Differential effect of components of the extracellular matrix on differentiation and apoptosis

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Background: Epithelial cells are closely associated with a basement membrane, but the intimate relationships that affect growth, differentiation and survival remain enigmatic. We have previously reported that granulosa cells adjacent to the basement membrane of the ovarian follicle have a higher degree of differentiation compared with cells located distal to the basement membrane. By contrast, granulosa cells distal to the basement membrane are the first to undergo apoptosis during follicular atresia. Moreover, growth of granulosa cells *in vitro* on a naturally produced basement-membrane-like extracellular matrix (ECM) enhances progesterone production and the cellular response to gonadotropic hormones by an undefined mechanism.

Results: To investigate the effect of the ECM on granulosa cell differentiation and death, primary granulosa cells were cultured on ECMs that lacked or contained bFGF (basic fibroblast growth factor). These otherwise identical ECMs were deposited by HR9 mouse endodermal cells, which do not synthesize bFGF, or by HR9 cells transfected with the bFGF gene. Both ECMs provided protection against apoptosis in serum-free medium, but only the bFGF-containing ECM maintained expression of the steroidogenic P450scc enzyme system and the production of progesterone. Moreover, culturing the cells on this ECM enhanced the expression of the 30 kDa steroid acute regulatory protein which plays a key role in steroid hormone biosynthesis. Laminin, but not fibronectin, was able to replace the ECM in protecting the cells from apoptosis, but not in maintaining steroidogenesis, whereas bFGF was able to enhance steroidogenesis without protecting the cells against apoptosis. Cells cultured on both ECMs or laminin had a well-developed actin cytoskeleton compared with cells cultured on non-coated dishes, which underwent apoptosis.

Conclusions: Cellular responses to ECM are mediated by the combined action of macromolecular constituents and regulatory molecules, such as bFGF, that are sequestered and stored in the ECM. ECM or laminin protects against cell death by interacting with specific integrin receptors and maintaining a well-developed actin cytoskeleton. ECM-bound bFGF provides differentiation signals for granulosa cells, which are in intimate contact with the ECM. Thus, a clear distinction can be made between the survival activity and the differentiation stimulus exerted by the ECM on epithelial cells.

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Background

Basement membranes and extracellular matrices (ECMs) are the natural substrates upon which cells migrate, proliferate and differentiate *in vivo*. Studies aimed at elucidating the mode of cellular responses to ECMs revealed that the ability of cells to respond to various growth and differentiation factors is determined to a large extent by their shape and orientation, which in turn are modulated by interactions between components of the ECM and specific transmembrane receptors called integrins [1–3]. Studies with mammary gland epithelial cells led to the identification of genes that are dependent upon the ECM for their transcription and to the discovery of ECM-response elements [3,4]. On the basis of these and other observations, it is recognized that the ECM plays an active and complex role in regulating the morphogenesis of cells that contact it, influencing their survival, migration, proliferation and metabolic functions. Cellular responses to the ECM seem to be mediated by the combined action of basement membrane macromolecules, such as collagen IV, laminin, nidogen/entactin and heparan sulfate-containing proteoglycans, and regulatory molecules, such as growth factors and enzymes, that are immobilized and stored in the ECM by attachment to its macromolecular

Figure 1

Flow cytometry analysis of primary granulosa cells cultured in serum-free medium on uncoated (control) or ECM-coated (HR9-bFGF/ECM) tissue culture dishes. Cells were maintained in culture for 24 h and DNA was stained using propidium iodide.

constituents, primarily to heparan sulfate proteoglycans (HSPG) [5,6].

Our studies on the control of cell proliferation and differentiation by the local environment focused on the interaction of cells with the ECM produced by cultured corneal endothelial cells (CE/ECM) [7,8], and by HR9 mouse endodermal carcinoma cells which lacked or contained basic fibroblast growth factor (bFGF): HR9/ECM or HR9bFGF/ECM, respectively [9]. The morphology and molecular composition of these ECMs closely resemble those of basement membranes *in vivo*. We found that vascular endothelial cells and other cell types plated on CE/ECM no longer required soluble FGF and/or other growth factors to proliferate and express their differentiated functions [10]. Our studies on ovarian follicular cells showed regulation of differentiation in preovulatory rat granulosa cells following 48 hours of culture on ECM [11,12]. Moreover, we observed a synergistic effect of the ECM and gonadotropic hormones on the differentiation of human granulosa cells *in vitro* [13–15]. Studies on the intact preovulatory ovarian follicle indicated that granulosa cell layers adjacent to the follicular basement membrane are significantly more differentiated than cells distal to the basement membrane, suggesting a role for the basement membrane in induction of differentiation *in vivo* [16,17].

Recent studies indicate that cell survival as well as proliferation depend on appropriate signals mediated by growth factors and/or adhesion molecules within the ECM [18,19]. In addition, cell attachment to ECM proteins *in*

vitro can regulate the apoptosis-related genes encoding interleukin-1-β-converting enzyme and Bcl-2 [20], as well as the cell-cycle inhibitors p21$^{WAF1/CIP1}$ and p27^{KIP1} [21]. Disruption of cell–matrix interaction induced apoptosis in human endothelial cells [22] and in kidney epithelial cells (MDCK) [23]. Apoptosis was induced by fibronectin in human hematopoietic cells, and by a laminin-derived multimeric synthetic polypeptide containing the sequence Tyr–Ile–Gly–Ser–Arg (YIGSR) in human fibrosarcoma cells [24]. However, the active component(s) of the ECM that protects against apoptosis remains enigmatic. Moreover, it is not clear whether the same ECM component(s) that affect cell proliferation and differentiation can also modulate apoptosis.

In the present study, we used highly steroidogenic primary granulosa cells cultured on various types of ECM, or on purified ECM components, for a comprehensive analysis of their differentiation and apoptotic responses. For the first time, we were able to distinguish clearly between the differentiation stimulus exerted by the ECM-resident bFGF and the anti-apoptotic activity exerted by bFGF-free ECM or laminin alone, but not by fibronectin.

Results
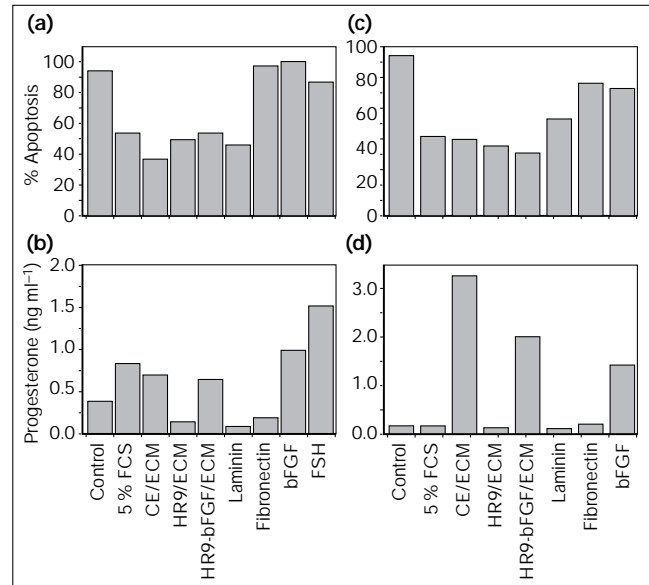### Effect of ECM on granulosa cell survival
Mature rat granulosa cells isolated from pre-ovulatory follicles underwent massive and rapid cell death after 24 hours in culture when plated on tissue culture plastic in the absence of serum. In contrast, cells cultured on dishes coated with the ECM deposited by bovine corneal endothelial cells (CE/ECM), attached rapidly to the surface and survived for at least 72 hours [11]. The protective effect of ECM on cell survival was studied by flow cytometry analysis of DNA content, using various types of ECM or pure isolated components of the ECM, such as laminin and fibronectin. When pre-ovulatory granulosa cells were cultured on uncoated dishes in the absence of serum, ~80 % of the cells contained a sub-G0 amount of DNA, indicating massive degradation of DNA characteristic of apoptosis [25–29]. Most of the remaining (~20 %) cells were in G0/G1 phase, while only 2 % were in G2/M phase (Fig. 1). In contrast, only 20 % of cells cultured on HR9-bFGF/ECM were in sub-G0 phase, while 70 % were in G0/G1 and 8 % in G2/M (Fig. 1).

To determine whether the adhesive property of the ECM was responsible for cell survival, as reflected by the significant increase in the number of cells arrested in G0/G1 phase, we tested the ability of poly-lysine-coated dishes to prevent apoptosis in the absence of serum. We found that, in spite of adhesion to the poly-lysine-coated surface, 80 % of the cell population were in sub-G0 phase, which was similar to the incidence of apoptosis in control cells grown on uncoated dishes (Fig. 2). Cells cultured on bFGF-free ECM deposited by mouse PF-HR9 endodermal cells

Figure 2
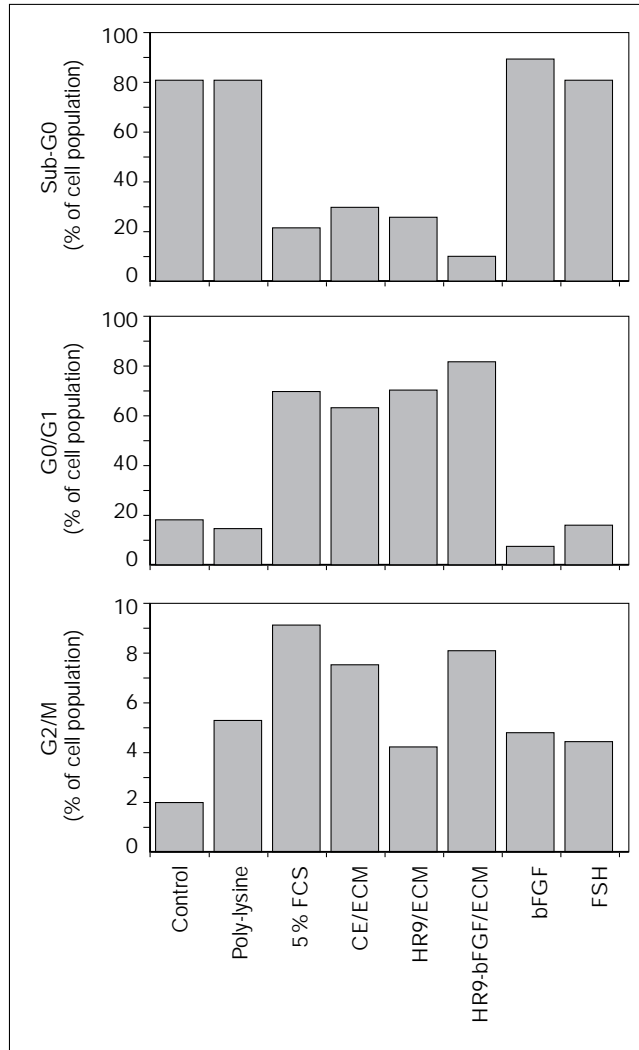
Cell-cycle analysis by flow cytometry of primary granulosa cells. Cells were cultured on different substrates or on uncoated tissue culture dishes in serum-free medium in the absence (control) or presence of ovine FSH (400 ng ml^{-1}) or bFGF (2 ng ml^{-1}). Cells were stained with propidium iodide before FACS (fluorescence-activated cell sorter) analysis. The data are from one representative experiment (of three) and the variation between different experiments did not exceed ± 15 %.

Figure 3

Effect of serum, ECMs, bFGF and gonadotropins on granulosa cell survival and steroid hormone production. Primary granulosa cells obtained from preovulatory follicles were cultured as monolayers for 24 h **(a,b)** or 48 h **(c,d)** on the various substrates or on uncoated dishes in the absence (control) or presence of 5 % FCS, 2 ng ml^{-1} bFGF or 400 ng ml^{-1} ovine FSH. At the end of the incubation period, media were collected for the progesterone assay and the cells were fixed with paraformaldehyde and stained with DAPI. Apoptotic nuclei were scored under the fluorescent microscope. Data are means of three experiments in which progesterone and apoptosis were determined in the same cultures, the variation between different experiments was less than 15 %.

(HR9/ECM) had a low incidence of apoptosis, similar to that of cultures grown on CE/ECM. bFGF-containing ECM deposited by HR9 cells transfected with the gene encoding bFGF (HR9-bFGF/ECM) [9] were similarly protected against granulosa cell apoptosis in a serum-deprived medium. In cultures grown on HR9/ECM or HR9-bFGF/ECM, only 10–20 % of cells were in sub-G0, while the vast majority (70–80 %) were in G0/G1, and 5–8 % were in G2/M phase (Fig. 2). These observations indicate that the protective activity of ECM against apoptosis induced by serum deprivation is not due to bFGF. The presence of 5 % fetal calf serum (FCS) produced a protective effect similar

to that induced by the various types of ECM. The protective effect of the various ECMs or serum on cell survival, which resulted in a pronounced elevation of G0/G1 cells and a reduction in sub-G0 cells, was highly significant when compared with cultures grown on uncoated dishes in the absence of serum ($p < 0.001$, $n = 3$; Student's t test). Because FSH (follicle-stimulating hormone) and bFGF are known to be potent stimulants of granulosa cell differentiation, we tested their effect on DNA content in cell populations maintained on regular tissue culture plastic. More than 80 % of the cells were found to contain sub-G0 quantities of DNA (Fig. 2), which indicated massive degradation of DNA characteristic of apoptosis [25–29]. Thus, bFGF or FSH had no protective effect against apoptosis induced by serum deprivation in granulosa cells.

Effect of ECM on steroidogenesis
ECM was previously found to enhance steroidogenesis *in vitro*, in both rat and human pre-ovulatory granulosa cells [11,13]. To examine the correlation between stimulation of steroidogenesis and cell survival by various types of ECM, cells were cultured in serum-free medium on different

Figure 4
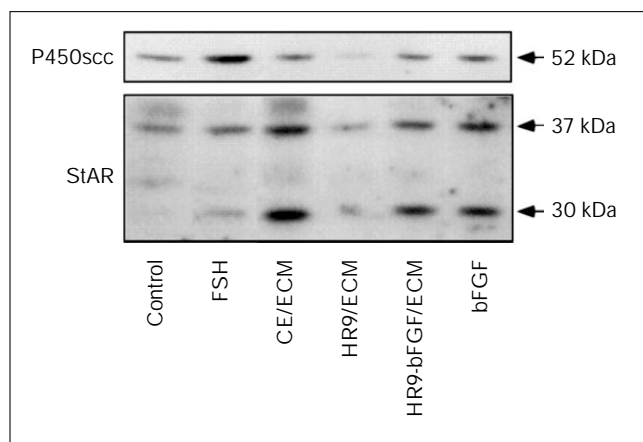
Expression of cytochrome P450scc and StAR in primary granulosa cells cultured on uncoated or ECM-coated dishes. Cells were cultured in serum-free media on various ECM substrates and on uncoated dishes in the absence (control) or presence of ovine FSH (400 ng ml⁻¹) or bFGF (2 ng ml⁻¹). Western-blot analysis of cell lysates was carried out. The 37 kDa and 30 kDa forms correspond to the precursor and the mature form of StAR protein, respectively.

matrices. The incidence of apoptotic cells visualized by DAPI (4′,6′ diamido-2-phenylindole hydrochloride) staining was correlated with the amount of progesterone secreted into the culture medium (Fig. 3). As shown in Figures 2,3, there was a close agreement between the levels of cell survival as measured by FACS (fluorescence-activated cell sorting) and by DAPI staining. Compared with control cells cultured on uncoated dishes, all the types of ECM studied — CE/ECM, HR9/ECM and HR9-bFGF/ECM — protected the cells from apoptosis induced by serum deprivation. Interestingly, laminin had a protective effect against apoptosis that was similar to that of the intact ECM; in contrast, fibronectin failed to provide significant protection (Fig. 3a). Although CE/ECM and HR9bFGF/ECM enhanced (~2-fold) progesterone production by granulosa cells after 24 hours in culture, relative to the basal level of progesterone production by cells cultured on uncoated dishes, HR9/ECM, which lacks bFGF, or pure laminin, both failed to do so (Fig. 3b). Moreover, compared with control cells seeded on plastic, progesterone production was clearly inhibited by HR9/ECM and laminin. Saturating doses of bFGF (2 ng ml⁻¹) or FSH (400 ng ml⁻¹) were unable to protect the cells from apoptosis, but they did enhance progesterone production by 3- and 5-fold above control levels, respectively, after 24 hours of stimulation (Fig. 3b). The protective effect against apoptosis by the various ECMs remained constant during 48 hours in culture (Fig. 3c). The differences in progesterone production by cells plated on ECMs containing bFGF (CE/ECM and HR9-bFGF/ECM), *versus* cells plated on laminin or on ECM lacking bFGF (HR9/ECM), were much more

pronounced, however, because of the continuous progesterone production by cells maintained on bFGF-containing ECMs (Fig. 3d). The synthesis of progesterone by cells stimulated with soluble bFGF alone did not increase during the second day of culture, probably because of massive cell death observed in these cultures (Fig. 3d).

Effect of ECM on expression of StAR protein and P450scc

Acute enhancement of progesterone production may be due to rearrangement of the steroidogenic organelles — mitochondria, smooth endoplasmic reticulum and lipid droplets [30]. We examined whether the ECM-induced enhancement in steroidogenesis was merely due to such rearrangement, or to *de novo* synthesis of the recently discovered steroidogenic acute regulatory protein (StAR), and/or the steroidogenic enzyme P450 side-chain cleavage (P450scc), which are among the limiting steps in steroidogenesis.

Using antibodies specific to P450scc and immunoblot analyses of cellular proteins derived from cultures grown on different substrates, we found that the 52 kDa P450scc enzyme was expressed in significantly higher amounts in cells grown on bFGF-containing ECMs (CE/ECM or HR9-bFGF/ECM), compared with cells grown on ECM devoid of bFGF (HR9/ECM; Fig. 4). Interestingly, an even higher level of P450scc was seen in cells treated with FSH alone, supporting the view that apoptosis and steroidogenesis can co-exist in the same cultures [31]. As for the StAR protein, the non-cleaved 37 kDa precursor, regarded as the active form in cholesterol import into the mitochondria [32], was more highly expressed in cells grown either on CE/ECM or HR9-bFGF/ECM, compared with cells grown on HR9/ECM devoid of bFGF. Expression of the 37 kDa protein was also higher in bFGF- or FSH-treated cells than in cells cultured on HR9/ECM or on uncoated dishes. More of the 30 kDa cleaved protein was present in cells grown on CE/ECM or HR9bFGF/ECM and in cells stimulated with bFGF, than in cells seeded on plastic or on HR9/ECM. FSH-treated cells had a relatively low amount of the 30 kDa protein in spite of a high amount of the 37 kDa protein (Fig. 4). This probably due either to slow conversion of the 37 kDa precursor to the 30 kDa mature protein, or to a rapid degradation of the mature form [32].

Effect of ECM on cell shape and organization of the actin cytoskeleton

Because modulation of the actin cytoskeleton may be involved in differentiation and apoptosis of steroidogenic cells, we examined the organization of the actin cytoskeleton in cells cultured on various ECMs (Fig. 5). After 24 hours of culture in serum-free medium, the cell bodies were reduced dramatically (Fig. 5a,h), probably due to pinching off the apoptotic bodies and shrinkage of cells, which are characteristic of the apoptotic process [33,34]. Phalloidin-bound actin was present in the cells in a diffuse and non-organized form. The condensed and fragmented

Figure 5

Effect of ECM components on survival and actin cytoskeleton development in granulosa cells cultured for 24 h. Primary rat granulosa cells obtained from pre-ovulatory follicles were cultured in serum-free medium either on uncoated tissue culture dishes (**a,h**) or dishes coated with CE/ECM (**b,i**), HR9-bFGF/ECM (**c,j**), HR9/ECM (**d,k**), laminin (**e,l**), or fibronectin (**f,m**). Cells were also incubated on uncoated dishes in the presence of 2 ng ml^{-1} bFGF (**g,n**). Cellular DNA was stained with DAPI (**a–g**) and the actin network of the same cultures was stained with phalloidin rhodamine and visualized under the fluorescent microscope (**h–n**). DNA condensation (asterisks) and fragmentation (arrowheads) is evident in the vast majority of cells cultured on plastic in the absence (a) or presence (g) of bFGF or on fibronectin (f). A high proportion of cells cultured on HR9-bFGF/ECM (c), HR9/ECM (d) or laminin (e) have normal nuclei moderately and homogeneously stained with DAPI. Only these cultures (i–l) have a well-developed network of actin filaments (arrows) in well spread cells. Cells grown on uncoated plastic dishes (h) or on fibronectin-coated dishes (m) have a round morphology with a diffuse pattern of actin (o). Scale bar = 20 μm.
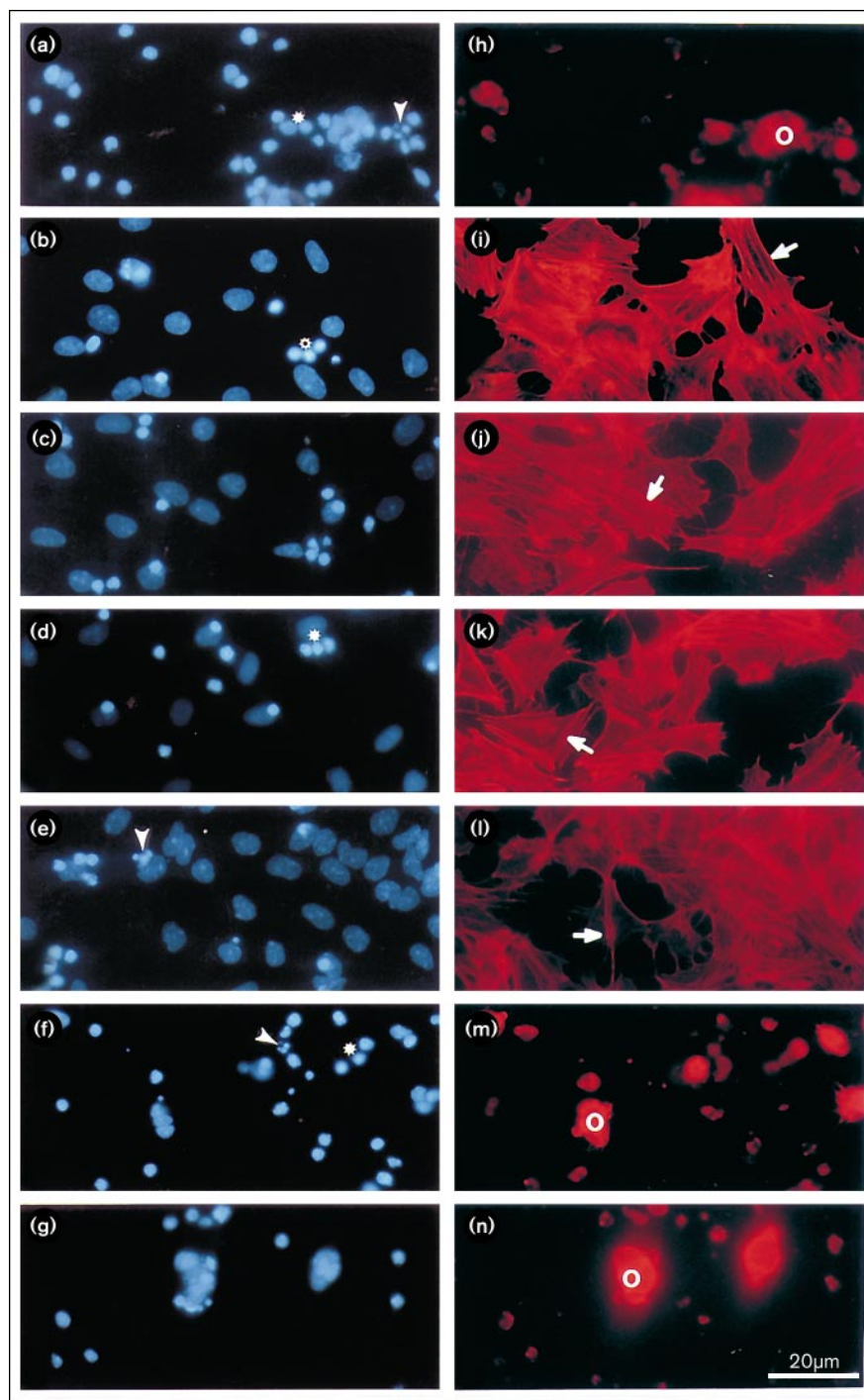
nuclear DNA found in the vast majority of the cells is also typical of the apoptotic process [35]. In contrast, the majority of cells cultured on CE/ECM or HR9-bFGF/ECM had normal nuclei homogeneously stained by DAPI (Fig. 5b,c), characteristic of non-apoptotic cells [36], while up to 30 % of the cells had apoptotic nuclei, in agreement with the FACS analysis. The non-apoptotic

nuclei were surrounded with a well-developed cytoplasm (data not shown) and an extensive fine network of actin filaments, which delineated the border of the cells which were well spread over the ECM (Fig. 5i,j).

Cells grown on HR9/ECM lacking bFGF had a morphology and well-developed actin cytoskeleton that was

similar to the cells grown on HR9-bFGF/ECM (Fig. 5d,k). Interestingly, cells maintained on pure laminin had the same morphological features as cells grown on a multicomponent naturally produced ECM (Fig. 5e,l). Cells maintained on fibronectin alone had numerous fragmented and highly condensed apoptotic nuclei (Fig. 5f). The actin cytoskeleton in these cells was poorly developed, reflecting a reduced cytoplasmic volume characteristic of cells grown on uncoated dishes (compare Fig. 5i,j with 5m). Cells exposed to soluble bFGF alone had a morphology and a poorly developed actin cytoskeleton characteristic of apoptotic cells (Fig. 5g,n).

After 48 hours in culture, differences in the organization of the actin cytoskeleton in non-apoptotic cultures, as compared with apoptotic cells grown on fibronectin or uncoated dishes, were much more prominent (data not shown). The actin cytoskeleton in the apoptotic cultures was poorly developed around a small volume of cytoplasm, while significant parts of the apoptotic nuclei were completely naked and did not contain any cytoplasmic remnants. In contrast, cells cultured on the various types of ECMs or on laminin had a well-developed actin cytoskeleton that was spread out more extensively than in cells cultured for 24 hours (data not shown).

Discussion

Granulosa cells, which nurse the egg and make up the main population of ovarian follicular cells, can serve as a convenient model for studying the effect of ECM on cell differentiation and cell survival. It was previously demonstrated that a gradient of differentiation exists in the intact follicle, where cells adjacent to the basement membrane are more differentiated than cells distal to the basement membrane [37]. Moreover, ECM produced by either corneal endothelial cells or by mouse endodermal cells was shown *in vitro* to enhance gonadotropin receptor formation, intercellular communication and progesterone production in granulosa cells obtained from pre-ovulatory follicles [11,13]. In the present work, we have demonstrated for the first time that the ECM may provide powerful protection against granulosa cell apoptosis induced by serum deprivation. Moreover, laminin, a single component of the ECM, can protect almost as effectively as the multicomponent basement membrane, whereas fibronectin failed to do so.

Recent studies show that ECM protects human endothelial cells and mammary epithelial cells from apoptosis [20,22]. However, there are some conflicting data on the active molecules in the ECM network that are responsible for its protective activity. Sugahara *et al.* [38] suggest that fibronectin can induce apoptosis in human hematopoietic cell lines *via* interaction with very late antigen 5. On the other hand, Zhang *et al.* [39] claim that fibronectin supports cell survival *via* α5β1 integrin which leads to up-regulation of Bcl-2 expression. Integrin-mediated cell

attachment regulates cell survival and proliferation in several cell types *in vitro* [22,23,40]. It has been demonstrated, for example, that integrin α2β3, whose expression is enhanced on angiogenic vascular cells, promotes a survival signal, because antagonists of this integrin cause unscheduled apoptosis of newly formed blood vessels [41]. Laminin was shown to have a moderate and transient effect which could not account for the entire effect of Matrigel ECM in preventing apoptosis of Sertoli cells [42]. We demonstrate that laminin can account for nearly the entire effect of three different types of ECM on granulosa cell viability. It appears that the growing family of different integrins that are recognized and activated by laminin and/or fibronectin can increase cell-survival rates, or promote apoptosis, in different cell types. Because, in the present study, fibronectin failed to prevent apoptosis of granulosa cells, integrin α6β4 or α6β1, which specifically interact with laminin [43], may play a key role in prevention of apoptosis in granulosa cells as well as in other cell types. The anti-apoptotic response of cells to fibronectin or laminin may differ among different cells and in different stages of development due to modulation in expression of various integrin receptors [44].

The detailed FACS analysis of DNA content allowed us to determine the cell-cycle distribution of granulosa cells cultured for 24 hours on various types of ECM. It should be noted that the presence of sub-G0 cells, as revealed by FACS analysis, is a reliable indicator of apoptotic cell death in various cell types [25–29]. In granulosa cells cultured on uncoated dishes in the absence of serum, most of the cell population was in sub-G0 phase which reflects the intensive DNA degradation characteristic of apoptosis [25–29]. The rest of the cell population was in the G0/G1 phase and only 2 % were caught in G2/M. Indeed, the proliferative activity of pre-ovulatory granulosa cells is known to be marginal [37,45]. In contrast, cells grown on different ECMs were mainly arrested in G0 phase, while the proportion of sub-G0 cells was reduced dramatically. Interestingly, the number of cells in G2/M phase increased significantly following plating on ECM, probably due to integrin-mediated growth-promoting signals and the possible mitogenic effect of laminin, which contains sequences similar to those of epidermal growth factor [46]. The protective effect of serum against apoptosis may result from the activity of soluble components of the ECM. The protective effect of ECM can not be explained merely by its induction of cell adhesion, because adhesion to poly-lysine was not able to protect against apoptosis. Also, soluble factors known to enhance granulosa cell differentiation, such as bFGF or FSH, did not protect against apoptosis in serum-deprived cultures, suggesting a unique role for ECM in the survival of highly differentiated granulosa cells. However, bFGF could show a moderate protective effect against apoptosis upon serum removal from cultures that were pre-incubated with serum for 24 hours

[36], suggesting that bFGF and serum factors can cooperate in controlling granulosa cell survival.

In this work, we demonstrate that ECM containing bFGF stimulated progesterone production, concomitant with a protective effect against apoptosis. In contrast, HR9/ECM, which lacks bFGF, protected against apoptosis, but was unable to enhance steroidogenesis; on the contrary, it even reduced progesterone production relative to control cells. The enhancement of steroidogenesis in cells grown on HR9-bFGF/ECM clearly indicates that the differentiation-stimulating activity of the ECM was exerted by bFGF that is sequestered and stabilized by heparan sulfate in the ECM [6,47]. We further documented here, and in a previous study [36] that bFGF by itself is a potent stimulator of steroidogenesis in pre-ovulatory granulosa cells, in spite of its inability to protect efficiently against apoptosis induced by serum deprivation. Therefore, we can distinguish for the first time between the survival activity and the differentiation stimulus exerted by ECM. These studies *in vitro* suggest that the differentiation-promoting activity of the follicular basement membrane on granulosa cells adjacent to a basement membrane may also be exerted by bFGF produced in the ovary [48] and sequestered by the ECM. In view of our present data, it is tempting to suggest that the inner layers of granulosa cells, distal to the basement membrane, are more sensitive to apoptotic signals than cells in close contact with the basement membrane where the anti-apoptotic signal of the basement membrane, and in particular of laminin, has a direct effect.

Steroidogenesis can be enhanced by the rearrangement of steroidogenic organelles containing steroidogenic enzymes, and by proteins that regulate cholesterol transport into the mitochondria; it may also be enhanced by *de novo* synthesis of the protein components of the steroidogenic machinery that are limiting factors in steroidogenesis. Our observation that ECM containing bFGF can maintain the expression of P450scc, which was markedly reduced in cells plated on ECM lacking bFGF, suggests that ECM-bound bFGF may affect the turnover of this enzyme. Moreover, the enhancement of StAR expression by CE/ECM and HR9-bFGF/ECM suggests that bFGF sequestered in the ECM may play an important role in up-regulating this key element and in regulation of steroidogenesis *in vivo*.

There is increasing evidence that reorganization of the actin cytoskeleton may play an important role during apoptosis. Recent observations suggest that fodrin (an analog of spectrin), which is a major component of the cortical cytoskeleton of most eukaryotic cells, is degraded during apoptosis in a variety of cells, including T-lymphoma cells [49]. Moreover, it is known that α-spectrin can be ubiquitinated [50] and is therefore susceptible to degradation by the 26S proteasome, and that cells deficient in spectrin tend to show a blebbing reminiscent of the morphology of apoptotic cells [51]. In addition, partial degradation of actin during apoptosis was recently observed in human leukemia U937 cells [52]. Most recent observations indicate that, in granulosa cells, detachment of the actin cytoskeleton from the inner face of the cell membrane and rearrangement of the actin filaments is a characteristic feature of cell apoptosis [31]. We clearly demonstrate that the integrity of the actin cytoskeleton is a dominant feature of non-apoptotic cells that are in close association with different types of ECM, and may therefore play an important role in cell survival and maintenance of the differentiated phenotype. Moreover, compared with intact ECM, laminin has a most pronounced effect on the integrity and development of the actin cytoskeleton; we therefore suggest that the interactions between laminin and specific integrin molecules play a key role in the maintenance and development of the actin cytoskeleton and in cell survival.

In conclusion, our work provides a clear distinction between two fundamental functions of the ECM: maintenance of cell survival, which is exerted by laminin, and stimulation of the differentiation of granulosa cells, which is exerted by ECM-resident bFGF.

Materials and methods
Cell culture
Primary granulosa cells of pre-ovulatory follicles were obtained from 25-day-old female rats treated with 15 IU PMSG [11]. Cells were plated in serum-free Dulbecco's modified Eagle's medium (DMEM):F12 (1:1), either on plastic tissue culture dishes, or various types of ECM, or on culture dishes coated with poly-lysine, laminin or fibronectin. Cells were maintained at 37 °C in 7 % CO_2 in humidified incubators.

Cultures of bovine corneal endothelial cells were established from steer eyes and maintained in culture as previously described [53]. PF-HR-9 cells derived from a differentiated mouse endodermal carcinoma [54] and PF-HR9 cells transfected with the bovine bFGF gene [9] were cultured as described [9].

Preparation of dishes coated with ECM
Cultures of bovine corneal endothelial cells were dissociated from stock cultures (passage 2–5), and plated (5×10^4 cells per dish) into 35 mm tissue cultures dishes as described above, except that bFGF was not added and 5 % dextran T-40 was included in the growth medium. 6–8 days after the cells reached confluency, the subendothelial ECM was exposed by dissolving the cell layer (3 min at 22 °C) with a solution containing 0.5 % Triton X-100 and 20 mM NH_4OH in PBS, followed by four washes in PBS [7,8,10]. The ECM remained intact, free of cellular debris, and firmly attached to the entire area of the tissue culture dish.

For preparation of HR-9/ECM, cells (10^5 per 35 mm dish) were seeded into tissue culture dishes coated with fibronectin (50 μg per dish) to achieve a firm adhesion of the ECM to the plastic substratum. Ascorbic acid (50 μg ml^{-1}) was added on days 2 and 4 and the ECM denuded 6–7 days after seeding the cells [9]. Major constituents of the HR-9/ECM were laminin, entactin, collagen type IV and heparan sulfate proteoglycans HSPGs [9,55]. We have demonstrated previously that wild-type PF-HR9 cells produce an FGF-free ECM (HR9/ECM); PF-HR9 cells transfected with the gene encoding bFGF deposited an identical ECM (HR9-bFGF/ECM) except for the inclusion of bFGF as a complex with HSPGs.

Coating of culture dishes with fibronectin, laminin and poly-lysine

Fibronectin and laminin diluted in PBS were used at a concentration of 25 μg ml⁻¹. Poly-lysine was used at a concentration of 1 mg ml⁻¹ in PBS. Tissue culture dishes were coated by incubation (1 ml per 35 mm dish) for 1 h at 24 °C, followed by three washes in PBS, as described [56].

FACS analysis of DNA content

Cells grown either on non-coated dishes or on ECM coated dishes for 24 h or 48 h were trypsinized, washed with 10 ml cold PBS and fixed in cold methanol (–20 °C) for 1 h. Subsequently, cells were centrifuged, resuspended in 1 ml cold PBS and stained for 15 min with 50 μg ml⁻¹ propidium iodide in presence of RNase A (100 μg ml⁻¹). Cells were analyzed in a fluorescence-activated cell sorter (FACSort; Becton Dickinson).

Fluorescence labeling of DNA and actin cytoskeleton

At the end of the culture period, medium was aspirated and stored at –20 °C for progesterone measurement. Cells were fixed (30 min, 24 °C) in 3 % paraformaldehyde/PBS (pH 7.4), washed intensively with PBS and permeabilized for 4 min with 1 % Triton X-100 in PBS, followed by further washing. The fixed and permeabilized cells were incubated for 30 min at room temperature with 0.5 μg ml⁻¹ of DAPI (4′,6′ diamido-2-phenylindole hydrochloride) and with 0.3 μg ml⁻¹ of rhodamine-labeled phalloidin. Cells were washed intensively with PBS and mounted in Mowiol.

Quantitative analysis of apoptosis

Microscopic examination of the cell cultures was carried out using a Zeiss Axioskop microscope in both phase and fluorescent modes. Four to seven random fields of each treated culture were photographed through a 40× objective lens in both modes. Apoptotic and total cell nuclei were counted on photographs (at 400× magnification). The percentage of apoptotic nuclei was calculated, scoring 150–300 cell nuclei per treatment.

Progesterone measurement

Progesterone accumulated in the culture medium was determined by radioimmunoassay [57,58]. Antibodies to progesterone were kindly provided by F. Kohen.

Immunoblot analysis

Cells were washed twice with cold PBS and incubated for 10 min on ice with cold lysis buffer containing 50 mM Hepes (pH 7.2), 150 mM NaCl, 2.5 mM MgCl₂, 1 mM EGTA, 1 mM PMSF, 10 μg ml⁻¹ leupeptin, 10 % glycerol, and 1 % NP-40. Lysates were heated (5 min, 95 °C) in sample buffer and were separated by 8 % SDS–PAGE followed by electrotransfer onto a nitrocellulose membrane overnight, on ice. The membrane was probed with rabbit anti-StAR antibodies followed by a horseradish peroxidase-conjugated second antibody and visualized using enhanced chemiluminescence. Reprobing of the nitrocellulose membrane with anti-P450scc antibodies was carried out as described earlier [58]. Antibodies to StAR were kindly provided by D.M. Stocco. Antibodies to P450scc were kindly provided by W.L. Miller.

References

1. Gospodarowicz D, Greenberg G, Birdwell CR: **Determination of cellular shape by the extracellular matrix and its correlation with control of cellular growth.** *Cancer Res* 1978, **38**:4155–4171.
2. Ingber DE: **Fibronectin controls capillary endothelial cell growth by modulating cell shape.** *Proc Natl Acad Sci USA* 1990, **87**:3579–3583.
3. Boudreau N, Meyrs C, Bissell MJ: **From laminin to lamin: regulation of tissue-specific gene expression by the ECM.** *Trends Cell Biol* 1995, **5**:1–4.
4. Damsky CH, Werb Z: **Signal transduction by integrin receptors for extracellular matrix. Cooperative processing of extracellular information.** *Curr Opin Cell Biol* 1992, **4**:772–781.
5. Ruoslahti E, Yamaguchi Y: **Proteoglycans as modulators of growth factor activities.** *Cell* 1991, **64**:867–869.
6. Vlodavsky I, Bar-Shavit R, Korner G, Fuks Z: **Extracellular matrix-bound growth factors, enzymes and plasma proteins,** In *Molecular and Cellular Aspects of Basement Membrane.* Edited by Rohrbach DH, Timpl R. Orlando, Florida: Academic Press; 1993:327–343.
7. Vlodavsky I, Liu GM, Gospodarowicz D: **Morphological appearance, growth behaviour and migratory activity of human tumor cells maintained on extracellular matrix vs. plastic.** *Cell* 1980, **19**:607–616.
8. Vlodavsky I, Folkman J, Sullivan R, Fridman R, Ishai-Michaelli R, Sasse J, Klagsbrun M: **Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix.** *Proc Natl Acad Sci USA* 1987, **84**:2292–2296.
9. Rogelj S, Klagsbrun M, Atzmon R, Kurokawa M, Haimovitz A, Fuks Z, Vlodavsky I: **Basic fibroblast growth factor is an extracellular matrix component required for supporting the proliferation of vascular endothelial cells and the differentiation of PC12 cells.** *J Cell Biol* 1989, **109**:823–831.
10. Gospodarowicz D, Delgado D, Vlodavsky I: **Permissive effect of the extracellular matrix on cell proliferation** *in vitro. Proc Natl Acad Sci USA* 1980, **77**:4094–4098.
11. Furman A, Rotmensch S, Kohen F, Mashiach S, Amsterdam A: **Regulation of rat granulosa cell differentiation by extracellular matrix produced by bovine corneal endothelial cells.** *Endocrinology* 1986, **118**:1878–1885.
12. Ben-Ze'ev A, Amsterdam A: **Regulation of cytoskeletal proteins involved in cell contact formation during differentiation of granulosa cell on ECM coated surfaces.** *Proc Natl Acad Sci USA* 1986, **83**:2894–2898.
13. Amsterdam A, Rotmensch S, Furman A, Venter EA, Vlodavsky I: **Synergistic effect of human chorionic gonadotropin and extracellular matrix on** *in vitro* **differentiation of human granulosa cells: progesterone production and gap junction formation.** *Endocrinology* 1989, **124**:1956–1964.
14. Amsterdam A, Rotmensch S, Ben-Ze'ev A: **Coordinated regulation of morphological and biochemical differentiation in a steroidogenic cell: the granulosa cell model.** *Trends Biochem Sci* 1989, **14**:377–382.
15. Amsterdam A, Plehn-Dujowich D, Suh BS: **Structure–function relationships during differentiation of normal and oncogene transformed granulosa cells.** *Biol Reprod* 1992, **46**:513–522.
16. Amsterdam A, Koch Y, Lieberman ME, Lindner HR: **Distribution of binding sites for human chorionic gonadotropin in the preovulatory follicle of the rat.** *J Cell Biol* 1975, **67**:894–900.
17. Zoller LC, Weisz J: **Identification of cytochrome P-450, and its distribution in the membrana granulosa of the preovulatory follicle using quantitative cytochemistry.** *Endocrinology* 1979, **103**:310–313.
18. Guadagno TM, Ohtsubo M, Roberts JM, Assoian RK: **A link between cyclin A expression and adhesion-dependent cell cycle progression.** *Science* 1993, **262**:1572–1575.
19. Ruoslahti E, Reed JC: **Anchorage dependence, integrins and apoptosis.** *Cell* 1994, **77**:477–478.
20. Boudreau N, Sympson CJ, Werb Z, Bissel MJ: **Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix.** *Science* 1995, **267**:891–893.
21. Fang F, Orend G, Watanabe N, Hunter T, Ruoslahti E: **Dependence of cyclin E-cdk2 kinase activity on cell anchorage.** *Science* 1996, **271**:499–502.
22. Meredith JE, Fazeli B, Schwartz MA: **The extracellular matrix as a cell survival factor.** *Mol Biol Cell* 1993, **4**:953–961.
23. Frisch SM, Francis H: **Disruptions of epithelial cell–matrix. Interactions induces apoptosis.** *J Cell Biol* 1994, **124**:619–626.
24. Kim WH, Schnaper HW, Nomizu M, Yamada Y, Kleinman HK: **Apoptosis in human fibrosarcoma cells is induced by a multimeric synthetic Tyr-Ile-Gly-Ser-Arg (YIGSR)-containing polypeptide from laminin.** *Cancer Res* 1994, **54**:5005–5010.
25. Darzynkiewicz Z, Bruno S, Del-Bino G, Gorczyca W, Hotz MA, Lassota P, Traganos F: **Features of apoptotic cells measured by flow cytometry.** *Cytometry* 1992, **13**:795–808.

26. Ormerod MG, Collins MKL, Rodriguez-Tarduchi G, Robertson D: **Apoptosis in interleukin-3 dependent haemopoietic cells: quantitation by two flow cytometric methods.** *J Immunol Methods* 1992, **153**:57–65.
27. Sun XM, Snowden RT, Skiletter DN, Dinsdale D, Ormerod MG, Cohen GM: **A flow-cytometric method for the separation and quantitation of normal and apoptotic thymocytes.** *Anal Biochem* 1992, **204**:351–356.
28. Telford W, King L, Fraker P: **Rapid quantitation of apoptosis in pure and heterogenous populations using flow cytometry.** *J Immunol Methods* 1994, **172**:1–16.
29. Fraker PJ, King LE, Elghanian DL, Telford WG: **Quantification of apoptotic events in pure and heterogenous populations of cells using the flow cytometer.** In *Methods in Cell Biology.* Academic Press; 1995, 46:57–76.
30. Amsterdam A, Aharoni D: **Plasticity of cell organization during differentiation of normal and oncogene transformed granulosa cells.** *Micros Res Tech* 1994, **27**:108–124.
31. Pitzer F, Dantes A, Fuchs T, Baumeister W, Amsterdam A: **Removal of proteasomes from the nucleus and their accumulation in apoptotic blebs during programmed cell death.** *FEBS Lett* 1996, **394**:47–50.
32. Stocco DM, Clark BJ: **Regulation of the acute production of steroids in steroidogenic cells.** *Endocr Rev* 1996, **17**:221–244.
33. Martin SJ, Green DR, Cotter TG: **Dicing with death: dissecting the components of the apoptosis machinery.** *Trends Biochem Sci* 1994, **19**:26–30.
34. Steller H: **Mechanisms and genes of cellular suicide.** *Science* 1995, **267**:1445–1449.
35. Williams GT, Smith CA, McCarthy NJ, Grimes EA: **Apoptosis: final control point in cell biology.** *Trends Cell Biol* 1992, **2**:263–267.
36. Aharoni D, Dantes A, Oren M, Amsterdam A: **c-AMP mediated signals as determinants for apoptosis in primary granulosa cells.** *Exp Cell Res* 1995, **218**:271–282.
37. Amsterdam A, Rotmensch S: **Structure–function relationships during granulosa cell differentiation.** *Endocr Rev* 1987, **8**:309–337.
38. Sugahara BH, Kanakura Y, Furitsu T, Kitayama H, Ishikawa J, Hashimoto K, *et al.*: **Induction of programmed cell death in human hematopoietic cell lines by fibronectin *via* its interaction with very late antigen 5.** *J Exp Med* 1994, **179**:1757–1766.
39. Zhang Z, Vuori K, Reed JC, Ruoslahti E: **The $\alpha 5\beta 1$ integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression.** *Proc Natl Acad Sci USA* 1995, **92**:6161–6165.
40. Montgomery AMP, Reisfeld RA, Cheresh DA: **Integrin $\alpha v\beta 3$ rescues melanoma cells from apoptosis in three dimensional dermal collagen.** *Proc Natl Acad Sci USA* 1994, **91**:8856–8860.
41. Stromblad S, Becker JC, Yebra M, Brooks PC, Cheresh DA: **Suppression of p53 activity and p21 WAF1/CIP1 expression by vascular cell integrin $\alpha v\beta 3$ during angiogenesis.** *J Clin Invest* 1996, **98**:426–433.
42. Dirami M, Ravindranath N, Kleinman H, Dym M: **Evidence that basement membrane prevents apoptosis of Sertoli cells *in vitro* in the absence of known regulators of Sertoli cell function.** *Endocrinology* 1995, **136**:4439–4447.
43. Hynes RO: **Integrins: versatility, modulation and signaling in cell adhesion.** *Cell* 1992, **69**:11–25.
44. Fujiwara H, Ueda M, Takakura K, Mori T, Maeda M: **A porcine homolog of human integrin $\alpha 6$ is differentiation antigen of granulosa cells.** *Biol Reprod* 1995, **53**:407–417.
45. Hsueh AJW, Adashi EY, Jones PBC, Welsh TH Jr: **Hormonal regulation of the differentiation of cultured ovarian granulosa cells.** *Endocr Rev* 1984, **5**:76–127.
46. Panayotou G, End P, Aumailley M, Timpl R, Engel J: **Domains of laminin with growth factor activity.** *Cell* 1989, **56**:93–101.
47. Saksela O, Moscatelli D, Sommer A, Rifkin DB: **Endothelial cell-derived heparan sulfate binds basic fibroblast growth factor and protects it from proteolytic degradation.** *J Cell Biol* 1988, **107**:743–751.
48. Tsafriri A, Adashi EY: **Local nonsteroidal regulators of ovarian function.** In *Physiology of Reproduction.* Edited by Knobil E, Neil JD. New York: Raven Press; 1994:817–860.
49. Martin SJ, O'Brien GA, Nishioka WK, McGahon AJ, Mahboubi A, Saido TC, *et al.*: **Proteolysis of fodrin (non-erythroid spectrin) during apoptosis.** *J Biol Chem* 1995, **270**:6425–6428.
50. Corsi D, Galluzzi L, Crinelli R, Magnani M: **Ubiquitin is conjugated to the cytoskeletal protein α-spectrin in mature erythrocytes.** *J Biol Chem* 1995, **270**:8928–8935.
51. Mohandas N, Chasis JA, Shohet SB: **The influence of membrane skeleton of red cell deformability.** *Semin Hematol* 1983, **20**:225–242.
52. Mashima T, Naito M, Fujita N, Noguchi K, Tsuruo T: **Identification of actin as a substrate of ICE and ICE- like protease and involvement of an ICE-like protease but not ICE in VP-16 induce U937 apoptosis.** *Biochem Biophys Res Comm* 1995, **217**:1185–1192.
53. Gospodarowicz D, Mescher AL, Birdwell CR: **Stimulation of corneal endothelial cell proliferation *in vitro* by fibroblast and epidermal growth factor.** *Exp Eye Res* 1977, **25**:75–87.
54. Chung A, Estes LE, Shinozuka HB, Braginski J, Lorz C, Chung CE: **Morphological and biochemical observations on cells derived from differentiation of the embryonal carcinoma cell line PCC4-F.** *Cancer Res* 1978, **37**:2072–2081.
55. Kramer HR, Vogel KG: **Selective degradation of basement membrane macromolecules by metastatic cells.** *J Natl Cancer Inst* 1984, **72**:889–899.
56. Vlodavsky I, Gospodarowicz D: **Respective involvement of laminin and fibronectin in the adhesion of human carcinoma and sarcoma cells.** *Nature* 1981, **289**:304–306.
57. Kohen F, Bauminger S, Lindner HR: **Preparation of antigenic steroid-protein conjugate.** In *Steroid Immunoassay.* Edited by Cameron EHD, Hillier SG, Griffiths K. Cardiff, Wales: Alpha Omego; 1975:11–23.
58. Breckwoldt M, Selvaraj N, Aharoni D, Barash A, Segal I, Insler V, Amsterdam A: **Expression of Ad4-BP/cytochrome P450 side chain cleavage enzyme and induction of cell death in long-term cultures of human granulosa cells.** *Mol Human Rep* 1996, **2**:391–400.