Inferring biological mechanisms from spatial analysis: Prediction of a local inhibitor in the ovary

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Female mammals are born with a lifetime's supply of oocytes individually enveloped in flattened epithelial cells to form primordial follicles. It is not clear how sufficient primordial follicles are maintained to sustain the reproductive lifespan, while providing an adequate supply of mature oocytes for ovulation. Locally produced growth factors are thought to be critical regulators of early follicle growth, but knowledge of their identity and source remains incomplete. Here, we have used a simple approach of spatial analysis of structures in histological tissue sections to identify likely sources of such regulatory molecules, narrowing the field for future screening for candidate growth factors or antagonists. We have quantified the relative spatial positions of primordial (resting) follicles and growing follicles in mice on days 4, 8, and 12 after birth, and calculated interfollicular distances. Follicles were significantly less likely to have started growing if they had 1 or more primordial follicles close by (within 10 μm), predicting that primordial follicles inhibit each other. This approach allows us to hypothesize that primordial follicles produce a diffusible inhibitor that prevents neighboring primordial follicles from growing. Such an approach has wide applicability within many branches of developmental and cell biology for studying spatial signaling within tissues and cells.

diffusing inhibitor | follicle growth | signal gradient

Spatial relationships play a key role in the development and regulation of biological functions in multicellular organisms. The analysis of imaging data therefore makes a vital contribution to many biological experiments. However, unlike, for example, the sophisticated quantitative analysis applied to high-throughput data, the analysis of spatial structures and patterns is usually still carried out in a largely qualitative manner. Thus, for instance, patterns of hair follicle spacing and orientation are compared on the basis of visual similarity, rather that explicit quantitative assessment e.g. (1–3). This makes it impossible to carry out statistical tests to determine whether differences between spatial patterns are significant or not. This, in turn, makes it difficult to deduce new mechanisms or to propose biological hypotheses from imaging data.

There is in fact a large literature on the descriptive quantification of spatial patterns (e.g., refs. 4–8). Although they are not in widespread use in cell and molecular biology, many of these techniques have been applied in specific biological examples. However, such techniques are primarily used in a diagnostic fashion, to classify images into two (or more) groups, such as diseased and healthy tissue. Such classification methods are poorly suited to the discovery of new biological mechanisms or functional relationships.

In this article, we introduce a new "inverse" approach to the analysis of spatial patterns and demonstrate its application to signaling in the mammalian ovary. By relating the properties of a structure (an ovarian follicle) to its distance from potential nearby sources of regulatory signals we provide strong evidence for a local diffusing inhibitor and determine its signaling range. This illustrates the power of our method in inferring new biological mechanisms from imaging data.

Female mammals are born with a finite number of oocytes that declines over their reproductive lifespan (9). During fetal life, or shortly after birth, oocytes become enveloped by flattened pregranulosa cells and form primordial follicles. Some of these follicles immediately start growing, a process that is marked by a change in the shape of the granulosa cells (from flattened to cuboidal), by the onset of granulosa cell division and by oocyte growth. Entry of primordial follicles into the growth phase continues throughout reproductive life. Its rate has to be carefully regulated to ensure a steady supply of mature follicles for ovulation, without prematurely exhausting the stock of oocytes (10).

It has long been recognized that the first follicles to grow are those situated more centrally in the ovary, at the boundary between the cortex and the medulla, whereas nongrowing primordial follicles generally reside in the outer cortex, just below the ovarian surface (11). The presence of such a pattern, which is common to a variety of mammals, suggests that initiation of follicle growth is a tightly regulated process, and that morphogen gradients may be involved. Very little is known about the mechanisms regulating such initiation. One hypothesis is that primordial follicles are held in a quiescent state by an inhibitory signal (10, 12) and that the rate of initiation is regulated by the local concentration of this inhibitor. It has been proposed that a logical source for such an inhibitor would be the growing follicles (Fig. 1*A*) (13, 14). If the number of growing follicles was large, the inhibitory signal would be stronger, reducing the rate of initiation. Conversely, if there was a lack of growing follicles, then the inhibitory signal would weaken, allowing more primordial follicles to initiate growth. Candidates for such inhibitory signals include Anti-Müllerian Hormone (AMH) (14) and activin (13), both members of the Transforming Growth Factor β (TGF-β) superfamily.

However, this hypothesis cannot explain the absence of global initiation of follicle growth in the neonatal mouse ovary, which initially lacks any growing follicles to produce the putative inhibitor. This implies the existence of another source of inhibition at this stage, and makes the mouse neonatal ovary a valuable model for the study of follicle growth initiation. Another potential source of inhibitor could be the primordial follicles themselves (15, 16) (Fig. 1*B*). Alternatively, the localization of nongrowing follicles to the outer cortex suggests that the source could be the ovarian surface epithelium (Fig. 1*C*).

We can discriminate between these competing hypotheses by observing that primordial follicles should generally be located close to the source of the putative inhibitor and growing follicles should lie further away. In this article we therefore develop a method of quantifying the distances between different classes of follicles and

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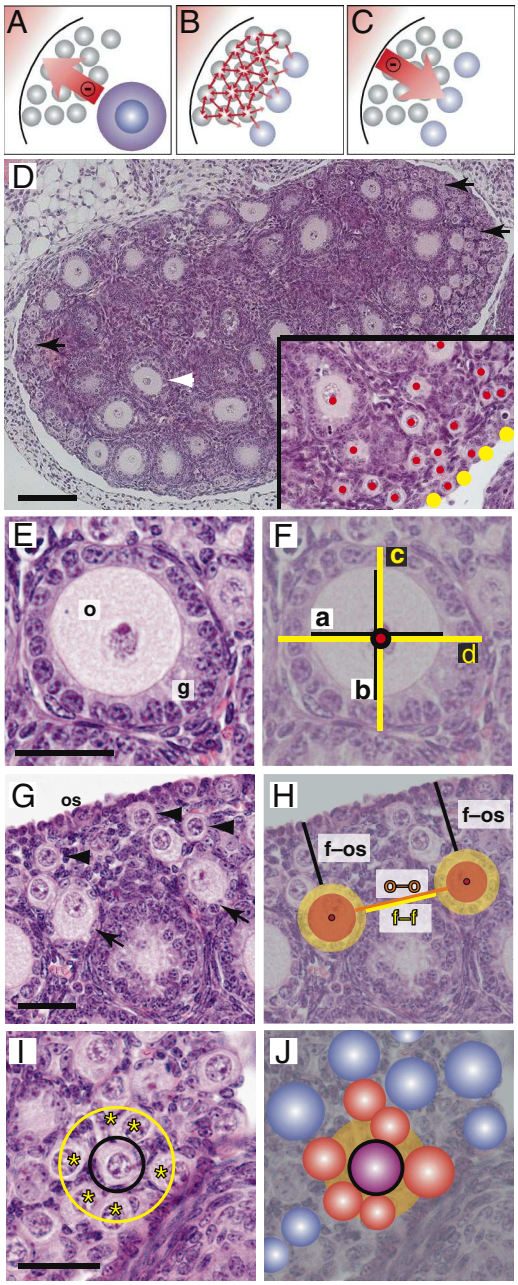

Fig. 1. Potential sources of inhibitor and spatial analysis of mouse ovary sections. (*A–C*) Hypothetical sources of a diffusing inhibitory signal from growing follicles (*A*), primordial follicles (*B*), or the ovary surface (*C*). (*D*) Middle section of day 12 mouse ovary with a number of primordial (black arrow) and growing (white arrow) follicles marked. (Scale bar: 100 μm.). Photomicrographs of all of the sections analyzed in this study are shown in Fig. S1. (*Inset*) magnified portion of image, with centres of follicles marked with red spots, and points on ovary surface marked with yellow spots. (*E*) Primary growing follicle with oocyte (o) surrounded by a single layer of cuboidal granulosa cells (g). (Scale bar: 30 μm.) (*F*) Measurements of center (red spot) of the same follicle and two perpendicular distances used to estimate oocyte (*a* and *b*) and follicle (*c* and *d*) diameter. (*G*) Primordial (arrowheads) and growing (arrows) follicles near ovarian surface (os). (Scale bar: 30 μm.) (*H*) Calculation of oocyte–oocyte (o–o), follicle edge to follicle edge (f–f) and follicle edge to ovary surface (f–os) distances, see Eqs. **1**–**3** in *SI Materials and Methods*. (*I* and *J*) Determination of the number of neighboring follicles that lie within 10 μm (yellow annulus) of the central target follicle (black ring). Follicles whose edges intersect this yellow region have a follicle to target follicle distance $d_{ij}^{(f)}$ (Eq. **3** in *SI Materials and Methods*) of 10 μm or less and are indicated with asterisks in *I* and shaded red in *J*. Follicles further than 10 μm from the target follicle are shaded blue. (Scaler bar: 30 μm.)

the various potential sources of a regulatory signal. We successfully apply our method to histological sections of mouse ovary at 3 time points (days 4, 8, and 12 post partum).

Results

For each day, the central section of 1 ovary of each of 2 mice was analyzed (Figs. S1 and S2). To localize the source of regulatory signals we determined the distances between every pair of follicles, and between each follicle and the ovarian surface epithelium. The developmental stage, x and y coordinates and dimensions of each follicle in the section were recorded (Fig. 1 *D–F*), allowing us to estimate the Euclidean distance between oocyte or follicle surfaces (Fig. 1 *G* and *H*). The distribution of developmental stages for each ovary is shown in Fig. S3*A*. In total, we analyzed 981 follicles, with the number in each section ranging from 115 to 219, giving rise to a total of 84,797 interfollicular distances (Fig. S3*B*). Mapping of the ovarian surface also allowed estimation of distance between follicles and the surface of the ovary.

This information was used to determine how many follicles at each developmental stage are within a set distance of any given follicle (Fig. 1 *I* and *J*). Preliminary exploration revealed a strong correlation between follicle stage and the presence of other follicles within 10 μm and this distance was therefore used for the remainder of the analysis. We used follicle-follicle distance (Fig. 1*H*), assuming that an inhibitor would be released from the surface of a source follicle and detected by receptors on the surface of the target follicle. It is also possible that the inhibitor would be produced by the oocyte, and/or detected by the oocyte. We thus also carried out the analysis for oocyte–oocyte distances, leading to similar conclusions (data not shown). We present the results of our analysis separately for each of days 4, 8, and 12, although the main conclusions are consistent across all 3 ages, and in particular follicle distributions on days 8 and 12 are remarkably similar. We also carried out the key analyses for each individual ovary (Fig. S4) and found no important differences between the 6 ovaries analyzed.

Effect of Neighboring Growing Follicles. If the first hypothesis that growing follicles inhibit initiation of growth (Fig. 1*A*) is correct, then primordial follicles should have 1 or more growing follicles in their proximity. Fig. 2*A* shows how the probability of a follicle initiating growth varies as a function of the number of growing follicles within 10 μm. We see that follicles with no growing neighbors are unlikely themselves to be growing, and as the number of growing neighbors increases, the follicle is itself more and more likely to be growing (the difference between 0 neighbors and 1 neighbor, and between 1 neighbor and 2 neighbors is significant with $P < 0.0001$ on each day). This provides strong evidence against an inhibitory signal produced by growing follicles as hypothesized in Fig. 1*A*. Indeed, it shows a stimulatory relationship between growing follicles and their neighbors. The most likely mechanism to explain this is that growing follicles produce a diffusing signal that exerts a stimulatory effect in their neighborhood. This result was unexpected and we discuss its interpretation below. However, because our main aim was to identify potential sources of inhibition, we turned our attention to the other possible origins of putative inhibitory signals (Fig. 1 *B* and *C*).

Effect of Neighboring Primordial Follicles. If the alternative hypothesis that primordial follicles themselves produce an inhibitor (Fig. 1*B*) is correct, then primordial follicles should have other primordial follicles nearby. This is precisely what we observe in our data (Fig. 2*B*). If a follicle has no nearby primordial follicles, it has an ≈90% chance of being a growing one. A single primordial follicle within 10 μm reduces this to ≈30–40% (significant difference for each day; $P < 0.001$) and 2 or more primordial neighbors bring the proportion down to 20% (significantly different from 1 neighbor on days 8 and 12; $P < 0.0001$; additionally on day 4 there is a significant difference between 2 and 4 neighbors; $P < 0.0001$ and on day 12 a

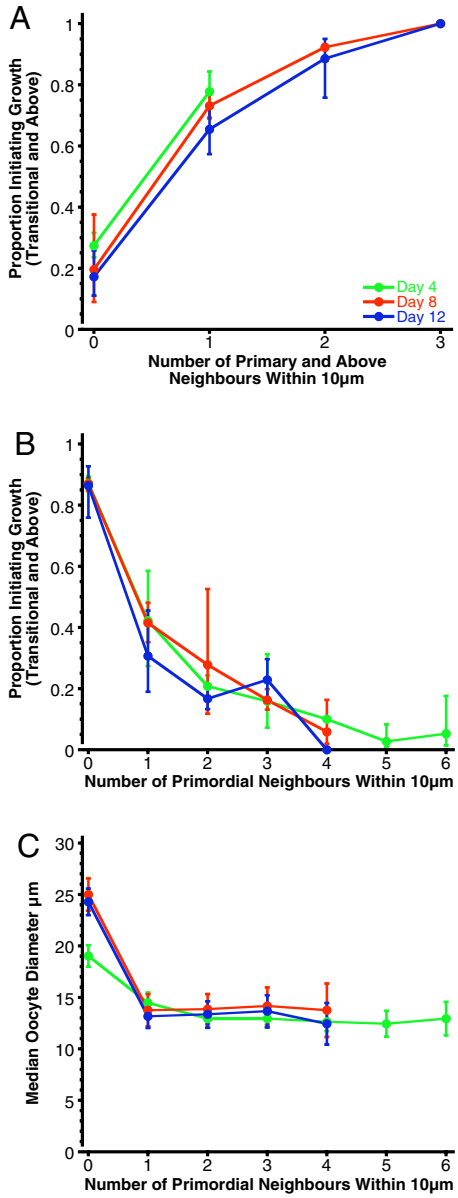

Fig. 2. Effect of number of primordial or growing follicle neighbors on follicle growth at 4, 8, and 12 days post partum. (*A*) Proportion of follicles initiating growth as a function of the number of growing (primary and above) neighbors within 10 μm. (*B*) Proportion of follicles initiating growth as a function of the number of primordial neighbors within 10 μm. (*C*) Oocyte diameter as a function of the number of primordial neighbors within 10 μm. Only oocytes with a visible nucleus are included. Fig. S5*A*, presents analogous results for follicle diameter. Error bars are 95% confidence intervals. A version of *A* and *B* plotted by individual ovaries is given in Fig. S4, and shows consistent behavior for all 6 ovaries that were analyzed. The raw data used to calculate the proportions in *A* and *B* are given in Tables S1 and S2, respectively.

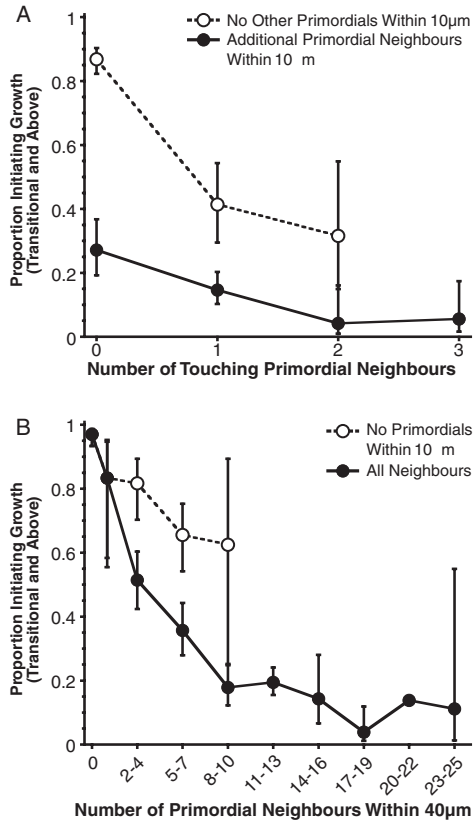

Fig. 3. Range of inhibitory effect of primordial follicles. Error bars are 95% confidence intervals. (*A*) Proportion of growing follicles as a function of touching primordial follicles. A follicle is defined to be touching if the center–center distance (calculated using Eq. **1** in *SI Materials and Methods*) is less than or equal to the sum of the estimated radii. (*B*) Proportion of growing follicles as a function of the number of primordial follicles within 40 μm.

significant difference between 2 and 3 neighbors; $P < 0.0001$). There is therefore a strong inhibitory relationship between nearby primordial follicles and the initiation of follicle growth.

In addition to using a categorical morphological classification of growing and nongrowing follicles, we carried out a similar analysis, using the oocyte diameter (Fig. 2*C*). As mouse follicles initiate growth, the oocyte itself starts growing. We see that oocytes in follicles with no nearby primordial neighbors have a significantly larger diameter than those with 1 or more neighbors; even a single primordial neighbor within 10 μm is associated with significantly smaller oocytes ($P < 0.0001$ for each day separately, using the Mann–Whitney test to compare the median oocyte diameter for follicles with zero neighbors against 1 neighbors, or against 1 or more neighbors).

Range of Signal. Figs. 2 *B* and *C* reveal a consistent relationship between reduced follicle and oocyte growth and the presence of nearby primordial follicles. This pattern predicts the existence of a previously unknown inhibitory signal, or signals, produced by primordial follicles, in line with the hypothesis shown in Fig. 1*B*. This is consistent with the observation that experimental reduction of the primordial pool in rats resulted in an increase in the proportion of growing follicles (17).

We next characterized the range of action of this inhibitor, and determined whether it was mediated by direct follicle-to-follicle contact or by a diffusing signal. Fig. 3*A* shows the likelihood of a follicle initiating growth against the number of touching primordial neighbors. Consistent with Fig. 2*B*, we see that increasing the number of touching primordial follicles has an inhibitory effect on follicle growth (Fig. 3*A*, dashed line). However, if there are additional primordial neighbors within 10 μm, the inhibitory effect of touching follicles is significantly enhanced ($P < 0.0001$) (Fig. 3*A*, solid line). This suggests that the inhibitory factor is not just mediated by direct follicle-follicle contact but can act over a distance.

To estimate a maximum distance over which this signal is effective, we plot the likelihood of growing against the number of primordial neighbors within a much greater distance, namely 40 μm (Fig. 3*B*). We see that primordial follicles within a 40-μm annulus

still have an inhibitory influence, but this is much weaker than over a distance of 10 μm. Thus, whereas ≈8–10 primordial follicles are required within a distance of 40 μm to reduce the probability of growing to ≈20% (Fig. 3*B*, solid line), between 2 and 3 are sufficient at a distance of 10 μm (Fig. 2*B*). This is emphasized when we analyze only those follicles that have no nearby primordial follicles within 10 μm. In this case 9 follicles within 40 μm only reduce the probability of growing to ≈65% (Fig. 3*B*, dashed line). This demonstrates that the inhibitory effect is weak beyond a distance of 10 μm.

Effect of Ovary Surface. Finally, if the ovarian surface epithelium is releasing an inhibitor (Fig. 1*C*), primordial follicles should be close to the surface with growing follicles situated deeper in the ovary. We first examined the average distance of follicles at each stage of development from the ovary surface (Fig. 4*A*). We observe a coherent pattern in which the primordial follicles are, on average, concentrated close to the ovary surface, with growing follicles at advancing developmental stages located further and further from the surface. We then calculated the proportion of growing follicles as a function of the distance from the surface (Fig. 4*B*). Less than 20% of the follicles within 20 μm of the ovary surface have initiated growth, but this proportion increases dramatically with increasing distance from the ovary surface (the difference in the proportions initiating growth between the 0- to 20-μm and 20- to 40-μm bands and between the 20- to 40-μm and 40- to 60-μm bands are all significant on each day; $P < 0.0001$). The consistency in this pattern between days 8 and 12 is quite striking. On these days, essentially all follicles that are >60 μm from the ovary surface are growing ones.

In addition to using a categorical morphological classification, we can also see how oocyte diameter varies with increasing distance from the surface of the ovary (Fig. 4*C*). On all 3 days, the median size of the oocytes in follicles within 40 μm of the ovary wall is uniformly small. Beyond this distance, there is a gradual increase in the diameter of oocytes on day 4, and a more marked increase on days 8 and 12. There is again consistency between days 8 and 12 up to a distance of 60 μm from the surface, suggesting regulation in the spatial pattern of follicle growth rather than a random process. One possible mechanism for this is a diffusing inhibitor released by the ovary surface, as illustrated in Fig. 1*C*.

Interaction Between Primordial Neighbors and the Ovary Surface. So far, we have shown that follicles are unlikely to grow if they are either close to the ovary surface, or close to other primordial follicles. These two factors are not independent. Follicles close to the ovary surface have a high probability of having at least 1 primordial neighbor (Fig. 5*A*). This probability declines with increasing distance from the surface, particularly on days 8 and 12. Conversely, follicles with no primordial neighbors are further from the ovary surface than those with at least 1 primordial neighbor (Fig. 5*B*). Again, this effect is more pronounced on days 8 and 12.

To quantify the relative strength of the putative inhibitory action of the ovarian surface epithelium and neighboring primordial follicles, we plotted the proportion of growing follicles at increasing distances from the ovary surface when they had no primordial neighbors, and when they had 1 or more primordial neighbors (Fig. 5*C*). The inhibitory effects of the ovary surface and of primordial neighbors are synergistic. The effect of the ovary surface on its own is weak, with ≈50% of isolated follicles within 20 μm of the surface being free to grow (Fig. 5*C*, green line). However, the presence of even 1 primordial neighbor within 10 μm reduces the probability of growing to 10% (Fig. 5*C*, red line). This strong effect of a single neighbor rapidly weakens as one moves away from the surface, so that beyond ≈40 μm of the ovary surface ≈75% of follicles with only 1 primordial neighbor are growing (Fig. 5*C*, red line). Primordial neighbors do appear to strongly inhibit growth away from the ovary surface, but only if there are at least 3 such neighbors (Fig. 5*C*, light blue line). Fig. 5*D* shows a complementary view, confirming that primordial neighbors have a much stronger inhibitory effect close to the ovary surface.

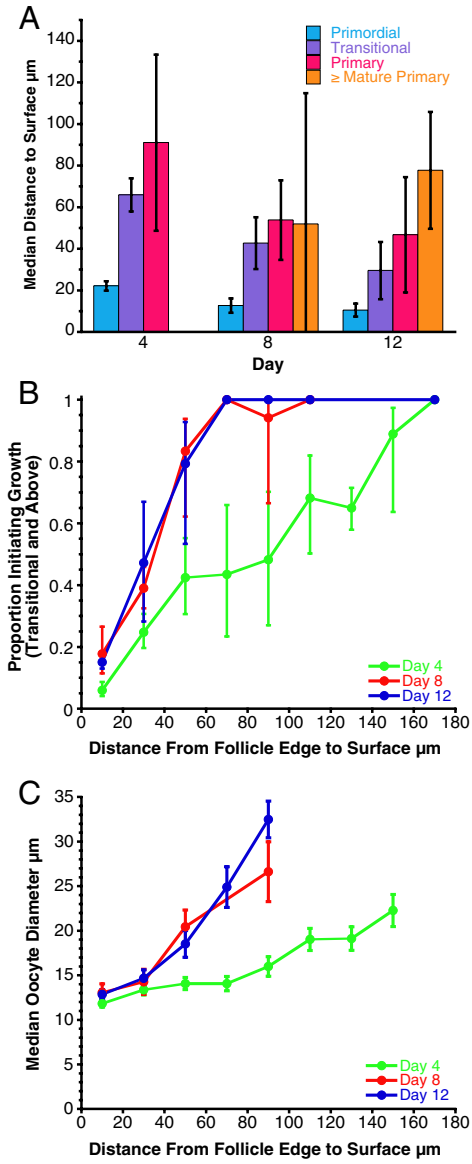

Fig. 4. Spatial pattern of follicle stage and oocyte size in relation to the shortest distance between the edge of the follicle and the ovary surface (f–os in Fig. 1*H*). (*A*) Median distance to the surface for follicles at different stages of development. (*B*) Proportion of growing follicles in successive bands of width 20 μm from the ovary surface. (*C*) Average oocyte diameter (measured only in oocytes with a visible nucleus) in successive bands of width 20 μm from the ovary surface. Fig. S5*B*, presents analogous results for follicle diameter. Error bars represent 95% confidence intervals.

Inhibitory Enhancement by Ovarian Surface. To summarize, our analysis predicts that the main inhibitory effect is due to production of an inhibitory signal by primordial follicles, with a range of ≈10 μm. However, this effect is substantially enhanced close to the ovary surface (≤ 20 μm). There are three possible explanations for this enhancement. If the inhibitory mechanism is mediated by a diffusing signal that cannot pass through the ovary surface and that is degraded relatively slowly, the signal will accumulate in a narrow region close to the surface, resulting in an increased level of inhibition. The plausibility of this effect is demonstrated by the

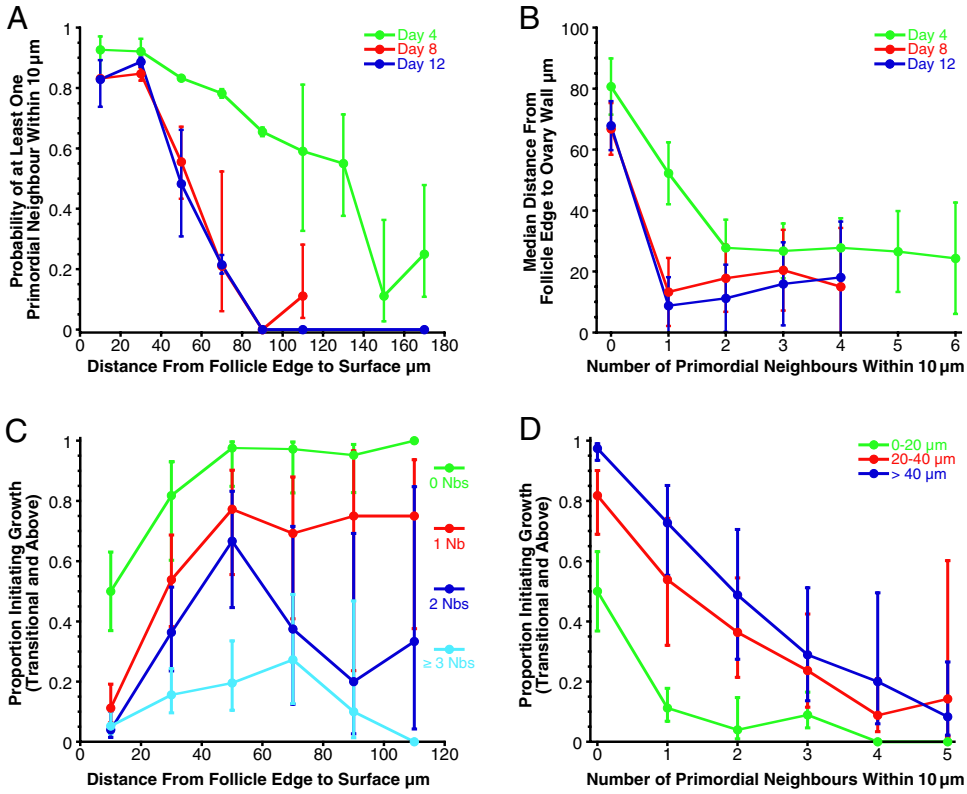

Fig. 5. Interaction between the effects of the ovary surface and nearby primordial follicles. Error bars are 95% confidence intervals. (*A*) Probability of having at least 1 primordial neighbor within 10 μm for follicles in successive bands of width 20 μm from the ovary surface. (*B*) Median distance from surface for follicles with different numbers of primordial neighbors. (*C*) Proportion of growing follicles in successive bands of width 20 μm from the ovary surface, classified by the number of primordial neighbors within 10 μm. Nb, neighboring primordial follicle. (*D*) Proportion of growing follicles in successive bands of width 20 μm from the ovary surface, broken down by the number of primordial neighbors within 10 μm. Error bars represent 95% confidence intervals.

simulation in Fig. 6. Alternatively, because the experimental data only includes follicles in a two-dimensional section, many follicles will have nearby neighbors above or below the section analyzed here. Because the density of primordial neighbors is higher near the ovary surface, the probability of having unobserved primordial neighbors is higher near the ovarian surface. This could lead to an apparent enhancement of the inhibitory effect. It would also explain why some 50% of follicles within 20 μm of the surface without primordial neighbors are not growing; it is simply that they are being inhibited by primordial neighbors in adjacent sections. Neither of the above effects require any additional assumptions beyond the production of a diffusing inhibitor by primordial follicles and would appear to be sufficient to explain the data in Fig. 5. A final, more complex, possibility is that in addition to the primordial follicles the inhibitor is also independently produced by the ovarian surface.

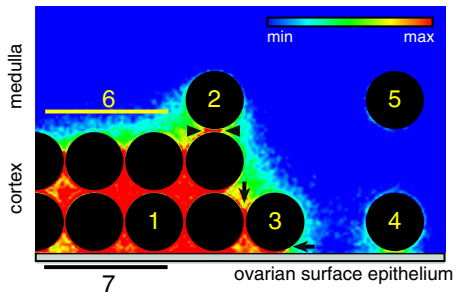

Fig. 6. A simulation of a diffusing inhibitory signal that is produced by, and detected by, primordial follicles (black), and that cannot pass through the ovarian surface epithelium (gray). Details of the simulation are given in *SI Materials and Methods* and it is illustrated in Fig. S6. There is a concentration gradient of inhibitor from high (red) to low (blue). Follicle 1 has 3 close primordial neighbors, exposing it to high levels of inhibitor, so that it is highly unlikely to grow. Follicle 2, at the inner surface of the cortical layer of primordial follicles, has only 1 close neighbor and is exposed to less inhibitor (black arrowheads), and hence is more likely to initiate growth. Follicle 3 also has 1 neighbor but is close to the ovarian surface, which is preventing the inhibitor from dispersing, hence increasing its concentration (black arrows). Follicle 3 is less likely to initiate growth than Follicle 2. Follicle 4 is isolated, but close to the ovary surface, so is exposed to more inhibitor than follicle 5, which is more centrally placed. Both are likely to start growing. Line 6 highlights the reduced concentration of inhibitor present at the inner surface of the cortex compared with that present just below the ovarian surface epithelium (line 7), suggesting a plausible model for why follicles at the cortex-medulla boundary start growing first.

Discussion

Using a method we developed, we have presented evidence for the existence of an inhibitory signal originating from primordial ovarian follicles in neonatal mice. Such an inhibitory signal may be important in preventing premature depletion of the primordial pool before puberty, during establishment of the adult ovarian morphology with its complete repertoire of interfollicular signals between quiescent, growing and preovulatory follicles. The identity of this signal remains unknown. Members of the TGF-β superfamily, in particular bone morphogenetic proteins (BMPs), are known to be important in the regulation of folliculogenesis and are widely, and differentially, expressed in all cell types of the ovary (18). TGF-βs can both stimulate and inhibit cell proliferation (19). Because primordial rat follicles do not express mRNA for any BMPs in either the oocyte or the granulosa cells (18), it seems unlikely that primordial follicles are inhibited directly by a primordial follicle-derived BMP. However, we cannot exclude the possibility that other TGF-βs, for example TGF-β1 or -β2 (20), play an inhibitory role.

An alternative scenario is that primordial follicles produce a BMP binding protein or antagonist that inactivates a stimulatory growth factor. A number of extracellular regulators of BMP action have been described, which modulate BMP action in a variety of cells. These antagonists include follistatin, noggin, gremlin, chordin, DAN, cerberus, myostatin, β-glycan, and PRDC1. Currently there is little information on their role in reproduction, although a few, including PRDC1 (which blocks BMP-2, -4, -6, or -7) (21), gremlin

(which blocks BMP-4) (22), and follistatin (which blocks activin) (18) have been identified in granulosa and interstitial cells in the ovary. There is evidence in other systems such as *Xenopus* development that BMPs do not themselves produce morphogen gradients, but rather that gradients of BMP antagonists react with constant levels of BMPs to produce a gradient (23). Such a mechanism would be consistent with the data we present here.

The additional finding that neighboring growing follicles appear to have a stimulatory effect on follicle development is intriguing. There are a number of possible mechanisms by which this could occur. First, growing follicles could start to produce an activator at the primary stage. One candidate is the oocyte-specific Growth Differentiation Factor-9 (Gdf-9), which is expressed from the primary stage onwards in mouse (24), and is known to stimulate granulosa cell mitosis (25, 26). Other possible candidates are the bone morphogenetic proteins BMP-4 and -7, which are first detected at the primary and secondary stages respectively (18) and stimulate initiation of follicle growth (27, 28). Activin is a further candidate; although it has been shown to be an inhibitor of preantral follicle development in adult mice (13), the same group and others have demonstrated stimulatory effects in prepubertal animals (29, 30). Alternatively, growing follicles may produce an antagonist to the inhibitor produced by primordial follicles, resulting in the stimulation of growth of other nearby follicles.

In conclusion, we have developed a new computational approach to the quantitative analysis of spatial data. By comparing the properties of a biological structure to the distances of nearby potential sources of regulatory signals, we show how to predict the existence, nature and range of previously unknown signals. We have demonstrated the value of this approach in the analysis of the initiation of follicle growth in the neonatal mouse ovary, where we have provided evidence for a hitherto unknown inhibitory signal.

The main drawback of our method is that it does not explicitly identify the signal in question. However, prediction of a putative signal, and its source, can inform and focus future experimental investigations. Several approaches can be used to ultimately pinpoint the relevant molecule. The sources of the signal (e.g., primordial follicles in the case here) can be isolated and analyzed using conventional molecular techniques, to identify highly expressed mRNA or protein.

Our approach is simple to implement, and is particularly suited to situations where either the identity of putative spatial signals is unknown, or their activity is not easy to measure directly. It has the advantage that it explicitly quantifies spatial effects rather than merely relying on subjective visual similarity between patterns. This approach can be widely applied to other spatial and patterning problems in physiology and molecular, cell, and developmental biology.

Materials and Methods

Bouin's fixed, paraffin embedded ovaries from female C57BL/6 mice (Harlan Olac) on days 4, 8, and 12 post partum ($n = 2$ per age group) were serially sectioned (5 μm) and stained with Haematoxylin and Eosin. Mice were housed in accordance with the Animals (Scientific Procedures) Act of 1986 and associated Codes of Practice. The largest section in each ovary was examined using an E600 microscope (Nikon). Each follicle was numbered, the developmental stage assessed and oocyte and follicle diameters were measured (*SI Materials and Methods*). Primordial follicles, surrounded by a single layer of flattened granulosa cells, were considered to be quiescent. Transitional follicles, with 1 or more cuboidal granulosa cells, were considered to have initiated growth, whereas follicles with 1 or more layers of granulosa cells were considered to be growing. Digital images of the entire section were imported into GraphClick (Arizona Software) and the x and y coordinates of the center of the oocyte of each follicle were obtained, allowing calculation of the Euclidean distance between the centres of pairs of follicles. Oocyte and follicle diameters allowed estimation of the distance between oocyte or follicle surfaces by subtracting the radius of each oocyte or follicle in the pair from the center–center distance (Eqs. **1**–**3** in *SI Materials and Methods*). The x and y coordinates of regular points along the ovarian surface were also obtained for calculation of distances between each follicle and the surface of the ovary (*SI Materials and Methods*).

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