pten$^{fl/fl}$ + AREG versus MII; NS (not significant) Pten$^{fl/fl}$,Zp3-CRE MII + AREG versus MII. See Supplementary Table 3 for statistics source data. Uncropped images of blots and gels are shown in Supplementary Fig. 7.
was observed in oocytes exposed to AREG while still in complex with EGF by western blot analysis (Fig. 5c,d) or phosphorylation was reminiscent of the AKT phosphorylation detected in somatic cells; conversely, this delayed phosphorylation could not place in the oocyte. CEOs were incubated in the presence of AREG; at the end of the incubation, the oocytes were denuded and used for secondary increase in AKT phosphorylation detected at 120–180 min of incubation with AREG (Fig. 5a,b). Because the delayed increase in AKT phosphorylation was reminiscent of the AKT phosphorylation detected in the oocyte 2–3 h after LH/hCG (human chorionic gonadotropin) stimulation, we investigated whether this secondary increase takes place in the oocyte. CEOs were incubated in the presence of AREG; at the end of the incubation, the oocytes were denuded and used for western blot analysis (Fig. 5c,d) or in situ detection by immunofluorescence (Supplementary Fig. 5). A delayed AKT phosphorylation was observed in oocytes exposed to AREG while still in complex with cumulus cells; conversely, this delayed phosphorylation could not be detected in DOs exposed to AREG (Fig. 5c,d and Supplementary Fig. 5). Variable AKT phosphorylation was observed in oocytes within the first 30 min, whether cultured as CEOs or denuded (Fig. 5d), a signal independent of AREG and probably caused by the mechanical denudation of the oocytes. These data suggest that AREG stimulation of CEOs causes a delayed transient increase in AKT phosphorylation in the oocyte. Pharmacological inhibition of PI(3)K with wortmannin or LY294002 blocked the AKT phosphorylation in oocytes when cultured in complex with cumulus cells in the presence of AREG for 2 h (Fig. 5e). Under these conditions, the AREG-dependent increased translation of the Tpx2 reporter was abolished, whereas the cell-cycle-dependent increase in reporter translation was not affected (Fig. 5f). Similar results were obtained with rapamycin (Fig. 5g and Supplementary Fig. 6), a mammalian target of rapamycin (mTOR) inhibitor.

It should be noted that, in the experiments reported above, the pharmacological inhibitors used block the PI(3)K–AKT–mTOR pathway both in somatic cells and in the oocyte. To determine whether the PI(3)K pathway function is required in the oocyte, we used a genetic model to test whether disruption of this pathway exclusively in the oocyte is sufficient to block the AREG-dependent increase in translation. To this aim, we used cumulus/oocyte complexes from Ptenfl/1;ZP3-CRE mice. In this genetic model, the Cre recombinase is under the control of an oocyte-specific promoter and the Pten (phosphatase and tensin homologue) gene is ablated only from oocytes and not from cumulus cells. Previous data with this model show that Pten inactivation causes a constitutive increase in AKT phosphorylation. In these oocytes, translation of the Tpx2 reporter is no longer dependent on AREG (Fig. 5h). This latter finding confirms that an intact PI(3)K pathway in the oocyte is necessary to translate the AREG signal from the soma.

**DISCUSSION**

Our findings demonstrate that translation of a subset of oocyte maternal mRNAs is under the control of somatic cell inputs acting through the PI(3)K–AKT–mTOR pathway. This regulation functions in concert with the translational control by meiotic cell cycle regulators and is involved in establishing the competence of oocytes to successfully develop as embryos.

Our in vivo and in vitro data document that exposure of somatic cell/oocyte complexes to the EGF-like growth factor AREG causes an increase translation of a subset of maternal mRNAs in the oocyte. This activation requires cell-to-cell communication, as it is lost in DOs. The use of alleles affecting Egfr and PI(3)K signalling in the somatic and germ cell compartments respectively confirms that AREG action requires Egfr expression in the soma and an intact PI(3)K signalling in the oocyte. Thus, Egfr signalling in the somatic cellular compartment is translated into PI(3)K activation in the contiguous cell compartment, the oocyte. Given the critical role for EGFR signalling in tumour growth, it is possible that the signalling across cells described here is also necessary for communication of transformed cells with the surrounding cellular environment.

Growth factor regulation of translation through the PI(3)K–AKT–mTOR pathway is established for somatic cells. However, the molecular mechanism underlying the specific regulation of a subset of transcripts in growth-factor-target cells remains controversial. Through target of rapamycin complex 1 (mTORC1) the PI(3)K pathway converges on regulation of S6 ribosomal protein and eukaryotic initiation factor 4E, a key rate-limiting initiation factor for cap-dependent translation. 4E-binding protein 1, one of the three 4E-binding proteins expressed in mammals, is phosphorylated by mTORC1, causing the dissociation from eukaryotic initiation factor 4E and the formation of an active initiation complex. Transcripts sensitive to mTOR regula-
We propose that mTOR signalling in oocytes controls translation whereas many of the growth factor targets in somatic cells are ribosomal proteins in the oocyte machinery are compromised, with meiosis becoming prone to abort. In the absence of somatic cells, these components accumulate at levels required for maturation, fertilization and embryo development reach optimal levels when oocyte developmental competence. As an extension of this concept, maternal mRNA translation and somatic inputs is essential for oocyte developmental competence. Our findings provide a molecular explanation for the widely reported observation that fully grown oocytes dissociated from the surrounding somatic cells may complete meiosis but it is not clear whether these sequences are indispensable. The mechanism have signature sequences at the 5' UTR, including a polypyrimide (5'TOP) or pyrimidine-rich translational element (PRTE) sequence, but it is not clear whether these sequences are indispensable. The mechanism of translational regulation we describe in the oocyte is clearly distinct. 5'TOP or PRTE sequences are not readily discernible in the 5' UTR of the prototype transcripts we have investigated in the oocyte.

A selective activation is retained when different luciferase reporters are injected in oocytes, even though they have an identical 5' UTR sequence. Conversely, the 3' UTR of these mRNAs is essential because the TPX2 3' UTR reporter recapitulates this regulation, whereas the 3' UTR of, for instance, cyclin B does not support this regulation. Elements present in the 3' UTR are necessary because a truncated TPX2 3' UTR is not sensitive to somatic cell signals. A further feature that distinguishes the effects we describe in the oocyte from those in somatic cells is that none of the oocyte targets of regulation identified are ribosomal proteins, whereas many of the growth factor targets in somatic cells are ribosomal proteins. In contrast, the regulation we describe takes place on a background of widespread destabilization and decreased translation of mRNAs coding for ribosomal proteins. The pharmacological and genetic analyses carried out indicate that the AKT/mTOR pathway is required for the AREG-dependent translational regulation in the oocyte.

We propose that mTOR signalling in oocytes controls translation through unique mechanisms that target the 3' UTR (Fig. 6). The possible role for the 5' UTR in this growth-factor-dependent regulation remains to be determined.

Correct mRNA translation and accumulation of corresponding proteins in the oocyte is critical for efficient meiotic cell cycle progression, fertilization and embryo development, and necessary to attain full developmental potential. Our findings provide a molecular explanation for the widely reported observation that fully grown oocytes dissociated from the surrounding somatic cells may complete nuclear maturation but are defective in fertilization and embryo development. Our findings support the idea that components required for maturation, fertilization and embryo development reach optimal levels when oocyte–somatic cell communication is preserved. In the absence of somatic cells, these components accumulate at levels sufficient for cell cycle progression but the robustness and stability of the oocyte machinery are compromised, with meiosis becoming prone to errors. This conclusion is supported by our observation that TPX2, a key component in spindle assembly, accumulates significantly less in the absence of somatic input, and spindle defects become more frequent in MII oocytes. In the same vein, decreased accumulation of Dazl, which may function upstream of TPX2 (ref. 22), probably destabilizes the network of RNA-binding protein required for maturation and embryo development. Thus, correct execution of the programme of maternal mRNA translation and somatic inputs is essential for oocyte developmental competence. As an extension of this concept, biomarkers monitoring this program, such as secreted proteins, should be of prognostic value for the fitness of an oocyte to develop into an embryo that successfully implants and sustains pregnancy to term.

**METHODS**

Methods and any associated references are available in the online version of the paper.

*Note: Supplementary Information is available in the online version of the paper.*

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**AUTHOR CONTRIBUTIONS**

J.C. developed the CEO translation assay and carried out the microarray experiments and some of the AKT assays; S.T. carried out the characterization of the Areg null phenotype; F.X. contributed with western blot studies and oocyte isolation for microinjection; C.-J.L. helped with immunostaining experiments and the microinjections in cumulus oocyte complexes; H.C. carried out the experiments on protein secretion and contributed to the writing of the manuscript. F.F. carried out microinjections in CEOs; K.H. contributed with the preparation of the translational luciferase reporters; C.O. and J.S.S. carried out the bioinformatic analysis of the microarray data. M.I.C. advised on data analysis and discussed results; M.R.-S. provided reagents and constructs and advised in the interpretation of the data; M.C. conceived the project, designed the experiments, analysed the data and wrote the paper.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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**METHODS**

Polysome isolation and microarray analysis. Four-week-old amphiregulin-null (C57BL/6J 129Sv) female mice and WT litter mates were used in the experiments. Forty-eight hours after PMSG injection, mice were stimulated with hCG for 0, 4 or 14 h, and GV, MI and MII stage oocytes were collected. Polysome-bound mRNAs were purified as detailed previously. In brief, oocytes were lysed with 1x PLB (30 mM Tris-HCl at pH 7.5, 100 mM NaCl, 10 mM MgCl2, 1% Triton, 1 mM dithiothreitol, 0.25 mM Na2VO4, 20 mM beta-glycerophosphate, 30 U ml−1 RNase inhibitor (USB Chemical), 10 μg ml−1 cycloheximide, plus protease inhibitor cocktail (Roche)). Oocyte lysates were centrifuged at 12,000 g for 10 min at 4°C. Supernatants were loaded on a 10 ml 15–50% sucrose gradient and centrifuged at 100,000 g for 130 min at 4°C. Polysome-bound RNAs were purified, reverse-transcribed and linearly amplified with WT-Ovation FFPE RNA Amplification System V2 (NuGEN). 5 μg complementary DNA was fragmented and hybridized with Affymetrix Mouse Genome 430.2 array chips.

**Microarray analysis.** Microarray data were normalized using the Robust Multi-array Average method. In this procedure, raw probe intensities are background-corrected, log transformed, and quantile normalized to facilitate comparison across arrays. Furthermore, for each gene an expression summary value is generated, which is based on a robust average of the normalized values for each of the probe sets targeting the gene. Robust Multi-array Average was carried out using the ‘affy’ library in Bioconductor and a custom chip definition file representing the latest RefSeq probe mapping to the mouse genome (Mouse4302_Mm_REFSSEQ, Version 16.0).0), downloaded from the Microarray Lab of the Molecular and Behavioral Neuroscience Institute, University of Michigan (http://brainarray.mbnl.med.umich.edu/Brainarray/Database/CustomCDF/CDF_download.asp). To determine genes differentially recruited to the polysome fraction in any of the four experimental groups (GV WT, GV Areg−/−, MI WT, MI Areg−/−) the Robust Multi-array Average-determined gene expression summaries for the four experimental groups were first compared using ANOVA. For the genes determined to be significant by ANOVA, t-tests were also carried out for pairwise comparisons of the four experimental groups, assuming equal variance. A gene was considered differentially recruited to the polysome fraction between two experimental groups if both the ANOVA P-value on all four groups and the t-test P-value on the two particular groups in question were less than a given cutoff. Cutoffs used were 0.001, 0.01 and 0.05. For the ontology analysis, transcripts recovered at levels significantly different in the polysomes (P < 0.05) and with a WT/Areg−/− ratio of at least 1.5 were included in the analysis. Enriched gene ontology terms were discovered using DAVID (refs 45,46). Enriched elements in the affected transcripts were discovered following published strategies (refs 47,48). Accession numbers for the overall study (GSE66600) and individual dataset are as follows: GSM1133470, GSM1133471, GSM1133472, GV stage oocyte, WT, three biological replicates; GSM1133473, GSM1133474, GSM1133475, MI stage oocyte, WT, three biological replicates; GSM1133476, GSM1133477, GSM1133478, GV stage oocyte, Areg−/−, three biological replicates; GSM1133479, GSM1133480, GSM1133481, MI stage oocyte, Areg−/−, three biological replicates.

**Real-time quantitative PCR.** Real-time qPCR was carried out using Power SYBR PCR master mix with an ABI 7900 real-time PCR system (Applied Biosystems). All primers were designed in two exons flanking introns to avoid amplification of genomic DNA (Supplementary Table 4). A dissociation curve analysis was carried out at the end of the amplification to verify the specificity of the primers. The polysome data were normalized to Elf4a1 and Actr3 transcript abundance, and the total transcript data were corrected for oocyte number. REST 2009 software was used to analyse the data.

**Oocyte culture and microinjection.** The culture medium used for most experiments was MEM (GIBCO), supplemented with 0.23 mM pyruvate, 75 μg ml−1 penicillin, 10 μg ml−1 streptomycin sulphate and 3 mg ml−1 BSA and buffered with 26 mM sodium bicarbonate. For oocyte isolation and microinjection, the medium was supplemented with 5 μM clostramide or 2 μM milrinone and buffered with 25 mM HEPES at pH 7.2, with sodium bicarbonate reduced to 6 mM.

**Reporter mRNA preparation and luciferase assay.** The RL reporter plasmids were constructed from pRl-TK vector (Promega). Generation of Dazl–Rluc, Tpz2–Rluc, Tex19.1–Rluc and cyclin B1–Rluc were carried out as described previously. All reporters contain a T7 promoter enabling in vitro transcription to synthesize mRNAs (Ambion). The FL control mRNA was polyadenylated with a Poly(A) tailing kit (Ambion). CEOS or DOs were injected with reporter mRNA (25 ng μl−1; RL containing the indicated 3′ UTR) together with 25 ng μl−1 polyadenylated FL RNA as a normalizing RNA, and incubated for 3 h in Meme/BSA containing 2 μM milrinone at 37°C with 5% CO2 before culturing in inhibitor-free medium to enable maturation. After 17 h of treatment in different conditions, oocytes were denuded and collected. Luciferase activities in the oocyte extracts were measured using a Dual Luciferase Reporter Assay Kit (Promega) and luminescence was detected with a SpectraMax L luminometer (Molecular Devices). Data are reported as ratios of RL and FL.

**In vitro fertilization.** Four- to five-week old female mice were used in the experiments. 48h after PMSG injection, mice were stimulated with hCG for 13 h. Superovulated CEOS were isolated in HTF and used in IVF. DOs were obtained by treating CEOS with 3 μg ml−1 hyaluronidase briefly. Fertilization was determined by monitoring pronuclear formation or by scoring the number of two-cell embryos.

**Western blot analysis.** Oocyte lysates were applied to SDS–polyacrylamide gel electrophoresis (10% Tris–glycine gels) and transferred to a polyvinylidene difluoride membrane. Membranes were blocked in 5% milk (TBS, pH 7.4) for 2 h at room temperature and incubated with primary antibody (1:500) overnight at 4°C. After washing in TBS-Tween 20 (0.05%), membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:2,000, catalogue no 31462, Pierce) or goat anti-mouse (1:2,000, catalogue no 31430, Thermo Scientific) for 1 h at room temperature. Signals were detected using Super Signal Dura (Pierce). Antibodies used were rabbit polyclonal anti-DAZL (ab34139, Abcam), rabbit whole antisera anti-TPX2 (NB500-179, Novus Biologicals), rabbit monoclonal anti-phospho-AKT (4060, Cell Signalling), rabbit monoclonal anti-phospho-S6 ribosomal protein (3564, Cell Signalling) and mouse monoclonal anti-tubulin (T6074, Sigma Aldrich). Ph(3)/IK inhibitors (LY294002 and wortmannin) were from Cell Signalling. Rapamycin (mTOR inhibitor) was from Sigma. INK128 (selective TORC1/2 inhibitor) was from SelleckBio.

**Immunofluorescent staining.** Oocytes were fixed in 2% paraformaldehyde in PBS for 30 min and permeabilized in 0.1% Triton X-100 in PBS for 15 min at room temperature. Oocytes were then washed and blocked in PBS containing 0.1% BSA and 0.01% Tween-20 for at least 15 min, and then stained with α-tubulin antibody (1:500 dilution; Molecular Probes) and Alexa Fluor 594 goat anti-mouse (1:500 dilution; Molecular Probes). Ten micromolar 4,6-diamidino-2-phenylindole dilactate (Molecular Probes) was added to the secondary antibody solution to stain DNA. Oocytes were mounted in CitiFLUOR (Anti-fadent Mounting Medium, AF3, Electron Microscopy Sciences) and imaged on a Leica TCS SP5 confocal microscope.

**Statistical analyses.** No statistical method was used to predetermine sample size. The experiments were not randomized. Experiments were repeated at least three times unless stated. The investigators were not blinded to allocation during experiments and outcome assessment. Data are expressed as the mean ± s.e.m. Statistical analysis was carried out using ANOVA, followed by Bonferroni post test for comparisons of multiple groups. For comparison between two groups, a paired t-test when appropriate or unpaired t-test with Welch correction was used. Association was tested with the Pearson correlation test. *P* < 0.05 was denoted as *, *P* < 0.01 as **, *P* < 0.001 as *** and *P* < 0.0001 as ****.

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Supplementary Figure 1  AREG-dependent stimulation of reporter translation is absent when meiotic reentry is prevented. A. Cumulus enclosed oocytes were injected with TPX2 3'UTR renilla luciferase reporter and cultured overnight in medium that allows maturation to MII or in medium that prevents maturation (2 μM milrinone) with or without AREG. At the end of the incubation oocytes were freed of surrounding cumulus cells and luciferase activity was measured in extracts from oocytes. The data are mean ± SEM of three to four independent experiments. * p <0.05 vs MII, ** p<0.01 vs MII+AREG. B. Cumulus enclosed oocytes were injected with TPX2 3'UTR renilla luciferase reporter (RLuc) and polyadenylated firefly reporter (FLuc) and cultured overnight as described above. Quantitative RT-PCR for RLuc and FLuc was performed from oocyte extracts. Exposure to Milrinone or AREG did not change the stability of the reporter. No significant differences between groups.
Supplementary Figure 2 AREG-dependent stimulation of reporter translation is absent when 3'UTR is truncated. A. Diagram of the renilla luciferase constructs injected to oocytes. B. Cumulus enclosed oocytes were injected with TPX2 3'UTR (1-1630) or TPX2 truncated 3'UTR renilla luciferase reporter and cultured overnight in medium that allows maturation to MII with or without AREG/EGF or in medium that prevents maturation (2 μM milrinone). At the end of the incubation oocytes were freed of surrounding cumulus cells and luciferase activity was measured in extracts from oocytes. The data are mean ± SEM of three independent experiments. * p <0.05 vs control.
Supplementary Figure 3 Enrichment in 3'UTR motifs in transcripts deregulated in the Areg-/- mice. Unique 3' UTR DNA sequence for all differentially translated transcripts (Supplemental Table 1 and 2) was downloaded from the mm9 assembly of the UCSC Genome Browser. The sequences were scanned for motifs with MEME-ChIP [47], searching only the given strand for any number of repetitions and allowing for motifs of length between 6 and 30 bases. Only motifs with e-values less than 0.01 are reported. For each motif discovered by MEME-ChIP, fasta sequences were downloaded and converted to RNA sequence, and the analogous RNA motif sequence logos were generated using WebLogo 3 [48]. The motif reported in the top panel corresponds to the motif recognized by the Drosophila Hrb87F or Hrb98DE protein. These proteins are homologous to the mammalian A/B-type HnRNP proteins involved in transport and stabilization of mRNAs.
Supplementary Figure 4  Phosphorylation of AKT (Ser473) in oocytes is dependent on the presence of somatic cells. A. Representative time course of AKT Ser473 phosphorylation in oocytes cultured as cumulus enclosed oocytes (CEOs). CEOs were incubated in medium with AREG and 2μM milrinone to prevent maturation. At each time point oocytes were freed of surrounding cumulus and used for Western blot analysis. Total AKT was used as loading control. A representative experiment of the three performed is reported. B. Representative Western blots of phosphorylated AKT in oocytes cultured as CEO or denuded as indicated times with or without AREG. Total AKT was used as loading control.
**Supplementary Figure 5** In situ detection of AKT phosphorylation in oocytes stimulated with AREG when in complex with cumulus cells. **A.** CEOs were cultured with or without AREG for 150 min; at the end of the incubation, oocytes were freed of cumulus cells and stained for phospho-AKT (green). **B.** Quantification of the intensity of the phospho-AKT from different experiments (mean ± SEM; n= 3). * p <0.05 vs CEO. The scale bar corresponds to 10 µm.
Supplementary Figure 6 mTOR inhibitors block the reporter translation stimulated by AREG. **A.** Representative Western blot of phosphorylation of ribosomal protein S6 (rpS6) in oocytes cultured as CEO in medium containing 50 nM of rapamycin (mTOR inhibitor) or 100nM of INK128 (selective TORC1/2 inhibitor) for 150min. Tubulin was used as loading control. **B.** Quantification of the intensity of the phospho-rpS6 immunoreactive band from different experiments (mean ± SEM; n= 3) * p <0.05 vs AREG.
Supplementary Figure 7 Uncropped images of the Western blots included in the main text showing the molecular weight markers. Dashed boxes indicate the portion of the gel included in the figure. The membrane used to generate Fig 1A, 2D and 2E were cut into two sections and developed with different antibodies. The black dotted line indicates the position of the cut. After development, the two sections of the blot were realigned and exposed together.