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Immunoneutralization of Growth Differentiation Factor 9 Reveals It Partially Accounts for Mouse Oocyte Mitogenic Activity[1]

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ABSTRACT

Paracrine factors secreted by oocytes play a pivotal role in promoting early ovarian follicle growth and in defining a morphogenic gradient in antral follicles, yet the exact identities of these oocyte factors remain unknown. This study was conducted to determine the extent to which the mitogenic activity of mouse oocytes can be attributed to growth differentiation factor 9 (GDF9). To do this, specific anti-human GDF9 monoclonal antibodies were generated. Based on epitope mapping and bioassays, a GDF9 neutralizing antibody, mAb-GDF9-53, was characterized with very low cross-reactivity with related transforming growth factor (TGF)β superfamily members, including BMP15 (also called GDF9B). Pep-SPOT epitope mapping showed that mAb-GDF9-53 recognizes a short 4-aa sequence, and three-dimensional peptide modeling suggested that this binding motif lies at the C-terminal fingertip of mGDF9. As predicted by sequence alignments and modeling, the antibody detected recombinant GDF9, but not BMP15 in a Western blot and GDF9 protein in oocyte extract and oocyte-conditioned medium. In a mouse mural granulosa cell (MGC) bioassay, mAb-GDF9-53 completely abolished the mitogenic effects of GDF9, but had no effect on TGFβ1 or activin A-stimulated MGC proliferation. An unrelated IgG at the same dose had no effect on GDF9 activity. This GDF9 neutralizing antibody was then tested in an established oocyte-secreted mitogen bioassay, where denuded oocytes cocultured with granulosa cells promote cell proliferation in a dose-dependent manner. The mAb-GDF9-53 dose dependently (0–160 μg/ml) decreased the mitogenic activity of oocytes but only by ~45% at the maximum dose of mAb. Just 5 μg/ml of mAb-GDF9-53 neutralized 90% of recombinant mGDF9 mitogenic activity, but only 15% of oocyte activity. Unlike mAb-GDF9-53, a TGFβ pan-specific neutralizing antibody did not affect the mitogenic capacity of the oocyte, but completely neutralized TGFβ1-induced DNA synthesis. This study has characterized a specific GDF9 neutralizing antibody. Our data provide the first direct evidence that the endogenous GDF9 protein is an important oocyte-secreted mitogen, but also show that GDF9 accounts for only part of total oocyte bioactivity.

follicle, granulosa cells, growth factors, ovary, ovum

INTRODUCTION

Growth and development of the somatic and germ cell compartments of the ovarian follicle occur in a coordinated and mutually dependent manner. While it is abundantly clear that endocrine and local ovarian hormones drive folliculogenesis, over the past decade there has been a large interest in paracrine factors secreted by oocytes and their role in the regulation of key granulosa cell processes. Oocyte control of folliculogenesis is important because altered expression of these oocyte paracrine factors has profound effects on fertility. Folliculogenesis fails in the absence of the oocyte, whether lacking due to genetic deficiencies or experimental ablation. It is now known that oocyte-secreted factors regulate folliculogenesis by modulating a broad range of activities associated with growth and differentiation of granulosa cells (reviewed, [1]). This knowledge has come largely from exploitation of an in vitro bioassay whereby denuded oocytes are cocultured with ovarian granulosa cells. Using this type of assay, it has become clear that oocyte-secreted factors modulate many granulosa cell activities, including proliferation [2–6], hyaluronic acid production and mucification [7, 8], steroidogenesis [9], inhibin-activin-follistatin synthesis [10, 11], and granulosa cell mRNA expression of luteinizing hormone receptor (LHR) [12] and kit-ligand [13]. By modulating some of these key regulatory elements, precise oocyte control of ovarian function via paracrine factors has broader implications for endocrine status and fertility (reviewed, [14]).

Despite the critical importance of these oocyte factors to fertility, their exact identity remains unknown. Members of the transforming growth factor-β (TGFβ) superfamily are the prime candidate molecules because of their capacity to mimic the actions of oocytes on granulosa cells in vitro (reviewed, [15]). For example, mouse cumulus cell mucification and expansion are absolutely dependent on oocyte-

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secreted factors, yet in the absence of oocytes, TGFβ1 and growth differentiation factor 9 (GDF9) are able to substitute for oocytes and enable cumulus cell expansion [7, 8, 16]. In fact, TGFβ1/β2 are able to completely substitute for oocytes in modulating many key oocyte-regulated granulosa cell functions, including regulating cumulus cell steroidogenesis, promoting granulosa cell proliferation, and enabling cumulus-oocyte complex (COC) expansion [6, 15, 17]. However, in all cases, TGFβ-neutralizing antibodies are unable to inhibit oocyte effects on cumulus/granulosa cells in vitro, demonstrating that these activities of oocytes can be mimicked, but cannot be accounted for, by TGFβ1/β2 [6, 15, 17].

Another member of the TGFβ superfamily, GDF9, is also a candidate as a key oocyte molecule regulating granulosa cell function. GDF9 is closely related to the bone morphogenetic proteins (BMPs) and activins and, in most mammals, is only expressed in gametes and in the hypothalamic-pituitary axis [18, 19]. Recent evidence suggests that GDF9 signals through known receptors and intracellular receptor-regulated signal transducers (SMADs) of the TGFβ superfamily. GDF9 binds the type II BMP receptor (BMPR-II) [20] and subsequently seems to activate the TGFβ type I receptor, also known as activin receptor-like kinase-5 (ALK-5) [21]. GDF9 intracellular effects appear to be mediated through the SMAD2 and SMAD3 signaling mediator proteins [22, 23], and in this respect, the effect of GDF9 is indistinguishable from the effect of the TGFβ's themselves. Like TGFβ1/β2, GDF9 is able to mimic oocyte-regulated granulosa cell activities, such as proliferation, steroidogenesis, kit-ligand, and inhibin synthesis [13, 16, 24, 25].

Apart from these important functions, there is a keen interest in GDF9 for two other reasons: first, in the ovary of most mammals, GDF9 is only expressed in oocytes; second, GDF9 is essential for normal follicle development and fertility. Inactivation of GDF9 through genetic deletion [26] in mice blocks folliculogenesis at the primary follicle stage. A natural GDF9 mutation in sheep also causes sterility and severely affects folliculogenesis [27]. Recent active immunization experiments against GDF9 in sheep have provided further clear evidence for an important role of this molecule in ovarian folliculogenesis and fertility in ruminants [28, 29]. Because the lesion in the GDF9 knock-out mouse ovary is so severe and because GDF9 is able to mimic the activity of oocytes in regulating granulosa cell functions, in the mouse, oocyte-paracrine actions are often equated with GDF9 activity [30]. However, it remains to be demonstrated to what extent oocyte paracrine actions on granulosa cells can be attributed to GDF9, or whether, like TGFβ1 and TGFβ2, GDF9 is simply able to mimic the actions of oocytes. To test the hypothesis that GDF9 is the key molecule responsible for the mitogenic effects of oocytes on granulosa cells, we generated a monoclonal antibody that recognizes a well-defined epitope and that specifically neutralizes GDF9 bioactivity in vitro. We then utilized this neutralizing antibody in the commonly employed oocyte-secreted factor bioassay in an attempt to characterize oocyte bioactivity through functional neutralization. Here we propose that GDF9 is indeed a key oocyte-secreted granulosa cell mitogen, but also that there remain other oocyte-secreted mitogenic molecules distinct from GDF9 that need to be characterized in further studies.

MATERIALS AND METHODS

Generation of Anti-Human GDF9 Monoclonal Antibodies and Their Epitope Mapping

The generation of anti-hGDF9 C-terminal mAbs used in this study has already been described in detail [22, 28]. Briefly, a synthetic peptide corresponding to a peptide sequence close to the C-terminus of human GDF9, VPAKYSPLSVLTIEPDGSIAYKEYEDMIATKC, was synthesized and coupled to a purified protein derivative of tuberculin [31]. Outbred Tyler Original (T/O) mice (Southend on Sea, Essex, U.K.) underwent an immunization regime over a 4-mo period. The animals were killed and the spleens removed for fusion to Sp2/0 murine myeloma cells, following standard procedures. The hybridoma supernatants were initially screened by ELISA against the peptide coated to Nunc immunoplates by standard protocol [32]. Reactive clones were expanded and recloned by limiting dilution. These were then rescreened against recombinant GDF9 and the best reacting clones selected prior to expansion and isotyping. As each clone was found to be IgG1, all were purified on a protein A column using a high-salt protocol [33] before assessment. Epitope mapping was performed by using 14-aa peptides attached to cellulose membranes (SPOT peptides) as previously described [34]. By using a 1-aa frameshift, the peptides (each 14-aa long) covered the entire human GDF9 C-terminal sequence used for immunization, enabling the identification of the minimal peptide sequence of the epitope binding to the various mAb clones.

In Silico Analyses

TGFβ superfamily C-terminal sequences were aligned using ClustalW [35], then manually adjusted. Epitope specificity was predicted using GoCore 3.2 (http://www.helsinki.fi/project/ritvos/GoCore) [36]. Protein modeling was performed by 3D-JIGSAW (http://www.bmm.icnet.uk/~3djigsaw/) [37]; then diagrams were produced using WebLab Viewer Lite and Swiss-PdbViewer (http://www.expasy.org/spdbv/) [38].

Immunoblot Analysis of Recombinant and Oocyte Proteins

Cross-reactivity of the mAb-GDF9-53 with various types of recombinant GDF9s and oocyte extracts was examined using Western blot analysis. Recombinant mouse GDF9 was generated in house (see below). *Escherichia coli*-produced recombinant ovine BMP15 [28] was generously donated by Prof. Ken McNatty, AgResearch, New Zealand. To generate oocyte-conditioned medium, immature oocytes were collected and denuded of cumulus cells as described below, then cultured for 20–24 h in 1.6-ml Eppendorf tubes in medium (see below) at 8–16 denuded oocytes (DO)/μl. Following culture, oocytes were separated from the medium and both stored at −80°C. Prior to electrophoresis, Laemmli buffer containing 104 mM dithiothreitol was added to oocytes or oocyte-conditioned medium and heated for 4 min at 100°C.

For GDF9 immunoreactive analyses, recombinant GDF9 proteins were detected using Amersham Biosciences' (Castle Hill, Australia) enhanced chemiluminescence (ECL) Western blotting detection reagents, whereas oocyte GDF9 proteins were detected using the more sensitive ECL Advance system (Amersham Biosciences). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for 1 h at room temperature, incubated overnight with anti-GDF9-mAb (recombinant GDF9/9B, 1 μg/ml; oocyte products, 5 μg/ml) followed by incubation with horseradish peroxidase-conjugated anti-mouse antibody (for recombinant GDF9/9B, 1:3000; for oocyte products, 1:200 000; Jackson ImmunoResearch Laboratories, West Grove, PA). Five percent milk in Tris-buffered saline with 0.1% Tween-20 (TBS-Tw) was used for blocking and preparation of antibody dilutions when ECL was used, whereas the supplied kit blocking reagent in TBS-Tw was used for ECL Advance.

Bioassay of Oocyte Mitogenic Activity

Experimental procedures used in this study for the bioassay of oocyte mitogens, including the collection, preparation, and coculture of mural granulosa cells (MGC) with DO, have been previously described for mouse [5] and bovine oocytes [4, 6]. This study was approved by local Animal Ethics Committees. Ovaries were collected from immature (23–32 days old) 129/SV mice 46 h after priming with 5 IU of equine chorionic gonadotrophin (eCG; Folligon, Intervet, Castle Hill, Australia). MGC and COC were collected by puncturing large antral follicles. COC were denuded of cumulus cells by vortexing COC for 3–4 min in collection me-

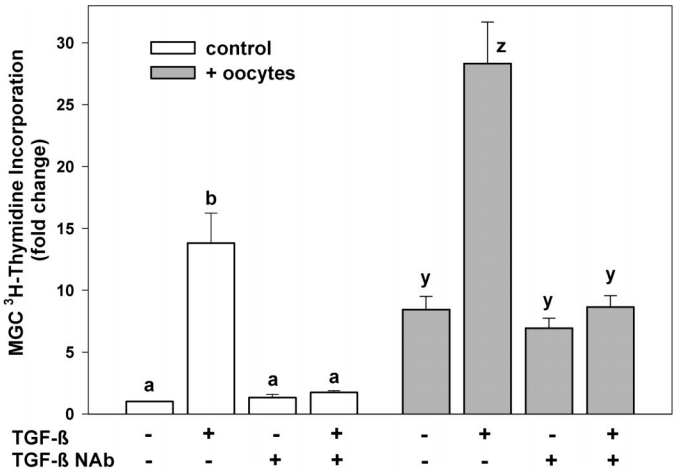

FIG. 1. Effect of neutralizing TGFβ bioactivity on MGC DNA synthesis in response to stimulation with TGFβ1 or denuded oocytes. MGC were cultured either alone, with 2 ng/ml rhTGFβ1, or with 16 oocytes/125-μl well, either in the presence or absence of 80 μg/ml of a TGFβ pan-specific polyclonal neutralizing antibody (TGFβ NAb). Bars represent relative mean values ± SEM expressed as a fraction of the control (MGC alone) from four replicate experiments. Bars with different superscripts within a group ($^{a-b}$ controls, $^{y-z}$ + oocytes) are significantly different ($P < 0.05$; two-way ANOVA). Addition of oocytes significantly increased [^{3}H]-thymidine counts (treatment main effect; $P < 0.001$), including within each treatment group relative to its respective no-oocyte control ($P < 0.05$).

dium. MGCs were collected by removing all debris from the ovary puncture dishes followed by centrifugation. MGCs and DOs were washed twice in protein-free culture medium: bicarbonate-buffered tissue culture medium-199 with supplements [5]. A small fraction of MGCs were dispersed for cell counts. MGCs were cultured at 2×10^5 cells/ml as clumps of cells rather than as dissociated cells. MGCs and DOs were transferred to individual wells of 96-well plates (Falcon, Franklin Lakes, NJ). Depending on the individual experiments, DOs, hormones, reagents, and media were added to the wells to give a final volume of 125 μl. Within each experiment, all treatments were carried out in triplicate or quadruplicate wells. Each experiment was replicated on 3–11 occasions. Cells were cultured in an atmosphere of 37°C, 96% humidity in 5% CO_2 in air for 18 h, followed by a further 6-h pulse of 15.4 kBq tritiated thymidine (^{3}H-thymidine; ICN, Costa Mesa, CA). Following culture, MGCs were harvested and incorporated [^{3}H]-thymidine was quantified using a scintillation counter as an indicator of the proportion of cells in S-phase, hence, providing an indication of the level of MGC DNA synthesis and proliferation [39].

Oocyte Bioassay: Experimental Reagents and Treatments

Four reagents were used to promote granulosa cell DNA synthesis: recombinant mouse GDF9, denuded oocytes, recombinant human TGFβ1, and recombinant human activin A (both from R&D Systems, Minneapolis, MN). Bioactive recombinant mouse GDF9 was produced in house in transfected human embryonic kidney-293H cells as previously described [22]. An approximation of mGDF9 concentrations in conditioned medium was obtained as previously described [22] using Western blot analysis of titrated mGDF9 against purified N-terminally tagged recombinant rat GDF9 [24]. Control conditioned medium was produced by culturing untransfected 293H cells (Gibco Life Technologies, Paisley, U.K.) under identical culture conditions used to produce the mGDF9. In neutralization experiments, all mitogens were pre-incubated with antibodies for 30 min in culture wells, prior to addition of MGC. The TGFβ pan-specific neutralizing antibody (R&D Systems) is a rabbit polyclonal antibody raised against multiple TGFβ antigens, including rhTGFβ1, pTGFβ1.2, pTGFβ2, and recombinant amphibian TGFβ5.

Data Analysis

Treatment effects on MGC [^{3}H]-thymidine incorporation were examined using either one-way or multivariate analysis of variance (ANOVAs) (nonparametric ANOVAs when the normality test failed) and differences between treatment means tested using Tukey-Kramer (pairwise comparisons) or Dunnett method (multiple comparisons versus a control) post hoc comparisons. A P value of <0.05 was considered statistically significant.

RESULTS

Effectiveness of the TGFβ Antibody at Neutralizing Oocyte Mitogenic Activity

Figure 1 illustrates that both oocytes and TGFβ1 stimulate ($P < 0.01$) MGC [^{3}H]-thymidine incorporation either alone or together in an approximately additive fashion. To determine if the mitogenic effects of oocytes on granulosa cells can be attributed to TGFβ, an attempt was made to neutralize oocyte-secreted factor(s) in vitro using a TGFβ pan-specific polyclonal antibody that neutralizes TGFβ1, β1.2, β2, β3, and amphibian β5. At a maximum neutralizing dose of 80 μg/ml, the TGFβ antibody was able to completely neutralize ($P < 0.05$), the stimulatory effects of TGFβ1, but not of oocytes ($P > 0.05$), on MGC [^{3}H]-thymidine incorporation (Fig. 1).

Characterization of a GDF9 Neutralizing Antibody

The choice of antibody clone to act as the GDF9-neutralizing antibody for this study was based on strong anti-GDF9 biological neutralizing capacity, high immuno-affinity to GDF9, and very low immunoaffinity to BMP15. A panel of four anti-hGDF9 C-terminal mAbs were screened and clone mAb-GDF9-53 was selected, proving more suitable than anti-GDF9–37 previously utilized for its specific immuno-affinity [22, 28]. Epitope mapping experiments using SPOT peptides revealed that mAb-GDF9-53 recognizes specifically a short 4-aa peptide sequence, EPDG, which is located approximately in the middle of the peptide sequence used for immunization. Alignment of C-terminal GDF9 and BMP15 aa sequences from a representative sample of vertebrate species (Fig. 2A) illustrated that the *EPDG* motif is very highly conserved between orthologs: 100% identity is observed in mammals and 75% identity in the frog and fish. In contrast, the epitope has a low level of similarity to the corresponding region in BMP15, including mouse BMP15. Three-dimensional modeling of the mature mouse GDF9 and BMP15 monomers suggests that the epitope lies at the C-terminal fingertip (Fig. 2B). Magnification of this region shows that, in BMP15, the two central mismatched amino acids protrude from the end of the finger. GoCore comparisons of the epitope region across selected TGFβ superfamily ligands (Fig. 2C) show the uniqueness of the motif at this site. With the exception of BMP15, none of the ligands analyzed share any single well-conserved amino acid from the motif.

As suggested by the modeling (Fig. 2B), mAb-GDF9-53 exhibited strong immuno-affinity for recombinant mouse GDF9 and very low cross-reactivity with ovine BMP15 (Fig. 3A). The immunoreactivity between recombinant GDF9 and mAb-GDF9-53 was completely blocked in the Western blot by a synthetically generated, short 8-aa peptide covering the binding epitope of mAb-GDF9-53 (data not shown). Using mAb-GDF9-53, GDF9 was detected in oocyte-conditioned medium and in oocyte extract, the vast majority as the proprotein form, with a molecular mass of 57 kDa, and a much smaller proportion as processed mature GDF9 (17.5 kDa; Fig. 3B).

Effectiveness of mAb-GDF9-53 at Neutralizing GDF9 and Oocyte Mitogenic Activity

All four anti-GDF9 mAbs screened showed strong biological neutralizing activity against recombinant mGDF9,

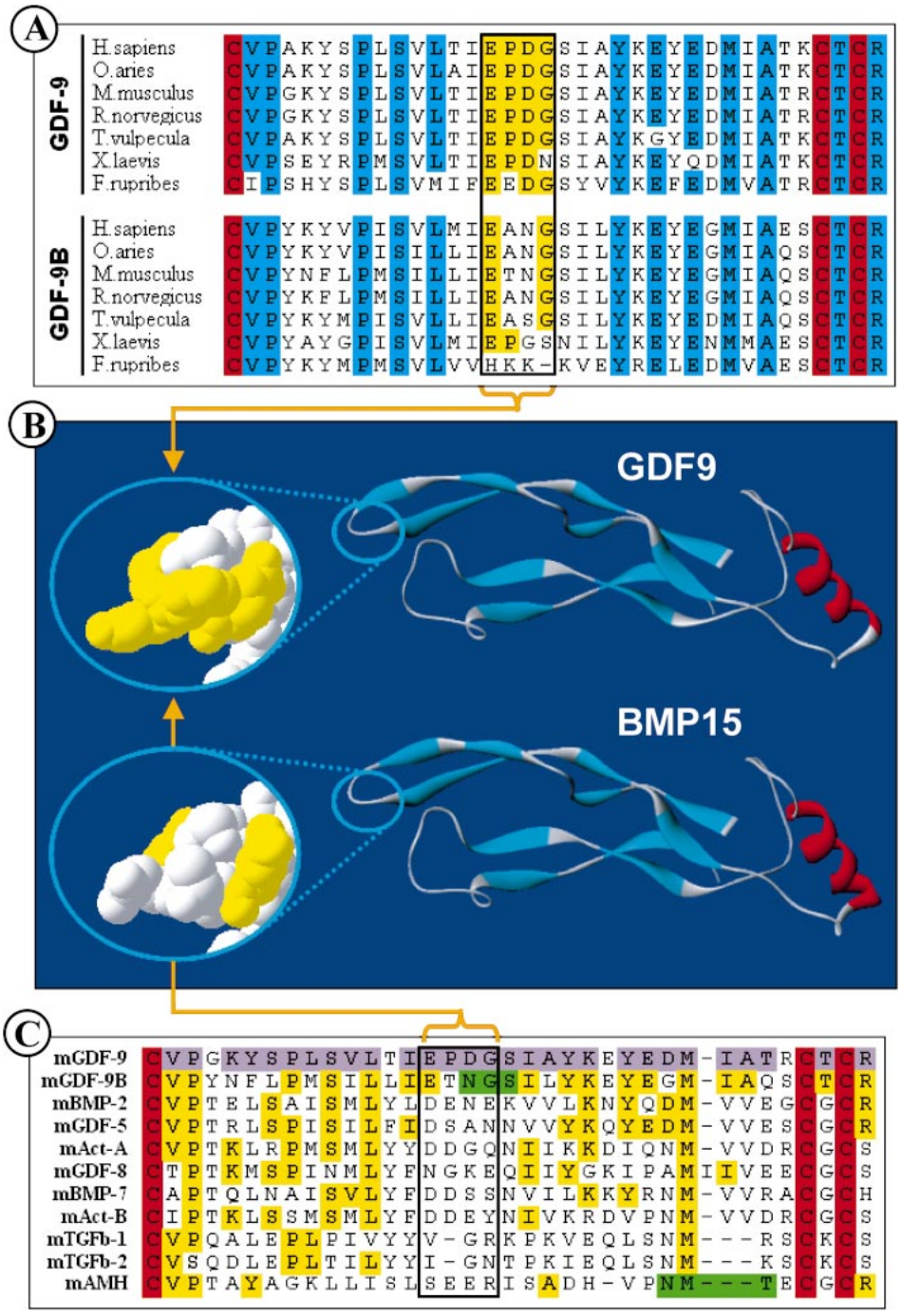

FIG. 2. Characterization of the mAb-GDF9-53 neutralizing antibody epitope: conservation across species, structural model, and uniqueness within the TGFβ superfamily. **A**) Alignment of the 35–36 aa from the C-termini of GDF9 and BMP15 from a number of vertebrate sequences. The alignments were made with ClustalW and then manually adjusted. The boxed motif is the epitope of mAb-GDF9-53, illustrating the high degree of conservation of this epitope across species and the low homology with BMP15. Color scheme: red, cysteines; blue, sites where GDF9 and BMP15 have identical aa for all species with at most one exception; yellow, aa within the anti-GDF9 epitope that match the EPDG sequence. **B**) The three-dimensional model of the mouse GDF9 and BMP15 monomers illustrating the protein backbone, the secondary structures, and the location of the anti-GDF9 epitope. The alpha helix is red and the beta strands are blue. The magnified region shows an atomic view of the epitope region with atom sizes in their relative proportions. The mAb-GDF9-53 epitope is yellow; for BMP15, the amino acids that match the epitope are yellow, but the central two amino acids do not match. **C**) A GoCore 3.2 analysis of the C-termini of mouse GDF9 and the TGFβ superfamily proteins shown. Displayed are the C-terminal 33–37 aa of the mouse sequence for each TGFβ superfamily member analyzed, ordered according to sequence similarity in their mature regions to GDF9. Purple regions indicate sites where GDF9 aa are fully conserved across known mammalian species. Yellow regions indicate sites for which another ligand shares an amino acid with GDF9 that is fully conserved in the known mammalian sequences of both proteins. The boxed EPDG motif, illustrating the binding epitope of mAb-GDF9-53 is a) unique in this region for all TGF-b family members displayed, b) fully conserved for GDF9 mammalian sequences, and c) has low homology with the other molecules within the epitope site. Cysteines are highlighted in red, potential N-linked glycosylation sites in green.

with anti-GDF9-53 the most potent, antagonizing 93% of activity averaged over seven experiments (Table 1). As predicted by the GoCore analysis (Fig. 2C), the biological neutralizing capacity of mAb-GDF9-53 was GDF9 specific; the antibody was ineffective at neutralizing the mitogenic activity of TGFβ1 and activin A (Table 1 and Fig. 4). Likewise, none of the other three mAbs antagonized TGFβ1 bioactivity (Table 1). Recombinant mouse GDF9 was a very potent stimulator of MGC DNA synthesis, dramatically increasing [^{3}H]-thymidine counts at low mitogen concentrations, as previously described in the rat [25]. The mAb-GDF9-53 antagonized, in a dose-dependent manner, the stimulatory effect of GDF9 on [^{3}H]-thymidine incorporation into MGC (Figs. 4 and 5A). The lowest dose of mAb tested, 2.5 μg/ml, was effective at causing a 75% reduction in [^{3}H] counts ($P < 0.01$). Similarly, the neutralizing actions of the mAb were not caused by general antagonist actions of the class of immunoglobulins, as human IgG had no inhibitory effect on GDF9-stimulated MGC DNA synthesis (Fig. 4).

All of the GDF9 monoclonal antibodies tested only partly antagonized the stimulatory effects of oocytes on MGC DNA synthesis (Table 1). Oocyte-stimulated MGC [^{3}H]-thymidine incorporation was decreased, in a dose-dependent manner, with increasing concentrations of anti-GDF9-53 (Fig. 5A). However, at a dose of 20 μg/ml, which eliminated effectively 100% of the recombinant GDF9 bioactivity, oocyte mitogenic activity was only attenuated by 25%. At very high doses of mAb-GDF9-53 (40–160 μg/ml), oocyte activity was only reduced to ~45% of control

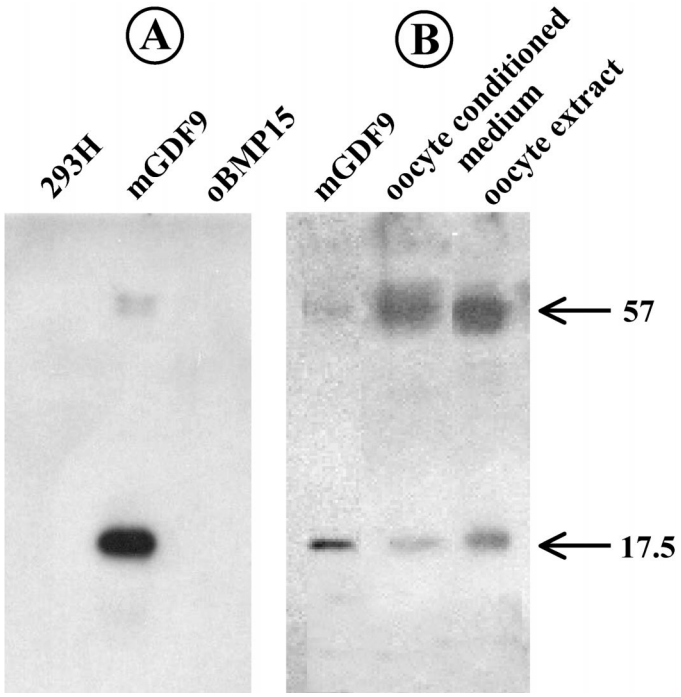

FIG. 3. GDF9 immunoblot analysis. **A**) Antibody specificity for GDF9 and (**B**) detection of oocyte-secreted GDF9. The recombinant proteins indicated (mGDF9 = mouse GDF9, oBMP15 = ovine BMP15) and oocyte extract or oocyte-conditioned medium were subjected to SDS-PAGE immunoblotting with the anti-GDF9 monoclonal antibody and detected using either ECL (**A**; recombinant proteins) or ECL Advance (**B**; oocyte products). The 57-kDa band is the GDF9 proprotein while the 17.5-kDa band represents the mature GDF9 monomer. 293H = control; conditioned medium from the untransfected human embryonic kidney 293H parent cell line.

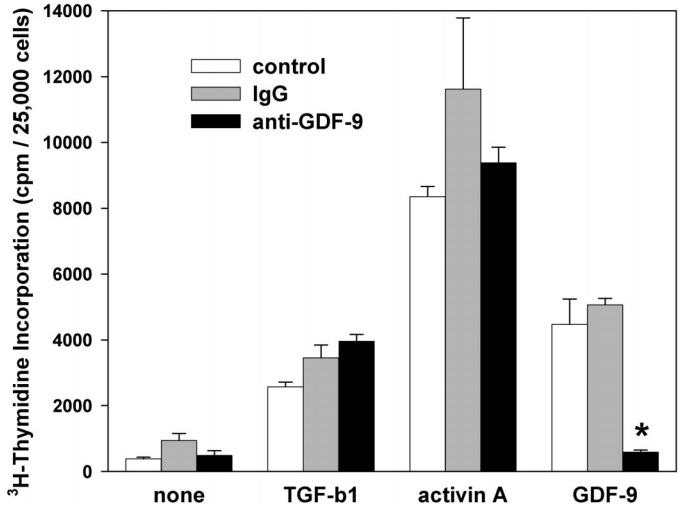

FIG. 4. Biological neutralizing specificity of anti-GDF9-53 with some members of the TGFβ superfamily. Mouse MGC were cultured with human TGFβ1 (0.5 ng/ml), human activin A (50 ng/ml), or mouse GDF9 (40 ng/ml), either in the absence or presence of a high neutralizing dose of 40 μg/ml of mAb-GDF9-53 or 40 μg/ml human IgG. Bars represent means ± SEM from at least triplicate wells and are representative of three replicate experiments. Asterisks represent significant neutralization ($P < 0.05$) caused by mAb-GDF9-53 relative to the control for that mitogen. Control = MGC alone or with treatment mitogen.

levels. To further exclude the possibility that the oocyte mitogen(s) could be in excess of the mAb, an experiment was conducted where the concentration of oocytes was decreased at a constant concentration of mAb. The anti-GDF9-53 at a high dose of 80 μg/ml caused the same degree of partial neutralization of oocyte bioactivity (~45%), regardless of oocyte density (Fig. 5B). Together, these data suggest that oocyte-secreted GDF9 promotes proliferation of MGC, but that only part of the total mitogenic activity of oocytes can be accounted for by GDF9.

DISCUSSION

In recent years, it has become increasingly clear that oocytes play a critical and multifaceted role in the development of ovarian follicles through the secretion of soluble paracrine growth factors. The primary recipients of these local oocyte factors are granulosa cells in preantral follicles and the specialized cumulus granulosa cells in antral follicles. Evidence, now from many different groups, on the many roles oocytes play in regulating granulosa cell functions comes in particular from in vitro oocyte-granulosa cell bioassays, similar to the one used in this study. Despite considerable effort and more than 14 yr of research in this area, the exact identity of these key oocyte molecules remain unknown. Regulatory molecules have still not been identified and purified from oocyte-conditioned medium, and attempts at neutralizing the activities of oocytes in the granulosa cell bioassay have not been successful. To date, the best candidate molecules are members of the TGFβ superfamily, in particular GDF9 (reviewed, [15]). Even though TGFβ1 and TGFβ2 are able to mimic the paracrine actions of oocytes on granulosa cells, these growth factors have been all but eliminated as candidate molecules. TGFβ1/β2 are highly unlikely to account for oocyte mitogenic activity (mouse [present study], cow [6]), oocyte-regulation of granulosa cell steroidogenesis [15], or oocyte-

TABLE 1. Biological neutralizing capacity of various anti-hGDF9 monoclonal antibodies.

Anti-hGDF9 clone[b]	Epitope	% Neutralization[a] GDF9 (80 ng/ml)	DOs (12 oocytes/well)	TGF-β1 (0.5 ng/ml)
Control (none)	—	0	0	0
IgG	—	−5	−6	5
mAb-53	EPDG	93	40	−4
mAb-37	LSVLTIEPDGSIAY	67	38	−12
mAb-19	LSVLTIEPDGS	73	17	−4
mAb-22	IEPDGS	75	22	−21

[a] Inhibition of GDF9-, oocyte-, or TGF-β1-stimulated MGC [^{3}H]-thymidine incorporation by various anti-hGDF9 monoclonal antibodies (or IgG control). Values are mean percent neutralization expressed relative to the control (mitogen with no antibody). Negative values indicate stimulation of MGC DNA synthesis.
[b] All IgGs at 80 μg/ml.

regulated cumulus cell mucification/expansion [15, 17]. This study was undertaken to determine if GDF9 accounts for the oocyte factor(s) that support folliculogenesis by promoting proliferation of follicular granulosa cells. The experiments reported here provide evidence that GDF9 is indeed an important oocyte-secreted mitogen, but that other molecule(s) additionally contribute to oocyte-induced granulosa cell proliferation.

Although in vivo immunization against GDF9 was recently documented [28], the current study is the first to describe an antibody with GDF9-neutralizing capacity in the oocyte-factor granulosa cell bioassay. The mAb-GDF9-53 was raised against a synthetic peptide fragment from the C-terminus of human GDF9. Epitope mapping revealed a short 4-aa binding motif, fully conserved across mammalian GDF9 sequences, yet with low homology at this epitope to related TGFβ superfamily members. Hence, as anticipated, the antibody is quite specific for GDF9 as it has no, or very low, immuno- and biological affinity for non-GDF9 TGFβ family members. Importantly for discriminating the biological actions of GDF9 from its closely related homologue BMP15 [40]; (also called GDF9B), the GDF9 neutralizing antibody also has very low affinity for BMP15. In vitro, this antibody specifically antagonizes the bioactivity of a fully processed recombinant mouse GDF9. Peptide modeling predicts that the antibody binds the C-terminal fingertip of GDF9. At this stage, it is unclear exactly how the antibody functionally antagonizes GDF9—it may interfere in some way with ligand interaction with either of the type-I or type-II receptors. Further experiments are underway to determine how the neutralizing mAb affects GDF9-receptor signaling.

This potent GDF9 antagonist was able to completely abolish recombinant GDF9 bioactivity and was able to neutralize ~45% of the mitogenic effects of oocytes. This is the first direct evidence that endogenous GDF9 accounts for part of the growth-promoting effects of oocytes on granulosa cells. Recently, various lines of evidence have implicated GDF9 as a key oocyte-secreted factor, particularly as addition of recombinant GDF9 to granulosa cells in vitro is able to mimic many of the demonstrated actions of oocytes on these cells [13, 16, 24, 25]. While the oocyte-mimicking actions of GDF9 on granulosa cells may provide some insight into the identity and actions of oocyte factor(s), this evidence is circumstantial. This is clearly illustrated by the fact that, just like GDF9, TGFβ1 and TGFβ2 are able to mimic a broad range of oocyte factors, yet these TGFβs are not the oocyte-secreted factors [present study, 6, 15, 17]. In the current study, we provide evidence that oocytes secrete GDF9 and that this molecule accounts for part of the mitogenic activity of oocytes: first, immunoreactive mature and unprocessed GDF9 was detected in oocyte-conditioned medium; and second, part of the proliferative effects of oocytes was antagonized by the GDF9-neutralizing antibody. It is notable that oocyte-granulosa cell cocultures have been exploited as an experimental tool for over a decade, yet this study is the first to positively identify and neutralize a protein in this medium. Interestingly, the most prevalent form of GDF9 detected in oocyte-conditioned medium was the unprocessed form. In general, members of the TGFβ superfamily act as homodimers of fully processed mature peptides, where the proregion is proteolytically cleaved at secretion. The biological significance of oocyte secretion of unprocessed GDF9 is unclear at this stage. This may be an artifact of the in vitro environment used to produce oocyte-conditioned medium, perhaps unprocessed GDF9 is biologically active as occurs with anti-Müllerian hormone, or maybe as speculated [13], other local signals induce protease activity that cleave and thereby activate the unprocessed GDF9.

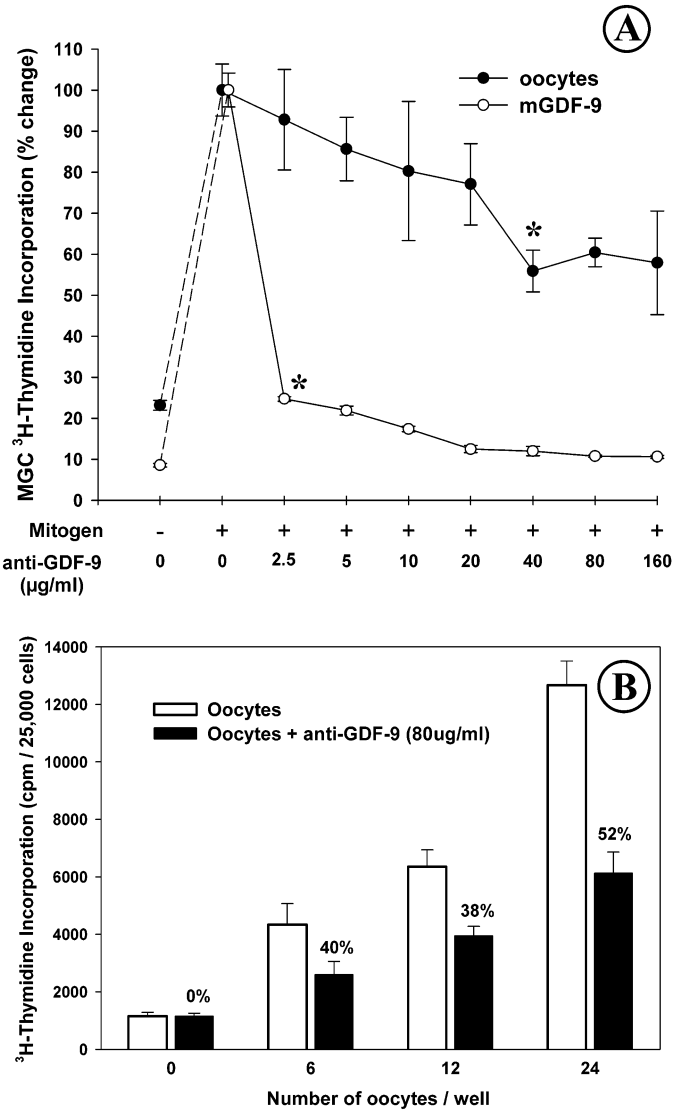

FIG. 5. Potency of anti-GDF9-53 at neutralizing oocyte- or GDF9-stimulated MGC DNA synthesis. **A**) Mouse MGCs were cultured either with or without denuded oocytes (12/125-μl well; closed circles) or 40 ng/ml recombinant mouse GDF9 (open circles), either in the absence or presence of increasing doses of mAb-GDF9-53. Points are means ± SEM from at least triplicate wells, expressed as a fraction of the control (mitogen with no antibody) and are representative of four replicate experiments. An asterisk represents the lowest dose of mAb-GDF9-53 that is significantly less than the 100% control ($P < 0.05$). **B**) Mouse MGCs were cultured with increasing numbers of denuded oocytes (0–24 oocytes/125-μl well) either in the absence or presence of mAb-GDF9-53 at a maximum dose of 80 μg/ml. After 24 h of culture, the labeled thymidine incorporated into cells was counted. Bars represent means ± SEM from at least triplicate wells and are representative of three replicate experiments. Where oocytes and antibody were present, the antibody significantly ($P < 0.05$) reduced [^{3}H] counts (percentages express the degree of neutralization caused).

While the results of this study indicate that GDF9 seems to account for approximately half of the mitogenic activity of mouse oocytes, the identity of the remaining half is unknown, although as outlined above, it is unlikely to be TGFβ1 or TGFβ2. BMP15 seems a likely candidate due to its many similarities to GDF9 (including sequence, struc-

tural, and expression profile) [41]. The notion that the molecules contributing to the mitogenic activity of oocytes are a combination of GDF9 and BMP15 (possibly in the form of a putative GDF9/BMP15 heterodimer) is supported by in vivo evidence from loss-of-function female animals. The GDF9 null mouse is sterile due to an early block in folliculogenesis [26], illustrating a clear requirement for GDF9 that cannot be compensated for by BMP15. The BMP15 null mouse is subfertile, with apparent defects in late folliculogenesis and ovulation [42]. This suggests an additional role for either BMP15 and/or a putative GDF9/BMP15 heterodimer in the mouse. There is an absolute role for both GDF9 and BMP15 in the sheep, as loss of function of either molecule through immunization [28] or through genetic deficiency [27, 43] leads to ovarian failure. Important in terms of the implications of these oocyte molecules to fertility is the observation that partial loss of GDF9 or BMP15 function in sheep, but not in mice, leads to increased fecundity [27, 28, 43]. Despite apparent strong circumstantial evidence for a combined role of both GDF9 and BMP15 as key oocyte paracrine regulators, other growth factor groups, e.g., the activins, fibroblast growth factors, and other BMPs, are likely to also contribute to the pool of oocyte-secreted factors.

The findings from this study provide strong support for the hypothesis that GDF9 is a potent oocyte-secreted granulosa cell mitogen and also that, in the mouse, there are likely to be multiple oocyte growth-promoting factors. This study has developed and exploited a highly specific GDF9 neutralizing antibody. This antibody should prove to be a powerful experimental tool for investigating other key granulosa cell processes under oocyte control.

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