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Follicle cell development is partly independent of germ-line cell differentiation in *Drosophila* oogenesis

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Summary. The developmental potential of the cells of the somatic follicular epithelium (follicle cells) was studied in mutants in which the differentiation of the germ-line cells is blocked at different stages of oogenesis. In two mutants, *sn*^{36a} and *kelch*, nurse cell regression does not occur, yet the follicle cells around the small oocyte continue their normal developmental program and produce an egg shell with micropylar cone and often deformed operculum and respiratory appendages. Neither the influx of nurse cell cytoplasm into the oocyte nor the few follicle cells covering the nurse cells are apparently required for the formation of the egg shell. In the tumor mutant *benign gonial cell neoplasm* (*bgcn*) the follicle cells can also differentiate to some extent although the germ-line cells remain morphologically undifferentiated. Vitelline membrane material was synthesized by the follicle cells in some *bgcn* chambers and in rare cases a columnar epithelium, which resembled morphologically that of wild-type stage-9 follicles, formed around the follicle's posterior end. The normal polarity of the follicular epithelium that is characteristic for mid-vitellogenic stages may, therefore, be established in the absence of morphologically differentiating germ-line cells. However, the tumorous germ-line cells do not constitute a homogeneous cell population since in about 30% of the analyzed follicles a cell cluster at or near the posterior pole can be identified by virtue of its high number of concanavalin A binding sites. This molecular marker reveals an anteroposterior polarity of the tumorous chambers. In follicles mutant for both *bgcn* and the polarity gene *dicephalic* the cluster of concanavalin A-stained germ-line cells shifts to more anterior positions in the follicle.

Key words: *Drosophila* – Oogenesis – Follicle cells – Egg shell – Ovarian tumor

Introduction

The *Drosophila* egg is a highly organized giant cell whose molecular complexity can now be gleaned as more and more molecular probes for specific gene products become available. Many genes have been identified that are required during oogenesis. Only a small number of these direct the formation of the anteroposterior and dorsoventral axial polarity of the developing follicle. The anteroposterior polarity is first apparent in the germarium when 1 of the 16 interconnected germ-line cells, the prospective oocyte, migrates to the posterior pole of the follicle (King 1970). The other 15 cells of the cluster develop into nurse cells. This migration is affected in the mutant *dicephalic* (*dic*) so that occasionally the pro-oocyte becomes locked between groups of nurse cells (Lohs-Schardin 1982; Frey et al. 1984). The altered follicular polarity in this mutant is also indicated by the presence of two micropyles at either egg pole. Of the rare embryos that develop from such eggs most produce anterior structures at both poles (Lohs-Schardin and Sander 1976; Lohs-Schardin 1982).

At about the time of oocyte migration the wild-type germ cell cluster becomes enveloped by somatic follicle cells and both cell types remain in close contact until the end of oogenesis. Indirect evidence for the importance of cellular interactions between germ-line cells and follicle cells was obtained from the analysis of genetic chimeras. Using this technique we have shown previously that *dic* gene activity is required in both the follicle cells and the germ-line cells (Frey and Gutzeit 1986). If any of the two cell types is *dic*$^-$ the mutant follicular phenotype may result. These findings argued for intercellular communication between germ-line cells and follicle cells already in the germarium. The cooperation of these cells is also evident from the experiments of Schüpbach (1987), who produced chimeras of the genes *gurken* (*grk*) and *torpedo* (*top*), respectively. Both genes affect the formation of the dorsoventral axis in the follicle as well as the embryo. One of the genes acts germline autonomously (*grk*) while the presence of the other gene (*top*) is required in the soma.

Using mainly morphological criteria, Margaritis (1985) was able to distinguish 11 different populations of follicle cells that finally produce the intricately patterned egg shell. Certain gene products are expressed only in specific follicle cell populations (e.g. Parks and Spradling 1987; Brower et al. 1981) and, recently, regulatory sequences have been identified that control the expression of a reporter gene in different follicle cell populations (Fasano and Kerridge 1988). Two groups of follicle cells are concerned with the formation of the anterior part of the egg shell, which is particularly complex in its organization. Both cell populations migrate to the anterior end of the oocyte during mid-vitellogenic stages and come to lie in the center of the follicle between nurse cells and oocyte. The first group of cells migrates at stage 9 (for stages, see King 1970) between the nurse cells from the anterior tip of the follicle to the anterior

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pole of the oocyte and constructs the micropyle during stages 12/13; the second population of follicle cells migrates centripetally between oocyte and nurse cells at stage 10B and later forms the respiratory appendages and the operculum of the egg shell.

The complex pattern of follicle cell differentiation particularly during the period of egg shell formation implies that these cells acquire specific position-dependent instructions. It is not known in which cells this information is generated nor how it is communicated between the different cell populations. The inferred signal transfer might be conducted by cell-surface molecules or via gap junctions that are present between germ-line cells and somatic cells until late stages of oogenesis (Giorgi and Postlethwait 1985).

In this communication we report some observations on the phenotypes of mutants, which indicate that the follicle cells may follow their normal developmental program for some time even when the differentiation of the germ-line cells is blocked at early or late stages of oogenesis.

Materials and methods

Fly stocks. The mutant *sn*^{36a} was obtained from M.D. Ferres (Madrid), and an allele of *kelch* (RF 41) from T. Schüpbach (Princeton). The mutant *bgcn* was obtained from E. Gateff (Mainz). This stock was 'cleaned' (1st and 3rd chromosomes exchanged with those of Oregon R wild-type flies and the stock was made isogenic) and used for the construction of the double mutant *bgcn*; *dic* (Strauß 1988).

Scanning electron microscopy (SEM). Ovaries of 4–7-day old mutant females homozygous for *sn*^{36a} or *kelch* were dissected in Robb's saline (Robb 1969). Follicles with clearly differentiated egg shell around the oocyte were carefully isolated with tungsten needles and the nurse cells removed. The oocytes were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 (0.5 h on ice) followed by 1% glutaraldehyde and 1% OsO_4 in the same buffer (1 h on ice). Dehydration of the samples, critical-point drying, and gold coating were carried out according to standard procedures.

Histological and ultrastructural analysis. The follicles were dissected in Robb's saline and fixed in the same way as those prepared for SEM. The follicles were dehydrated and embedded in Epon. Semithin sections (1 μm) were stained with 0.4% toluidine blue in 0.4% disodium tetraborate. For details concerning the preparation of the material for electron microscopical analysis see Zissler and Sander (1973).

Histological demonstration of concanavalin A (ConA) binding sites. Ovaries were fixed in 3% paraformaldehyde (Taab) in Sørensen buffer pH 7.4 containing 3.5% sucrose and embedded in diethyleneglycol distearate (Polysciences) as described by Capco et al. (1984) and Capco and McGaughey (1988). The embedding medium was removed by *n*-butanol, the sections were rehydrated in a series of decreasing concentrations of ethanol and finally washed with phosphate-buffered saline (PBS). The sections were incubated in 0.5 mg/ml ConA (Sigma) in PBS at 4° C overnight, washed thoroughly and finally covered with 0.1 mg/ml horseradish peroxidase (Polysciences) in PBS for 1 h. The peroxidase was visualized using chloronaphthol as a substrate for the bound enzyme.

Results

Egg shell formation without prior nurse cell regression

In a number of maternal mutants oogenesis is blocked at the beginning of nurse cell regression: an allele of *singed* (*sn*^{36a}; Bender 1960), some alleles of the *ovarian tumor* (*otu*) locus (Storto and King 1988), and several mutants isolated by T. Schüpbach (personal communication) including the *kelch* allele studied here. Apparently the process of nurse cell regression requires the activity of several genes. The phenotypes of all these mutants differ in a number of details.

In *sn*^{36a}, stage-10 follicles look morphologically normal (Fig. 1a) but at later stages of development the differentiation of the germ cells becomes asynchronous: while the nurse cells do not regress and retain a structure diagnostic for stage 10, the oocyte passes through stages 12–14 and develops into a small egg cell (Fig. 1b). During these stages the follicle cells execute their normal developmental program: they synthesize the egg shell layers and the border cells form the micropyle, which is often visible in the light microscope (Fig. 1b). As in normal development the *sn*^{36a} follicle cells flatten during the last stages of oogenesis (compare Fig. 1a and b) and finally degenerate. The phenotype of *kelch* is very similar.

The nurse cell cap at the anterior end of the small *sn*^{36a} or *kelch* egg was carefully removed so that the egg shell could be studied in more detail by SEM. The typical differentiations of the egg shell like the micropylar cone, operculum, and respiratory appendages are present in both *kelch* (Fig. 1d) and *sn*^{36a} eggs, but compared to a wild-type egg shell (Fig. 1c) morphological anomalies are apparent: the respiratory appendages are usually flattened and fused and the operculum forms almost at right angles to the long axis of the egg. This morphological deformation is presumably due to the presence of the nurse cells, which prevent the egg from assuming its normal shape. Similarly deformed egg shells may also form in *dic* follicles in which nurse cells do not regress (Bohrmann and Sander 1987). The tiny *kelch* egg is not laid, so that this and similar mutants classify as female steriles. In another *kelch* allele (Steward and Nüsslein-Volhard 1986) the centripetal migration of the follicle cells at stage 10B does not take place and hence the anterior structures of the egg shell are not formed (Schüpbach, personal communication).

Follicle cell differentiation in a germ cell tumor mutant

In the mutant *benign gonial cell neoplasm* (*bgcn*), which was discovered by Gateff (1982), the germ-line cells continue to divide mitotically and form a tumor. In contrast to normal development, in which 16 germ-line cells in each wild-type follicle remain interconnected via cytoplasmic bridges, *bgcn* germ-line cells almost always undergo complete cytokinesis. In electron micrographs and by phalloidin staining (for method see Warn et al. 1985) we have only rarely seen intercellular bridges between neighbouring germ-line cells of tumorous chambers. Mutant follicle cells

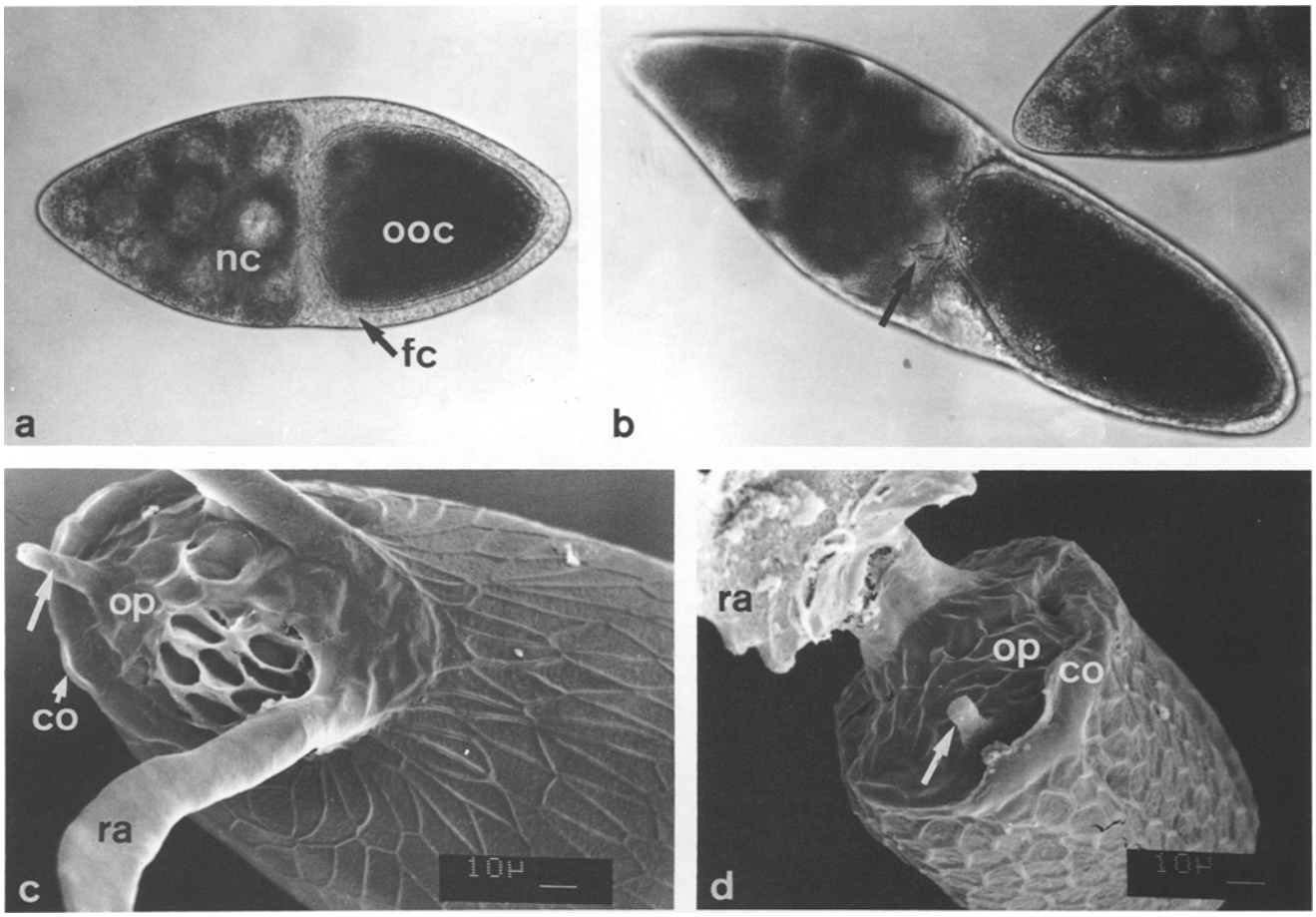

Fig. 1. a, b Isolated follicles from homozygous sn^{36a} flies. **a** Stage-10B follicle with normal gross morphology (*nc* nurse cells; *fc* follicle cells; *ooc* oocyte). **b** Older sn^{36a} follicle, note that the normal regression of the nurse cells did not take place, yet the oocyte developed to stage 12/13 as shown by the thinning of the follicle cells as compared to stage 10 (**a**) and by the formation of the egg shell (*arrow* points at micropylar cone). Follicles of the mutant *kelch* show a similar phenotype. Scanning electron micrographs of the anterior part of a wild-type and a *kelch* egg shell are shown in **c** and **d**, respectively. The nurse cell cap at the anterior end of the *kelch* egg has been removed to show the differentiations of the anterior egg shell. The micropylar cone (*arrows* in **c** and **d**) is well developed in the mutant while the respiratory appendages (*ra*) are fused and malformed. The operculum (*op*) and the collar (*co*) are formed in the mutant but, in contrast to wild-type eggs, the anterior part is flattened, which is presumably due to the persistence of the nurse cells at the anterior pole. The wild-type egg was photographed from the dorsal side while the *kelch* egg, because of the altered egg shape, is viewed from the ventral side

appear to function normally in parcelling up separate clusters of germ-line cells in the germarium. Finally, a chain of tumorous *bgcn* follicles is produced in the ovariole and each of these is connected with its neighbours by stalk cells.

Later functions of the follicle cells that are characteristic for vitellogenic stages of oogenesis could be recognized in some *bgcn* chambers. In normal development the follicle cells of stage-9 follicles synthesize large amounts of vitelline membrane material, which is deposited in the form of 'vitelline bodies'. In 1-µm sections of Epon-embedded follicles this material stains intensely with toluidine blue and appears as a row of dots around the oocyte. At stage 10 the vitelline bodies merge and the material spreads evenly over the oocyte surface. Vitelline membrane deposits are present in some *bgcn* chambers and the same observation has been made in follicles of the gonial tumor mutant *fused* (King et al. 1978). In rare cases the morphology and spatial organization of *bgcn* follicle cells also resemble those of stage-9 follicles, in that the cells at the posterior pole become highly columnar while progressively decreasing in height towards the anterior pole. Around the germ-line cells in the posterior half of the follicle, where normally the oocyte is located, the follicle cells have secreted vitelline membrane material (Fig. 2a). In some areas near the posterior pole the vitelline membrane is unusually thick; in more anterior positions vitelline bodies are present which are characteristic for stage-9 follicles. Mutant follicles with vitelline membrane deposits were analysed ultrastructurally. Figure 2b shows an example with a particularly thick vitelline membrane secreted locally by some follicle cells. The dark-staining material between follicle cells and vitelline membrane is presumably endochorionic material as judged by its ultrastructural appearance (Fig. 2b, arrows). In normal development synthesis of the endochorion begins at stage 12 of oogenesis. Apparently, these *bgcn* follicle cells express gene products that are normally required at the end of oogenesis. The mutant follicles grow for some time but finally show signs of degeneration in histological sections, like abnormally staining cytoplasm or the formation of pycnotic nuclei.

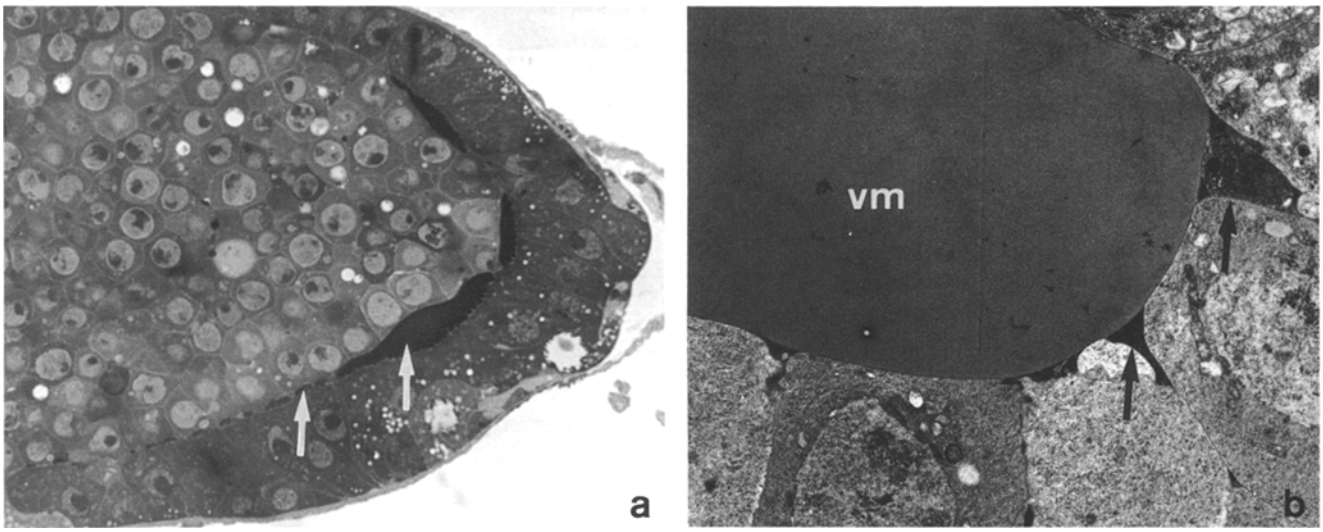

Fig. 2. **a** 1-μm section of toluidine-blue-stained *bgcn* follicle (embedded in Epon). Note the epithelium of columnar follicle cells, which surround the posterior part of the follicle. The epithelium decreases in thickness towards the anterior end of the follicle. The follicle cells have secreted vitelline membrane material (*white arrows*). Towards the anterior end the vitelline membrane deposits ('vitelline bodies') become smaller and are more or less regularly spaced, similar to the situation in wild-type stage-9 follicles. **b** Electron micrograph showing large vitelline membrane (*vm*) deposits in a *bgcn* follicle. The darker-staining material (*arrows*) is presumably secreted endochorionic material. Magnification ×4450

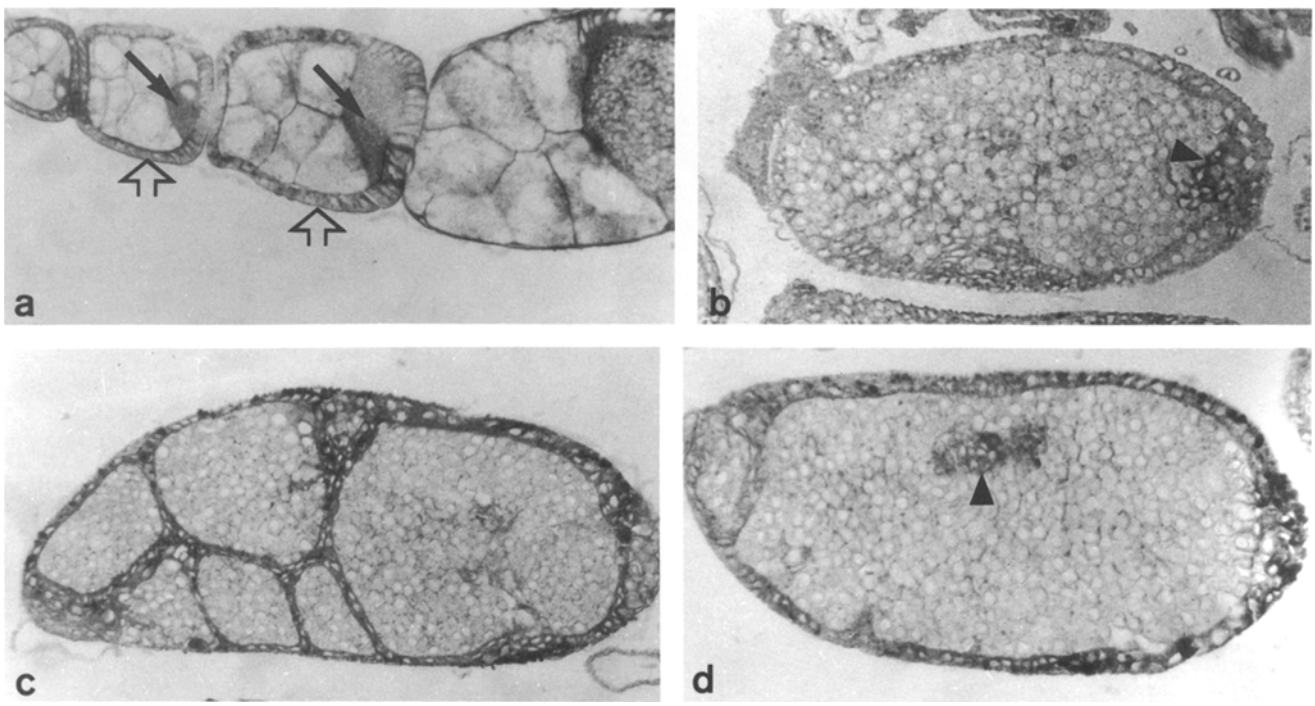

Fig. 3a–d. Concanavalin A binding sites visualized by peroxidase using chloronaphthol as substrate. Diethyleneglycol distearate sections (about 5 μm) through ovarioles (posterior ends to the right with reference to the maternal body). In wild-type follicles (**a**) the ooplasm stains most strongly (*arrows*); also the follicle cells (*open arrows*) give a positive reaction while the nurse cell cytoplasm is only lightly stained. In the tumorous *bgcn* chambers (**b**) all germ-line cells stain lightly except for a small cell cluster at the posterior pole (*arrowhead*). The tumor cells are enveloped by a dark-staining follicular epithelium. In the double mutant *dic*; *bgcn* (**c, d**) the follicle is often partitioned by immigrating follicle cells (**c**). The dark-staining cell cluster is located in the center of the largest group of germ-line cells (**d**) and not at the posterior pole as in (**b**)

Our morphological studies gave no evidence for the differentiation of the tumorous germ-line cells into oocyte-like or nurse-cell-like cells. However, histochemically two different cell populations among the germ-line cells can be identified by studying the distribution of ConA binding sites. The bound lectin was visualized by the glycoprotein horseradish peroxidase, which is specifically bound by the ConA multimer. Both in wild-type and *bgcn* follicles the follicle cells were intensely stained and could easily be identified (Fig. 3). Amongst the wild-type germ-line cells only the

oocyte contains large numbers of ConA binding sites (Fig. 3a), which may reflect the accumulation of carbohydrates in this cell. The nurse cells in wild-type follicles possess few ConA binding sites and stain only lightly as compared to the oocyte or the somatic follicle cells. The applied histochemical technique did not allow us to analyze whether the respective cells already acquire their characteristic staining properties in the germarium. However, in *bgcn* follicles we were able to distinguish two different populations of germ-line cells with respect to the number of ConA binding sites: in about 30% of the tumorous chambers clusters of cells stained more intensely than the other germ-line cells. In 16 of 52 follicles, analyzed by serial frozen sections, such dark-staining cell clusters were found, and in all cases the cluster was located at or near the posterior end of the *bgcn* follicle (Fig. 3b). No correlation between the presence and position of the cell cluster and the differentiation of the follicular epithelium was noticed. In control sections the incubations with ConA or ConA and peroxidase, respectively, were omitted; in these cases *bgcn* and wild-type follicles (prior to stage 13) showed no staining.

To test if the polar position of the dark-staining cells is under the control of genes that are required for the establishment of the anteroposterior polarity of wild-type follicles we constructed the double mutant *dic*; *bgcn*. In 17 of 37 analyzed follicles, a cluster of dark-staining cells was found. They were located at variable but consistently more central positions than those of *bgcn* follicles (compare Fig. 3b with c, d). In *dic*; *bgcn* follicles none of the identified ConA-labelled cell clusters was located at the posterior pole (with reference to the ovariole polarity).

In double-mutant follicles, groups of centripetally migrated follicle cells were seen between the tumor cells in semithin sections, so that the latter became subdivided into groups of varying size. The largest group including the cell cluster marked by ConA (Fig. 3c, d) is usually located at the posterior end of the follicle. The migration of follicle cells can best be demonstrated by making use of the intensive staining of these cells with the ConA/peroxidase technique. This unexpected migration seems to be due to the defective *dic* gene activity, since in semithin sections of *dic* follicles very thin follicle cells can often be identified between adjacent nurse cells, a process which does not occur in wild-type follicles (H.G., unpublished observation). This feature of the *dic* phenotype was observed only in stage 10B follicles. Hence the observed immigration of follicle cells in *dic*; *bgcn* follicles gives further evidence for the late expression of follicle cell functions in tumorous chambers. It is conceivable that clones of cells with the same surface properties are encompassed by immigrating follicle cells; unfortunately, suitable molecular markers to test this supposition are not available.

Discussion

In normal development the nurse cells regress at stage 10B and their cytoplasm streams into the rapidly growing oocyte (Gutzeit and Koppa 1982). Inhibitor studies, the analysis of time-lapse films, and in toto staining of microfilaments suggest that microfilament activity in the nurse cells is required for this process (Gutzeit 1986). The phenotypes of the mutants *kelch* and sn^{36a} indicate that the respective genes may affect directly or indirectly microfilament activity. The differentiation of a complete though somewhat deformed egg shell in *kelch*, sn^{36a} and mutants with similar follicular phenotypes shows that neither the influx of nurse cell cytoplasm nor the 50–100 follicle cells covering the nurse cells are required for this process. All other follicle cell populations take part in egg shell formation (Margaritis 1985).

Much of the particularly complex anterior part of the egg shell is produced by the border cells and the cells migrating centripetally between oocyte and nurse cells at stage 10B. As a result of this migration, a continuous follicle cell layer is formed around the oocyte, which is only interrupted by the four intercellular bridges between the oocyte and the neighbouring nurse cells. Do the two follicle cell populations migrate because the direct contact of the follicle cells with the oocyte is a prerequisite for the differentiation of the egg shell? For *Drosophila* follicles this is a possibility, but in other species this is clearly not the case. In the fungus gnat *Bradysia tritici* follicle cell migrations do not take place, and the micropyle is synthesized by a small group of follicle cells at the anterior pole of the follicle long before nurse cell regression is completed (F. Wenzel, personal communication). Hence these follicle cells have no direct contact to the oocyte and no signal can be transmitted between these cell types via gap junctions or cell-surface receptors.

Are the tumorous *bgcn* germ-line cells all identical and distributed at random in the follicle? Our results suggest that this may not be the case. Staining the tumor cells with ConA/peroxidase revealed a cell cluster close to the posterior pole and hence the chambers have an anteroposterior polarity that is not morphologically apparent. The posterior position of ConA-stained cell(s) is shared by wild-type and *bgcn* follicles, and hence the same mechanism for establishing the follicle's polarity may operate. In wild-type germaria the prospective oocyte migrates to the posterior pole of the 16-cell cluster. In *bgcn* but not in *dic*; *bgcn* germaria this migration may also take place. That cell migration of the pro-oocyte is not necessarily linked to the morphological differentiation of this cell is shown by mutants in which follicles with 16 nurse cells but no oocyte develop. A cell with four ring canals, which is a characteristic feature of the oocyte, was found in a posterior position in more than 90% of the analyzed follicles in three different mutants of this type (Frey 1986).

The germ-line cell differentiation in *bgcn* follicles is blocked prior to stage 1 while in some cases the developmental program executed by the follicle cells corresponds to that of late vitellogenic stages. In normal development, synthesis of the vitelline membrane takes place during stages 9 and 10A and endochorion formation during the following final stages of oogenesis (Margaritis 1985). From data on the length of the developmental stages (King 1970) it can be calculated that in *bgcn* the developmental asynchrony of germ-line cells and follicle cells is of the order of 3 days. Both cell types also develop asynchronously in follicles of sn^{36a} and *kelch*, but in these cases the time difference between the differentiation programs of follicle cells and germ-line cells amounts only to a few hours. These observations indicate that the follicle cells have a certain autonomy regarding the timing of their developmental program. Interestingly, the transcripts of the *period* gene, which plays a role in the generation of biological rhythms, are present in the follicle cells but not in the oocyte or nurse cells (Liu et al. 1988).

Are the germ-line cells able to develop without envelop-

ing follicle cells? During the first stages of oogenesis the presence of follicle cells may be crucially important for oogenesis, since no mutants have been described in which the follicle cells are absent during the first stages of oogenesis. In contrast, agametic flies with ovaries lacking germ-line cells can be produced experimentally or by mutation. In the paedogenetic dipteran *Heteropeza pygmaea* vitellogenic follicles may grow to some extent without follicle cells; such 'denuded' follicles may develop further and embryogenesis may be initiated in this parthenogenetically developing species (Went and Junquera 1981). However, circumstantial evidence suggests that the presence of the follicle cells is essential for the generation or maintenance of polarity. Denuded *Heteropeza* embryos are spherical and no visible egg axis is formed. Some temperature-sensitive alleles of *suppressor of forked* lead to follicle cell death in late previtellogenic *Drosophila* follicles when mutant flies are cultured at the restrictive temperature for several days (Jürgens 1977; Wilson 1980); in case of the mutant *l(1)su(f)*$^{mad-ts}$ the germ-line cells become spatially disorganized concomitant with the disappearance of the follicle cells (Gutzeit and Heinrich 1981).

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