

# Rearrangement of extracellular matrix during cluster formation by human luteinising granulosa cells in culture

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## ABSTRACT

Human ovarian granulosa cells were cultured on a basement membrane preparation (Matrigel) to investigate the role of extracellular matrix components in granulosa cell cluster formation. Time-lapse videomicroscopy of these cultures revealed a rapid aggregation of cells which was initiated during the first 2–4 h of culture so that by 8 h most of the granulosa cells were incorporated into clusters. Further amalgamation then occurred with the transfer of cells along ‘bridges’ between combining clusters. The clustering process, which was complete by about 24 h, was accompanied by reorganisation of matrix which was visualised by immunolabelling of laminin. Clustering cells appeared to gather matrix which became distributed around the clusters. Confocal microscopy showed matrix to be present over the surface of each cluster as well as around the base apparently anchoring the aggregate to the culture surface. Results suggest the potential for active rearrangement of matrix by granulosa-derived cells during corpus luteum development.

*Key words:* Ovary; ovulation; corpus luteum.

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## INTRODUCTION

The development of the corpus luteum following ovulation results in increased circulating progesterone and other steroids during the luteal phase of the menstrual cycle in women. This steroidal environment influences the uterine tube and uterus and, in a conception cycle, is critical for the successful development of the early embryo (Robinson et al. 1999). The establishment of the corpus luteum is characterised by extensive tissue remodelling as granulosa cells enlarge, differentiate and rearrange in relation to infiltrating cords of theca cells and invading capillaries. It is now recognised that tissue remodelling is invariably associated with changes in extracellular matrix (ECM) and this has been reviewed for the ovary (Luck, 1994). Of particular interest in the corpus luteum, is the apparent establishment of thin basement membrane-like structures around luteinising granulosa cells of rats (Matsushima et al. 1996) shown by immunostaining for collagen IV and laminin, suggested in an earlier study by the demonstration of a pericellular location of laminin in

murine corpora lutea (Wordinger et al. 1983). The expression of ECM proteins in bovine corpora lutea provides extra evidence for the importance of matrix components in this species (Zhao & Luck, 1995). It is possible that changes in the stability of luteal ECM are pivotal in determining whether the tissue is maintained or undergoes regression, and that an important function of the large granulosa-derived luteal cell is the abundant production of tissue inhibitor of metalloproteinase (TIMP) which serves to increase matrix stability (O’Sullivan et al. 1997; McIntush & Smith, 1998). Another role for ECM could be in the local, pericellular sequestration of heparin-binding growth factors, such as the larger forms of vascular endothelial growth factor (Park et al. 1993). This may be important in the spatial relationship of luteinising granulosa cells and invading endothelial cells during vascularisation.

The origin of the pericellular matrix associated with luteinised granulosa cells in the corpus luteum is not known. It is known that preovulatory granulosa cells actively synthesise basement membrane components (Rodgers et al. 1996) and the extracellular matrix in

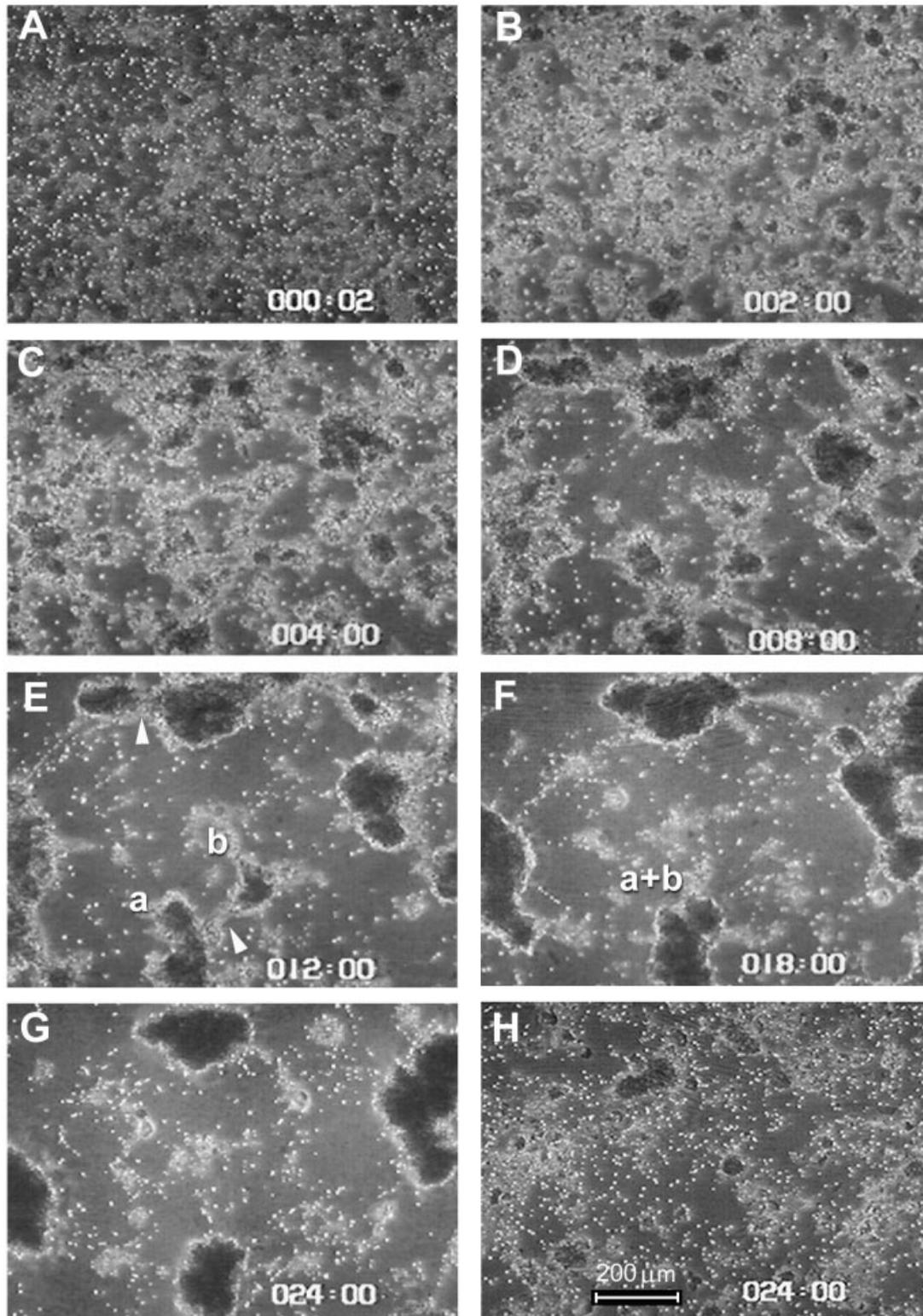


Fig. 1. Time-lapse videomicroscopy of human granulosa cells in culture showing progressive clustering on Matrigel over a 24 h period (A–G). The culture times at which individual images were captured from the video recording are shown as 'hours:minutes'. A 'bridging phenomenon' between amalgamating clusters is indicated by arrowheads (E). An example of amalgamation of clusters is given by clusters 'a' and 'b' (E) merging to give 'a+b' (F). The final appearance at 24 h of a control culture without Matrigel is also shown (H).

the corpus luteum could therefore be made by the granulosa-derived cells following ovulation. Alternatively, it could be derived from other cells (e.g.

endothelial cells) in the corpus luteum. In any event, it is possible that rearrangement of available matrix occurs as remodelling proceeds. Although it is

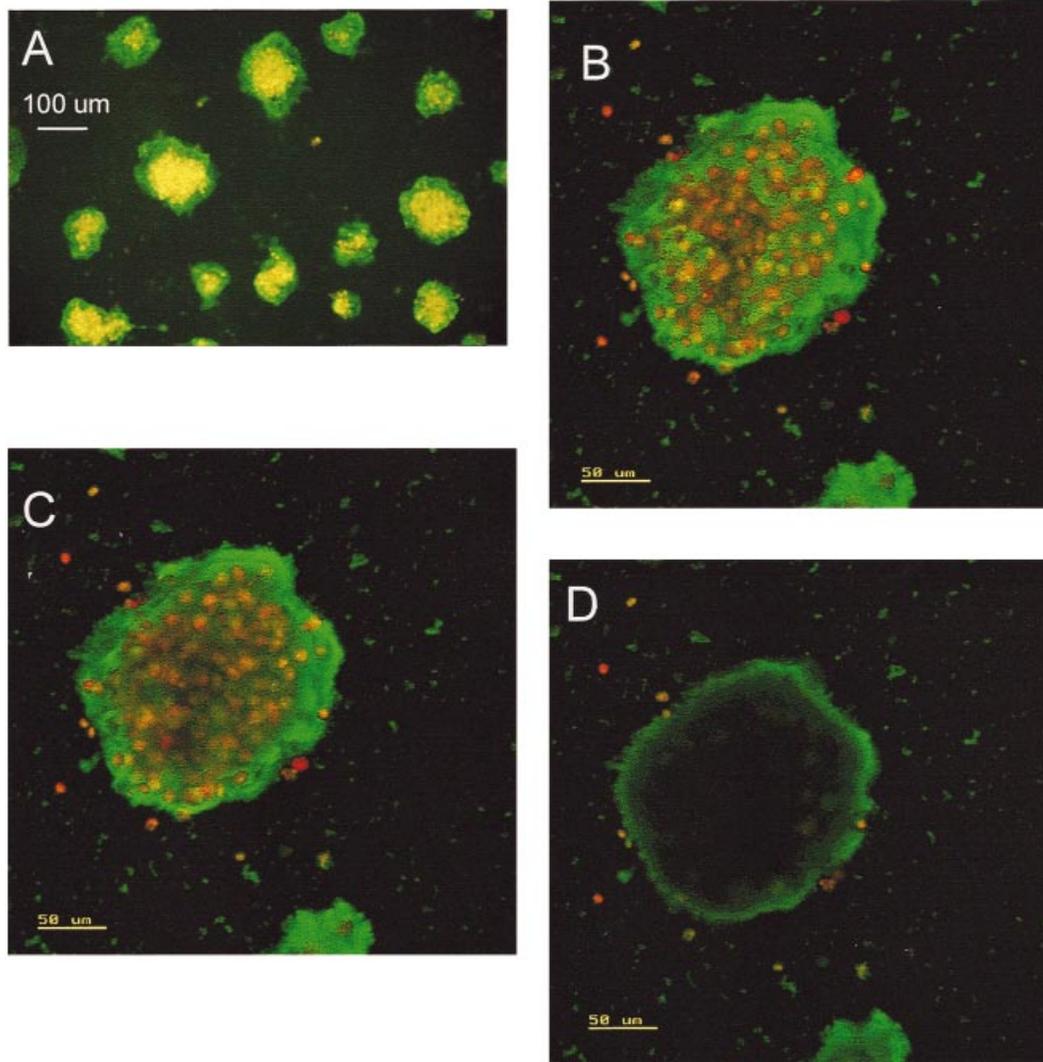


Fig. 2. (A) Granulosa cell clusters after 48 h of culture fixed and immunolabelled for laminin- $\beta_1$  (shown as green). Nuclei were counterstained with propidium iodide (shown as yellow) in this preparation. Areas between the clusters were largely cleared of laminin which had become gathered around the granulosa cell aggregates. (B–D) Confocal images of a cluster processed as in A showing laminin as green and nuclei shown as orange in this preparation. The complete cluster (overall height, 109  $\mu\text{m}$ ) was visualised as 23 equal virtual slices and the complete series combined to give a projection image (B) showing laminin over the upper surface of the aggregate. The lower 12 virtual slices were combined to give a view of the lower part of the cluster (i.e. as if the top were removed) and shows laminin around the sides (C). The first virtual slice adjacent to the culture surface (D) shows a ring of laminin at the base of the cluster.

documented that a number of cell types are able to rearrange extracellular matrix *in vitro* (Neville et al. 1991; Coger et al. 1997) it is over a longer time period than would reflect the processes occurring during corpus luteum formation.

Development of cell culture systems for granulosa cells that mimic their development *in vivo* must take account of the cell-matrix interactions expected in the corpus luteum. One approach is to culture cells with basement membrane components in the form of the commercial preparation, Matrigel, providing cell contact with ECM molecules including collagen IV and laminin (Richardson et al. 1992). A characteristic feature of this cell culture system is the formation of

cell clusters on the matrix. We now report a time-lapse video study of the clustering process which, combined with immunocytochemistry of laminin distribution, provides evidence that this involves a major re-organisation of the matrix around the clusters.

#### MATERIALS AND METHODS

##### *Patients*

Follicular aspirates were obtained at egg collection for IVF. Briefly, the patient protocol involved pituitary downregulation with gonadotrophin releasing hormone agonist and stimulation of follicular growth with human menopausal gonadotrophin. Final matu-

ration of follicles was initiated by hCG and eggs were collected 34 h later (Jenkins et al. 1991). All follicles over 15 mm in diameter were aspirated. Patients with known ovarian pathology (such as endometriotic deposits on the ovary or polycystic ovary disease) were excluded. Patients in the study included those with tubal blockage, those where the cause of infertility was attributed to the male partner and those acting as 'egg donors'.

#### *Granulosa cells*

Aspirates from each patient were combined and granulosa cells prepared according to a method previously described (Richardson et al. 1992). Briefly, the preparation involved enzymic dispersion and centrifugation over 45% Percoll (Pharmacia, St Albans, UK) to remove red blood cells. The culture medium used for preparation and culture was a mixture (50:50) of Ham's F12 and Dulbecco's modified Eagle's medium supplemented with glutamine (2 mM), penicillin (50 mg/l), streptomycin (60 mg/l), amphotericin (2.5 mg/l), insulin (6.25 mg/l), transferrin (6.25 mg/l), selenious acid (6.25 µg/l), bovine serum albumin (1.25 g/l) and linoleic acid (5.35 mg/l). Cells were counted using a haemocytometer. Cell viability, as assessed by trypan blue exclusion, was routinely over 90%.

#### *Videomicroscopy*

For this study, 10 ml of cell suspension (diluted to approximately  $2 \times 10^5$  cells/ml) were added to a thin layer of Matrigel (Strattech, Luton) formed by gelling 1 ml of Matrigel covering a culture area of 25 cm<sup>2</sup> in a 50 ml culture flask. The flask was gassed with 5% CO<sub>2</sub> in air, sealed and placed on an inverted phase-contrast microscope fitted with an enclosed chamber maintained at 37 °C. The cultures were video-recorded for 24 h using a JVC Newvicon colour camera (TK-800U) linked to a JVC time-lapse video cassette recorder (BR-9000U) and a JVC colour video monitor. Images were captured at specific time points and digitised. Before printing, the images were processed to remove video interlacing, to adjust contrast and to add labels.

Control cultures were grown in flasks without Matrigel. Propidium iodide, at a final concentration of 1 mg/l, was added to some unfixed cultures, both those grown on Matrigel and control cultures without Matrigel, to assess the number of dead cells present (Webster & Stewart, 1997).

#### *Immunocytochemistry*

Granulosa cells, at  $2 \times 10^5$  cells/ml, were cultured in 24-well culture dishes containing sterile glass coverslips (diameter, 10 mm) coated with 60 µl Matrigel (total incubate volume, 1 ml). After 48 h, medium was removed and cells fixed in ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min. After rinsing in PBS and blocking in 10% goat serum in PBS for 30 min, cultures were exposed overnight at 4 °C to a goat antirat laminin-beta-1 antibody (MAB1928; Chemicon, London) used at a dilution of 1:60 in the 10% goat serum. Following 3 washes with 0.2% bovine serum albumin in PBS, cultures were treated for 1 h at room temperature with a fluorescein-conjugated goat antirat IgG which was F(ab)<sub>2</sub> fragment specific (Strattech) diluted 1:100 in the 10% goat serum. After further washing in the 0.2% albumin in PBS, cultures were counterstained briefly with propidium iodide (1 mg/l). Cultures were either photographed using an inverted microscope with fluorescence attachment or viewed under the confocal microscope using aqueous immersion.

#### RESULTS

At the beginning of the culture period, the granulosa cells appeared as an evenly distributed layer of single cells with occasional small clusters (Fig. 1A) that were also present in the original cell suspension. After 2–4 h (Fig. 1B–C) the start of active cluster formation was seen, so that by 8 h (Fig. 1D) most of the granulosa cells were incorporated into clusters which were of various sizes. In the next period up to 18 h (Fig. 1E–F), amalgamation of clusters took place. In this process, active cell transfer occurred along bridges, or columns, of cells apparent between amalgamating clusters (labelled in Fig. 1E). By 24 h (Fig. 1G) the clustering process appeared to reach completion with the vast majority of granulosa cells now being present in discrete, densely packed, large clusters. No further merging of clusters occurred between 24–48 h of culture. A small minority of cells remained unassociated with clusters. Granulosa cells plated directly onto the plastic surface of the culture plate (Fig. 1H) did not form the large discrete clusters evident on Matrigel.

The clustering phenomenon occurred over a wide range of cell concentrations ( $10^4$ – $10^6$  cells/ml investigated), although the dilution of the granulosa cell suspensions resulted in smaller clusters. Over the short-term culture period, the use of additional

gonadotrophin (added as 100 ng/ml human chorionic gonadotrophin) had no noticeable effect on clustering. Within cell clusters cell viability was high with only occasional cells not excluding propidium iodide. This contrasted with the condition of cells cultured for 48 h without extracellular matrix where the majority of nuclei stained with propidium iodide.

The visualisation of matrix distribution through immunocytochemical labelling for laminin  $\beta_1$  subunit revealed that the clustering process had resulted in the gathering of laminin around the clusters (Fig. 2A). Laminin had been cleared from areas between the clusters. More detailed analysis by confocal microscopy of the distribution of laminin around the clusters showed that laminin was present over the top surface of the cell aggregates (Fig. 2B). A reconstruction using only the lower virtual slices generated by the confocal microscope (Fig. 2C), showed laminin around the sides of the cluster which appeared to stretch down to form a ring (Fig. 2D) in contact with the culture plate.

## DISCUSSION

The novel use of time-lapse videomicroscopy during the early stages of culturing isolated human ovarian granulosa cells has revealed a rapid aggregation of the granulosa cells which is well underway by 4 h of culture. This contrasts with the findings of Coger et al. (1997) who used time-lapse videomicroscopy to study aggregation of cultured hepatocytes and found that clustering proceeded over a longer period of several days. Thus, although clustering on Matrigel may be a general phenomenon shown by epithelia-derived cells (discussed by Coger et al. 1997) there appears to be considerable variation in the speed of clustering shown by different cell types. The time-lapse technique has also enabled the observation of a 'bridging' phenomenon (Fig. 1) apparent as cells actively transfer from small 'pre-clusters' to larger neighbouring clusters. This suggests that there is a controlled directionality to the clustering phenomenon rather than a random gathering of cells. Taken together our results show a rapid and controlled clustering of granulosa cells in culture which is consistent with the requirement for rapid, controlled rearrangement of granulosa cells in early corpus luteum formation *in vivo*.

Immunolabelling of laminin was used in the present study, making the assumption that movement of this individual but predominant constituent of Matrigel reflects the overall changes in matrix disposition. We found that by 48 h of culture, granulosa cells had formed cell aggregates surrounded by a culture surface

cleared of matrix (Fig. 2A). Moreover, the upper surfaces of the granulosa cell clusters were covered in a layer of matrix visualised in our study using confocal microscopy (Fig. 2B). A similar observation was made by Neville et al. (1991) in a study of mammary epithelial cell clustering. We suggest that as the cells aggregate they gather the matrix, which may involve a form of 'snowballing' as coated cells converge into clusters. There may also be some extra rearrangement of matrix to form the outer surface coating of matrigel described.

It has been suggested by Neville et al. (1991) that compared with other culture surfaces such as collagen, Matrigel is easily deformed and rearranged by cells. Certainly, decreasing Matrigel deformability through cross-linking with glutaraldehyde, inhibits clustering of hepatocytes (Coger et al. 1997). This has led us to the view that if a cell type has an innate ability to cluster, Matrigel may facilitate this process as it is readily rearranged. We propose that the clustering of granulosa cells described in the present report reflects a natural tendency for these cells to cluster, rather than a process specifically induced by extracellular matrix. It follows that the culture system described in this report provides a valuable model for more detailed studies of the cellular and matrix reorganisation which occurs as the corpus luteum is established after ovulation.

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