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Original Article

GH_3 tumor pituitary cell cytoskeleton and plasma membrane arrangement are determined by extracellular matrix proteins: implications on motility, proliferation and hormone secretion

Erika Azorín[1], Beatriz Romero-Pérez[1], Carmen Solano-Agama[1], María T de la Vega[1], César G Toriz[1], Blanca Reyes-Márquez[1], Sirenia González-Pozos[3], Víctor H Rosales-García[3], Margarita González del Pliego[4], Myrna Sabanero[5], María E Mendoza-Garrido[1]

[1]Department of Physiology, Biophysics and Neuroscience, CINVESTAV-IPN, Mexico City, Mexico; [2]Department of Cellular Biology, CINVESTAV-IPN, Mexico City, Mexico; [3]Central Laboratories, CINVESTAV-IPN, Mexico City, Mexico; [4]Department of Embryology, Medical Faculty, UNAM, Mexico City, Mexico; [5]Guanajuato University, Guanajuato, Gto., Mexico

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Abstract: The extracellular matrix (ECM) influences different physiological and pathophysiological aspects of the cell. The ECM consists in a complex network of macromolecules with characteristic biochemical properties that allow cells to sense their environments inducing different signals and changing cell behavior. The purpose of the present study was to evaluate the participation of different ECM proteins in cell morphology and its implication on motility, proliferation and hormone secretion in GH_3 cells, a tumor pituitary cell. GH_3 cells were cultured with a defined medium on collagens I/III and IV, fibronectin and laminin. GH_3 cells express α2 integrin subunit *de novo*. The cells responded to the ECM proteins with differentiated cell surface morphologies and membrane protrusions. A rounded shape with small membrane blebs, weak substrate adhesion and high motility was observed in cells on C I/III and fibronectin, while on C IV and laminin cells were viewed elongated and adhered. Differences on actin cytoskeleton, cytoskeletal-associated vinculin and phospho-MLC showed that ECM proteins determine the cytoskeleton organization. Cell proliferation showed dependency on the ECM protein, observing a higher rate in cells on collagen I/III. Prolactin secretion was higher in cells with small blebs, but an unchangeable response to EGF was obtained with the ECM proteins, suggesting is a consequence of cortical actin arrangement. We ascribe the functional differences of the GH_3 cells to the cytoskeletal organization. Overall, the data showed that ECM plays a critical role in GH_3 cells modulating different cellular comportment and evidenced the importance of the ECM composition of pituitary adenomas.

Keywords: GH_3 cells, ECM proteins, actin cytoskeleton organization, α2 integrin sub-unit, cell membrane organization, proliferation, Prl secretion

Introduction

It is well known that cell adhesion to the extracellular matrix (ECM) profoundly influences the major cellular programs of growth, migration, differentiation, and apoptosis [1-3]. Cell adhesion to the ECM is accomplished by specific membrane receptors. The major ECM adhesion receptors are integrins, a large family of αβ heterodimer trans-membrane proteins that link the ECM to the cytoskeleton and activate many intracellular signaling pathways [4]. Integrins recognize short peptide motifs of the ECM proteins, and the ligand specificity depends on both sub-units of a given αβ heterodimer, whereas, inside the cell, integrins establish a linkage with the cytoskeleton [5]. The overall effect of this interaction is to modulate the cell shape and polarity, cytoplasmic organization and cell motility [5, 6]. Besides these cellular responses to an adhesion challenge, growth factors and adhesion complexes collaborate in downstream signaling pathways [7, 8]. It is well known that cell adhesion is required for full acti-

vation and signaling of growth factor receptors [8, 9]. It is important to remark that variations in cellular responses are specific to cell type. In endocrine tissues, the ECM plays a crucial role in cellular organization and function [10-12]. In the pituitary, collagen fibrils form a varied framework throughout the gland [13], and provide structure to the connective tissue, and type IV collagen and laminin are elements of the basal lamina [14, 15]. Fibronectin is observed with a different deposition pattern, diffusely in patches, and in human pituitaries and adenomas correlates with fibrous matrix [16, 17]. However, few studies exist in which the participation of the ECM in the physiology of the gland has been analyzed. According to Horacek et al. [18], cultured pituitary cells secreted more prolactin (Prl) when a complex ECM (Matrigel) was used instead of laminin alone. Interestingly, cells that adhered to Matrigel exhibited rounded shapes and formed clusters, whereas a fibroblastic shape was observed when laminin was used as substrate [18]. On the other hand, infantile pituitary cells cultured over collagen type I/III without serum and stimulated with EGF exhibit F-actin organization in stress fibers, while absence of stress fibers are observed in adult cells [19]. Using a well-characterized tumor pituitary cell line, the GH_3 cell line, Elias et al. [20] observed a more flattened shape and an increase in Prl secretion when cells were cultured on Matrigel, contrary to normal pituitary cells [18]. But, laminin alone inhibits GH_3 proliferation and prolactin secretion [21]. All the antecedents showed differences in pituitary secretory cells response to isolated ECM proteins or complex mixtures of it. Likewise, no studies have been conducted in GH_3 cells to evaluate the relationship between the different responses elicited by ECM proteins, present in the pituitary connective tissue or basal lamina. The purpose of the present study was to analyze the role of different ECM protein components, collagens type I/III and IV, and with fibronectin or laminin, in GH_3 cell morphology, actin cytoskeletal arrangement and migration, and their influence in cell proliferation and secretion. A GH_3 cell corresponds to a pituitary somatotrope cell, synthesizes and secretes GH and, to a lesser extent, Prl. The analysis of GH_3 integrin sub-units expression evidenced α2 sub-unit presence, which is absent in normal rat pituitary cells, showing a change in integrin expression with the acquisition of a tumor phenotype. In summary, it was found that ECM proteins themselves modulate the actin cytoskeleton and plasma membrane organization together with changes in cellular patterns in GH_3 tumor pituitary cells, as proliferation and basal hormone secretion. These results showed the relationship of the morphology determined by the cell interaction with MEC proteins in the cell function of a tumor pituitary secretory cell. However, further investigation is needed in order to know the intracellular pathways that specifically activate each ECM protein, allowing the GH_3 cells to organize their cytoskeleton and modify their cellular processes.

Materials and methods

Materials

Collagen type I/III (95%/5%, from calf skin) and collagen type IV (Native, from Engelbreth-Holm-Swarm mouse sarcoma) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA); laminin was obtained from Invitrogen (Natural mouse, from Engelbreth-Holm-Swarm mouse sarcoma, Life Technologies Corp., Carlsbad, CA, USA); fibronectin and EGF were purchased from Roche Diagnostics GmbH (Werk Penzberg, Penzberg, Germany). Culture medium F12, fetal calf serum and horse serum were obtained from Gibco (Life Technologies Corp., Carlsbad, CA, USA). Rabbit antibodies against integrin subunits were purchased from Chemicon (Merck-Millipore, Billerica, MA, USA). An antibody against vinculin was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Mouse monoclonal antibodies against phosphorylated Erk 1/2 and phosphorylated myosin regulatory light chain (MLC) were purchased from Cell Signaling (Danvers, MA, USA). The radioimmunoassay kit for rat-Prolactin was generously provided by Dr A. F. Parlow from the National Hormone & Peptide Program, Harbor-UCLA Medical Center, USA. Monoclonal antibody against actin was generously provided by Dr M. Hernández, CINVESTAV. ^{125}I and [^{3}H]-thymidine were purchased from Amersham (GE Healthcare Bio-Science Corp., Little Chalfont, Buckinghamshire, UK). Rhodamine-conjugated phalloidin (TRITC-phalloidin) was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA), and secondary fluorescein-conjugated antibodies were obtained from Molecular Probes (Life Technologies Corp., Carlsbad, CA, USA). The rest of the chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

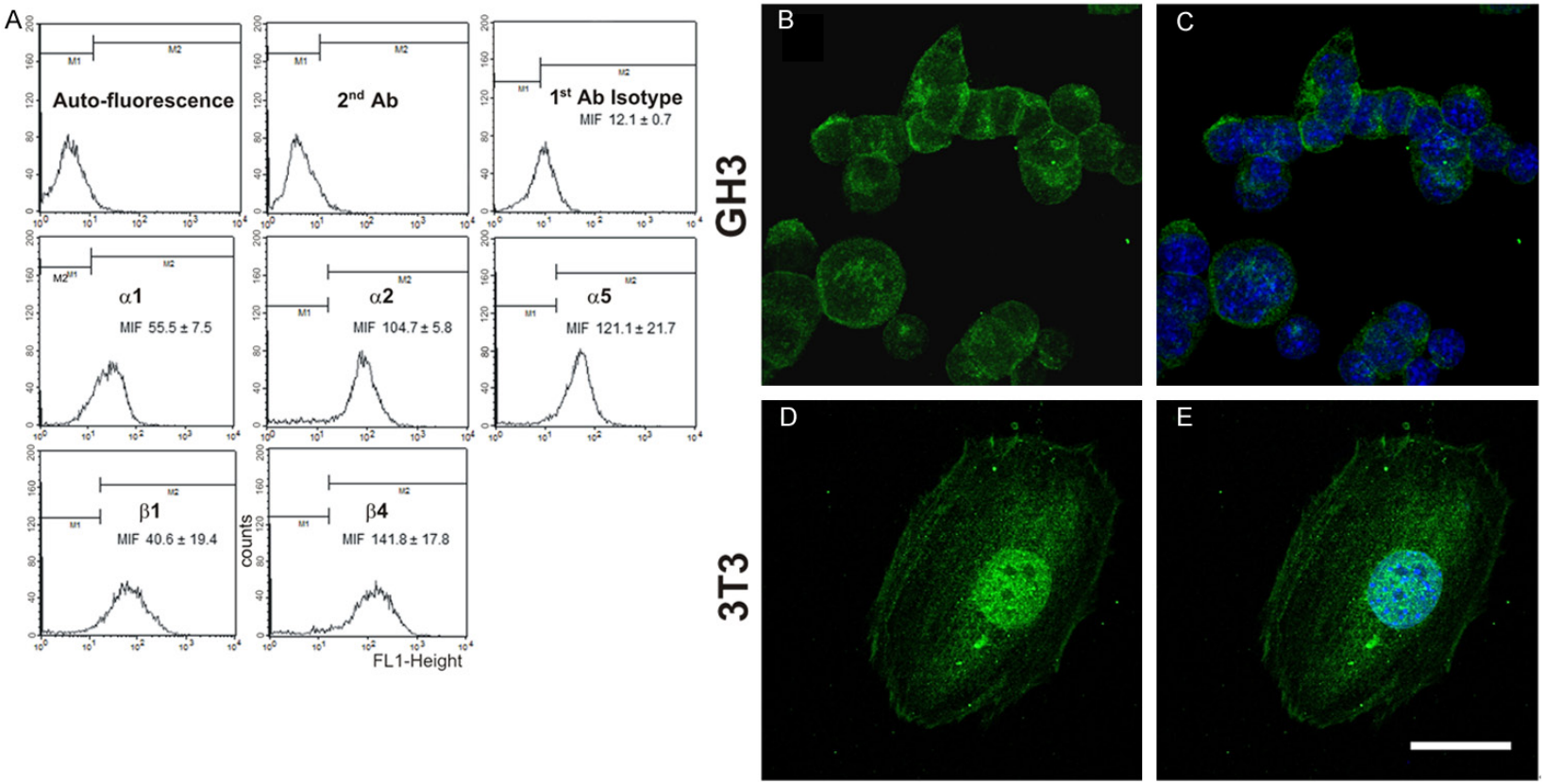

Figure 1. Integrin sub-units expression and α2 sub-unit localization in GH_3 cells. Cells cultured in standard culture condition were detached and assayed for immunocytochemistry for α1, α2, α5, β1 and β4, and further analyzed by flow cytometry (A). 10,000 cells per experimental condition were analyzed. Graphs correspond to representative single-parameter histograms obtained for each integrin sub-unit and data inside the graph correspond to the mean ± SEM of the median intensity of fluorescence (MIF) of at least three experiments. Data shown inside the figures corresponded to the mean fluorescence intensity values of four different experiments. The first three histograms correspond to GH_3 auto-fluorescence, unspecific binding of secondary antibody and primary antibody iso-type control. An immunolocalization of α2 integrin sub-unit was performed in GH_3 cells (B and C) and NIH 3T3 cells (positive control, D and E). Cells were fixed and labeled with a poly-clonal anti-α2 integrin sub-unit and stained with a FITC-conjugated secondary antibody (green) and the nucleuses were stained with DAPY (blue). Bar = 25 μm.

Table 1. Measurement of cell area of GH_3 cells cultured with DEF medium overdifferent ECM proteins

Culture period		24 h	48 h	72 h
ECM protein		Cell surface (μm^2)		
C I/III	25% percentile	60.0	59.0	73.4
	Median	71.1	71.5	89.4
	75% percentile	85.0	84.6	106.4
C IV	25% percentile	54.0	72.2	71.5
	Median	69.0	87.0***	87.1
	75% percentile	102.9	107.5	107.7
C/F	25% percentile	56.0	62.6	71.2
	Median	69.4	79.0***,+++	91.8*,+
	75% percentile	86.0	101.9	119.6
C/L	25% percentile	76.8	73.0	95.8
	Median	94.2***,+++,&&&	95.0***,+,&&&	123.5***,+++,&&&
	75% percentile	122.6	127.1	164.6

Measurement of cell area of GH_3 cells cultured with DEF medium over different ECM proteins. After 24, 48 or 72 h culture period the cells were fixed, stained and observed at the microscope with a 40× objective in order to obtain the cellarea of 600 cells per experimental condition. ***p<0.001 and *p<0.05 with respect to C I/III, +++p<0.001 and +p<0.05 with respect to C IV, &&&p<0.001 respect to C/F (Kruskal-Wallis test nonparametric ANOVA followed by a Dunn's multiple comparisons test).

Cell culture

Rat pituitary GH_3 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in Ham's F10 medium supplemented with 15% horse serum and 2.5% fetal calf serum. For experiments, cells were detached with EDTA (0.3 mg/ml in PBS) and re-suspended in a defined medium (DEF) of Ham's F12 containing 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml triiodothyronine, 60 ng/ml corticosterone, 6 ng/ml progesterone, 1 mg/ml bovine serum albumin (BSA, fraction V), 16 μg/ml putrescine, and 2.9 μg/ml sodium selenite. Cells were cultured for different periods at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Cytofluorometric analysis

Immunofluorescence assays were performed for the following integrin sub-units: α1, α2, α5, β1 and β4. GH_3 cells were suspended, and aliquots of 3×10^4 cells were fixed for 10 min with 1.5% formaldehyde followed by a 10 min incubation with methanol at 4°C. Then, the cells were blocked with 50 mM NH_4Cl for 10 min followed by a second blocking period with 1% IgG-free serum albumin for 30 min. The cells were then incubated with the primary antibody diluted in 0.1% albumin and 0.03% saponin in PBS overnight at 4°C. Then, the cells were incubated with FITC-conjugated secondary antibody diluted in 0.1% albumin and 0.03% saponin in PBS for 60 min at room temperature. The antibody concentrations were as follows: 1:400 for β4; 1:700 for α1 and β1, 1:800 for α2; and 1:1,000 for α5. The secondary antibody concentration used was 1:400. The cells were observed using a fluorescence-activated cell sorter (FAC'S CALIBUR, Beckton Dickinson, USA) and analysed by the Summit V4.3 program (Dako Inc., Carpinteria, CA, USA). Rat anterior pituitary cells were used as a negative control for α2 and positive controls for α1, α5 and β1 [22, 23] and MDCK cells were used as a negative control for β1 (the antibody does not react with dog protein). MA104 cells were positive for β4 and significantly less so for β1 (Figure S1).

Cell morphology and cell surface measurements

Cells were cultured in 24-multiwell plates (Costar, Corning Inc., Corning, NY, USA) containing a cover-glass at the bottom previously coated with 3-aminopropylthriethoxy-silane to ensure the attachment of the different ECM proteins to the glass. The cover glasses were covered with type I/III collagen (C I/III, 6.3 μg/cm^2), type IV collagen (C IV, 2 μg/cm^2), C I/III plus fibronectin as a connective tissue mixture (C/F, 6.3 μg/cm^2 and 2.6 μg/cm^2, respectively), C IV plus laminin as a basal lamina mixture (C/L, 2 μg/cm^2 and 1 μg/cm^2) or poly-lysine

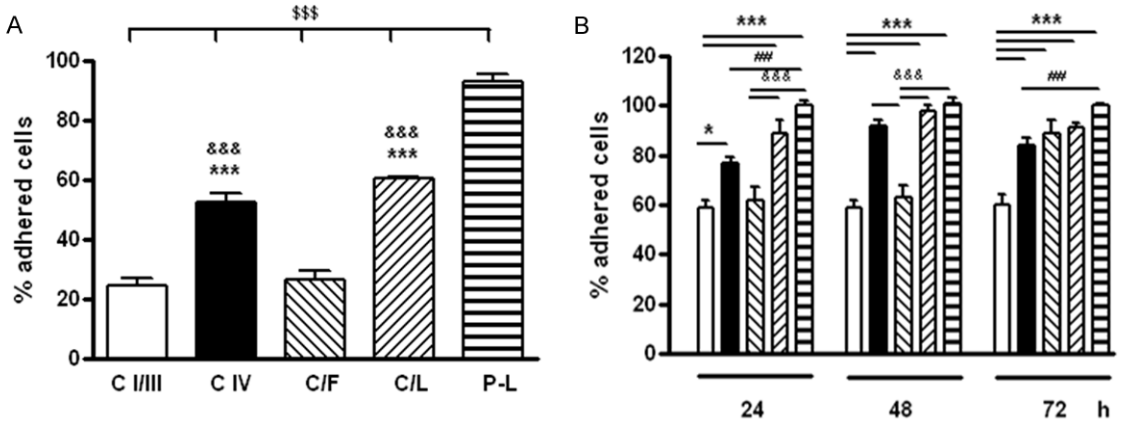

Figure 2. GH_3 cells adhesion capacities to different ECM proteins. Cells were cultured with DEF medium on C I/III (open bars), C IV (filled bars), C/F (left dashed bars), C/L (right dashed bars) or P-L (horizontal lines bars) during 60 min (A) or 24, 48 and 72-h (B) by duplicate, and the amount of cells were determined by the MTT assay. After the different culture periods, a set of cultures was washed to obtain the amount of adhered cells and the other set correspond to the amount of total cells seeded of each ECM protein used as substrate. Data corresponds to the per cent of adhered cells with respect to total cells of each ECM protein group. *p<0.05, and ***p<0.001 with respect to C I/III group; ##p<0.01 with respect to C IV; &&&p<0.001 with respect to C/F (ANOVA test followed by Tukey- Kramer multiple comparison test).

(P-L, 0.1 mg/ml). Aliquots of 3×10^4 cells were seeded over the cover glasses and cultured in DEF medium for 48 h. A serum-stimulated group (Ser) was cultured with 10% horse serum plus 5% fetal bovine serum in the presence of C I/III. After the end of each period, cells were fixed with 4% paraformaldehyde in PBS for 20 min followed by toluidine blue O staining and were mounted with an aqueous mounting medium. Cells were photographed using a Zeiss IIIRS microscope with a 500x magnifying power. Cell surface measurements were recorded using a light microscope (BMAX/ Olympus, Japan) using a 40x objective. The images were captured by a video camera (Ikegami CCD, Japan), and the projected areas of the adherent cells were determined using the LeicaWin software (Leica, Germany). At least 600 cells from 3 different experiments were measured. A set of cells was incubated in the same experimental conditions but was processed for electron microscope scanning. The cells were fixed with 2.5% glutaraldehyde in PBS for 60 min, followed by 1% OsO_4 in PBS for 60 min, were dehydrated with ethanol gradients and dried to a critical point in a Samdri 780A (Tousimis Research Co., USA). Then, the dried cells were shaded with gold particles and observed using a scanning electron microscope (JSM-6510LV, Jeol, Japan).

Actin cytoskeletal and vinculin arrangement

For these observations, cells were cultured over cover glasses coated with ECM proteins for 48 h. A group of cells cultured over C I/III were stimulated with 2 ng/ml lysophosphatidic acid (LPA) or 50 ng/ml EGF. Then, cells were fixed and permeabilized with 4% paraformaldehyde in PBS with 5 mM $MgCl_2$ (PBS-$MgCl_2$), 5% sucrose 3 mM EGTA with 0.2% Triton-X100 for 3 min. Then, the fixation was finished with a 4% paraformaldehyde in PBS-$MgCl_2$ for 30 min. Nonspecific binding was blocked with 1% IgG-free albumin in PBS-$MgCl_2$ plus 0.03% saponin. Cells were incubated with a primary antibody against vinculin 1:400 for 2 h at room temperature followed by incubation with a FITC-secondary antibody 1:250 for 60 min. Then, cells were stained for actin-F with 0.1 μM TRITC-phalloidin for 7 min and mounted for further analysis (Vectashield mounting medium, Vector, USA). The arrangement of the actin cytoskeleton and vinculin was visualized using a confocal microscope (TCS-SP 2, Leica, Germany).

Time-lapse observation of GH_3 cells

Aliquots 1.5×10^6 GH_3 cells were placed in 60 mm culture Petri dishes covered with the different ECM proteins and filled with DEF medium

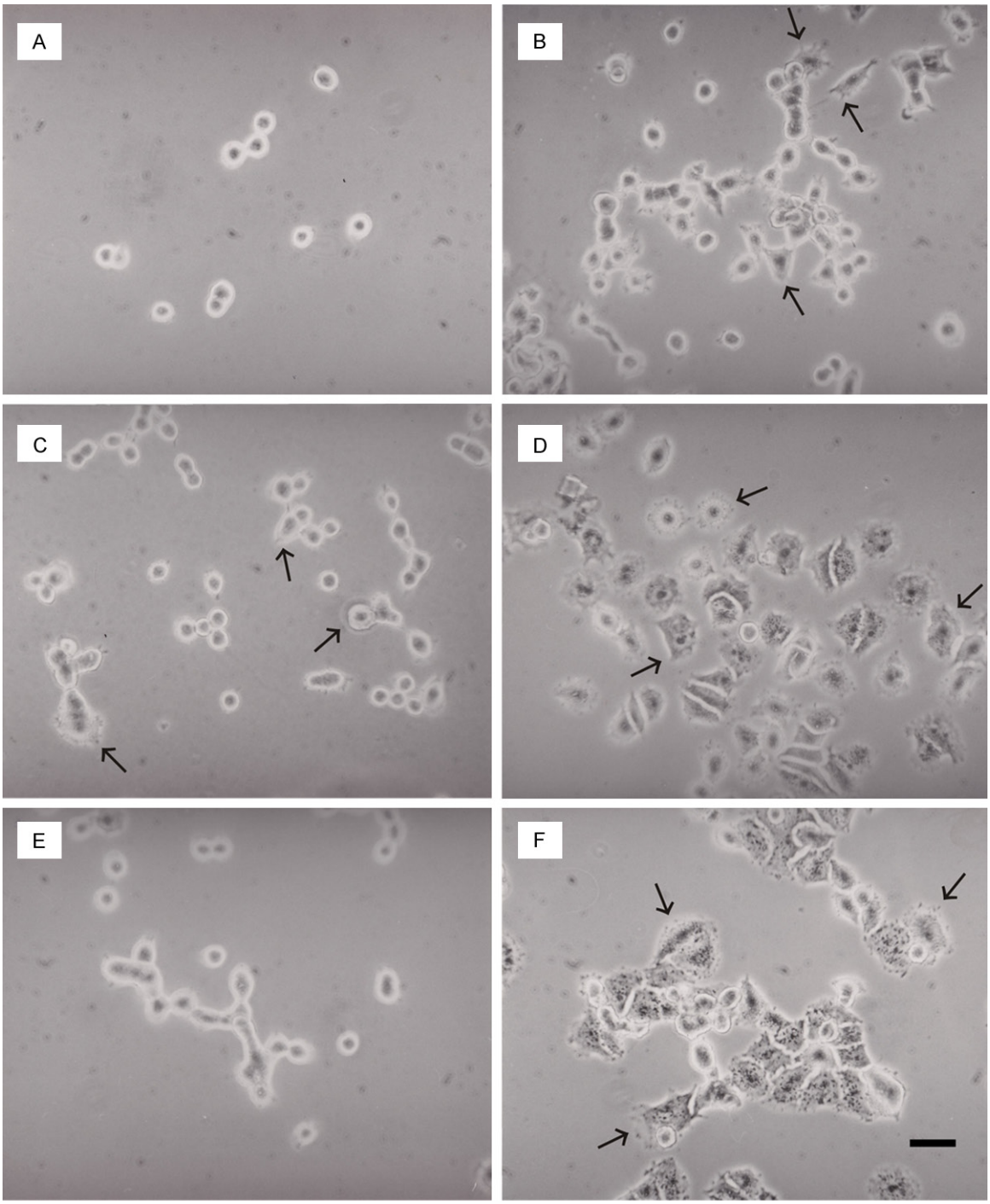

Figure 3. GH_3 cells shapes and membrane processes when cultured on different ECM proteins. Cells were cultured in DEF medium on C I/III (A), C IV (B), C/F (C), C/L (D), or P-L (E) and after 48-h were fixated and stained. A group of cells on C I/III were cultured with a serum enriched medium (F). Cells develop membrane processes depending to the ECM protein they adhere (thin arrows). Bar = 25 μm.

added with 3 mg/ml phenol red, previously equilibrated with the culture incubator atmosphere. The Petri dishes were sealed with parafilm (Pechiney, USA) and placed in a thermic isolated chamber with a thermostat unit (TempModul S1 D, Zeiss, Germany) on an inverted microscope (Axio Observer D1, Zeiss, Germany) with a phase contrast objective 40x. Time-lapse recorders were obtained using a digital camera (AxioCam MRE, Zeiss, Germany)

Table 2. Measurement of motility in GH_3 cells cultured over different ECM proteins

ECM protein	Total displacement (a.u)	Net displacement (a.u.)	Velocity (a.u./sec)
C I/III	447 ± 25	172 ± 18	12.2 ± 1.1
C IV	190 ± 6***	10 ± 1***	4.1 ± 0.1***
C/F	490 ± 57+++	72 ± 21**,++	13.6 ± 2.4+++
C/L	171 ± 5***,&&&	62 ± 1*,+++	3.9 ± 0.1***,&&&

The motility parameters of GH_3 cells were obtained from the lapse videos of cells cultured over C I/III, C IV, C/F or C/L in DEF medium, or C I/III in serum enriched medium during the first four hours of culture. ***p<0.001, **p<0.01 and *p<0.05 with respect to C I/III, +++p<0.001 and ++p<0.01 with respect to C IV, and &&&p<0.001 with respect to C/F (Kruskal-Wallis test nonparametric ANOVA followed by a Dunn's multiple comparisons test.

and AxioVision Rel.4.8 (Zeiss, Germany). Images were processed using the software Image J 1.47v and the Manual Tracking and Chemotaxis and Migration Tool 2.0 (Wayne Rasband, NIH, USA). Cultured cells were observed during the first 4 h after seeding Observations were performed in triplicate.

Cell adhesion assay

Experiments were performed using 96-multiwell plates covered with the different ECM proteins. GH_3 cells aliquots (2.0-1.5×10^5 cells) were seeded in each well with culture medium added with 2.5% fibronectin extracted FBS [24] and cultured for 60 min, or cultured in DEF medium for 24, 48 and 72 h. A serum control was also performed. Experiments were performed in duplicate; one to obtain the total cell number and the other to obtain the adhered cell number. The cells adhered were those attached to the bottom after they were rinsed. The colorimetric method of tetrazolium salt (MTT) mitochondrial conversion to formazan was used to assay the cells. 3-[4, 5-dimethylthiazol-2-yl]-2, 5-di-phenyl tetrazolium bromid (MTT, Sigma-Aldrich Co., St-Louis, MO, USA) was used at 5 mg/ml.

Immunoblot analysis

GH_3 cells (1.2×10^6 cells) were seeded in 60 mm diameter culture dishes previously covered with the different ECM proteins and cultured in defined medium. Forty eight hours later, the proteins associated with the actin cytoskeleton (detergent-insoluble fraction) were separated from the cytosol fraction (detergent-soluble). Cells attached to the different substrates were treated with a PHEM-Triton buffer (60 mM PIPES, 25 mM HEPES, 10 mM EDTA, 2 mM $MgCl_2$, 10 mM NaF, 10 mM Na_3VO_4, 20 mM β-glycerophosphate, a cocktail of protease inhibitors (Complete, Roche Diagnostics GmbH, Werk Penzberg, Penzberg Germany), 2 mM PMSF, and 0.1% Triton X-100, pH 7.4) and scraped. After 10 min on ice, the scraped cells were centrifuged at 16,000 g for 3 min. The supernatant corresponded to the detergent-soluble fraction, and the pellet corresponded to the detergent-insoluble fraction. The pellet proteins were extracted in a RIPA buffer (50 mM TRIS, 150 mM NaCl, 1% Triton X-100, 1% deoxycholic acid, 2% SDS, 10% glycerol, 20 mM NaF, 2 mM Na_3VO_4, and protease inhibitors, pH 7.4). The protein content in the fractions was determined using the micro-BCA protein assay (Thermo Scientific Pierce, Rockford, IL, USA). Cytosol and cytoskeletal-associated protein fractions were subjected to SDS-PAGE and Western blot analysis. A monoclonal antibody against vinculin was used at a 1:3,000 dilution, and an antibody against actin was used at a 1:2,000 dilution. In order to analyze the differences in activated myosin, we measured phosphorylated MLC in GH_3 cells in the experimental condition mentioned above and used a polyclonal antibody at 1:3,000. MLC was extracted using the trichloroacetic acid/urea method [25]. However, in the experiments performed for the analysis of EGF receptor activation, the cells were cultured in the same manner, but 60 min previous to the EGF stimulation, cells were incubated in medium 199 plus 0.1% BSA followed by the addition of 50 ng/ml EGF. After 10 min, cells were lysed for Western blot analysis, and Western blots were probed for phosphorylated Erk 1/2 (1:3,000). Actin was used as a loading control for the samples.

Cell proliferation assay

Aliquots of 3×10^5 cells/well were seeded in a 24-multiwell plate. The bottom of each well was covered with a different ECM protein or polylysine. The cells were cultured in a defined medium for 48 h. At the end of the culture period, the medium with non-attached cells was discarded and the cells stimulated with 10%

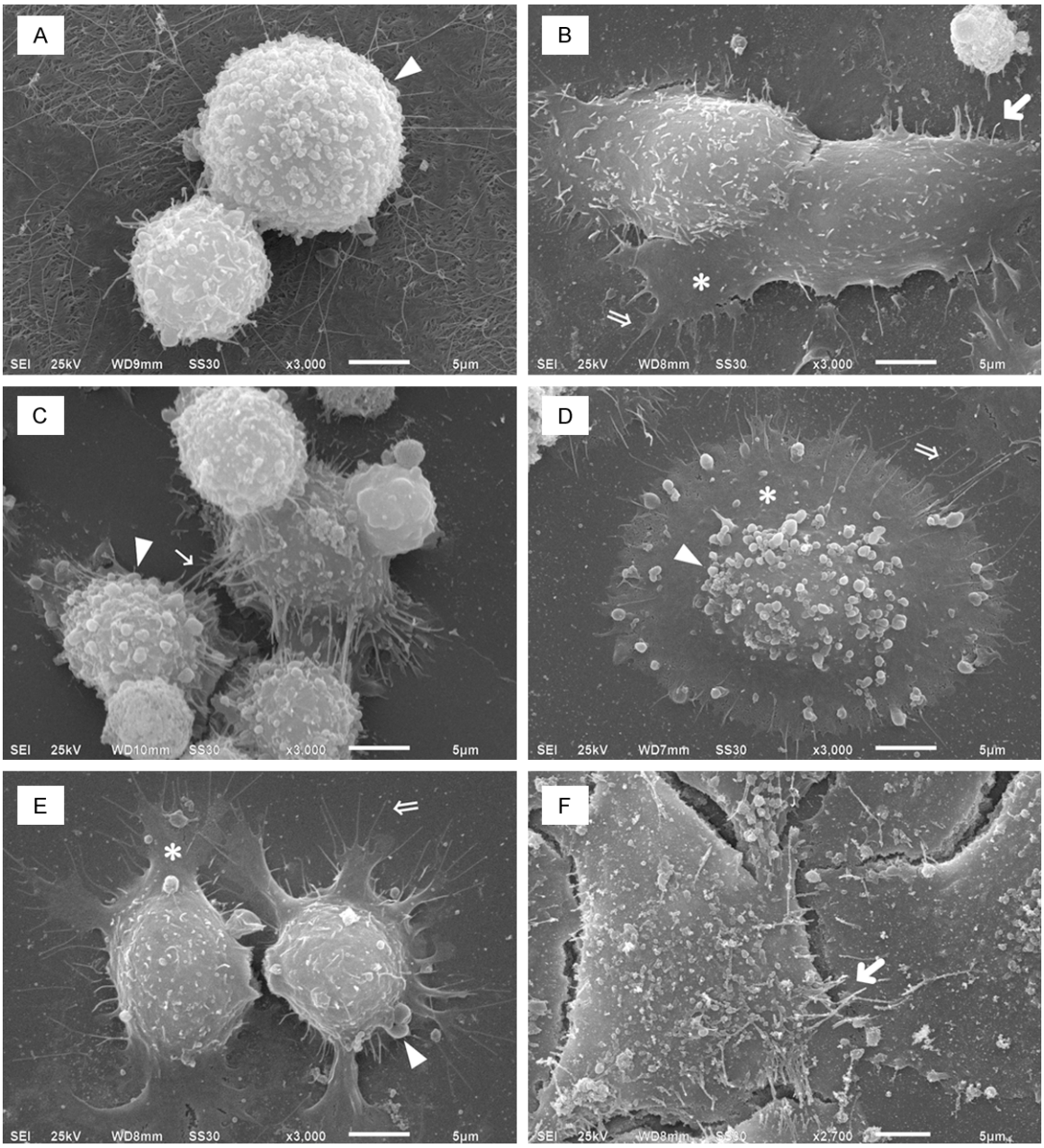

Figure 4. GH_3 cells shapes and membrane processes when cultured on different ECM proteins. Cells were cultured in DEF medium on C I/III (A), C IV (B), C/F (C), C/L (D), or P-L (E) and after 48-h were assayed for electron microscopy scanning. A group of cells on C I/III was cultured with a serum enriched medium (F). Depending in which ECM proteins GH_3 cells attached they develop different membrane organizations as blebs (arrowheads), membrane projections between (thin arrows), finger projections (thick arrows), filopodia (empty arrows) or lamellipodia (asterisk). Bar = 5 μm.

horse serum and 5% fetal calf serum for 24 h. Four hours before the stimulation period ended, 1 μCi/ml [^{3}H]-thymidine was added. The incorporated thymidine was obtained using the trichloroacetic acid method, and the samples were measured in a scintillation counter (LS 6500, Beckman Coulter, USA). An aliquot of the NaOH cell lysate was obtained for further protein quantification.

Radioimmunoassay (RIA)

GH_3 cells (1.5×10^6 cells/well) were seeded in a 24-multiwell plate previously covered with ECM proteins, as in the other experimental proto-

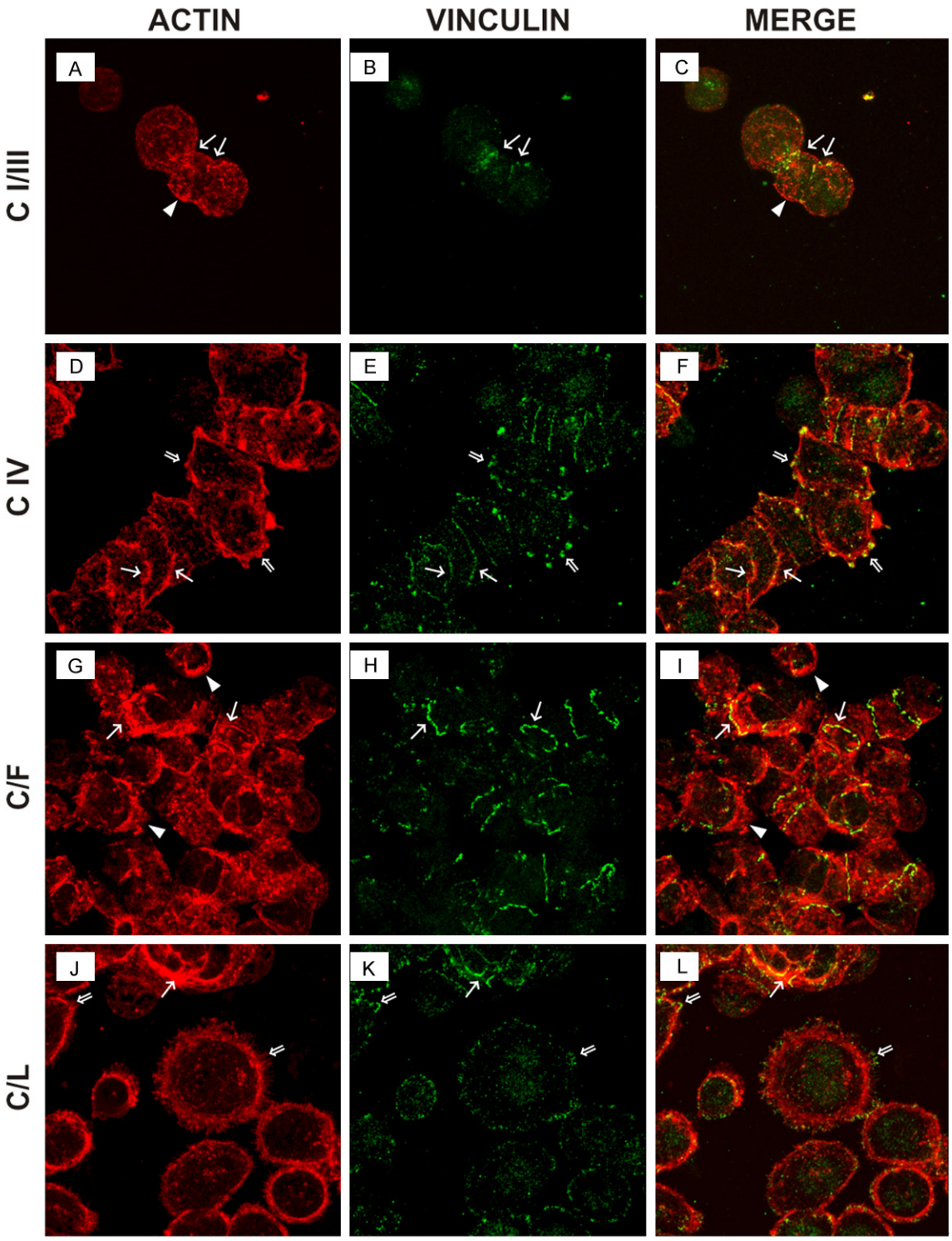

Figure 5. Actin cytoskeleton organization and vinculin distribution GH_3 cells cultured on different ECM proteins. Representative images of cells cultured on C I/III (A-C), C IV (D-F), C/F (G-I) or C/L (J-L) with DEF medium for a 48-h culture period. Cells were fixed, and labeled with a mono-clonal anti-vinculin and stained with a FITC-conjugated secondary antibody (green). Actin fibers were stained with TRITC-phalloidin (red). Cells exhibit actin fibers in cortical belt (arrowheads), and cell-cell boundaries (thin arrows) and adhesion complexes (empty arrows). Bar = 15 μm.

cols, and cultured in defined medium with or without 50 ng/ml EGF. After 24, 48 or 72 h, the medium from a set of cultures was obtained and harvested for further Prl quantification by

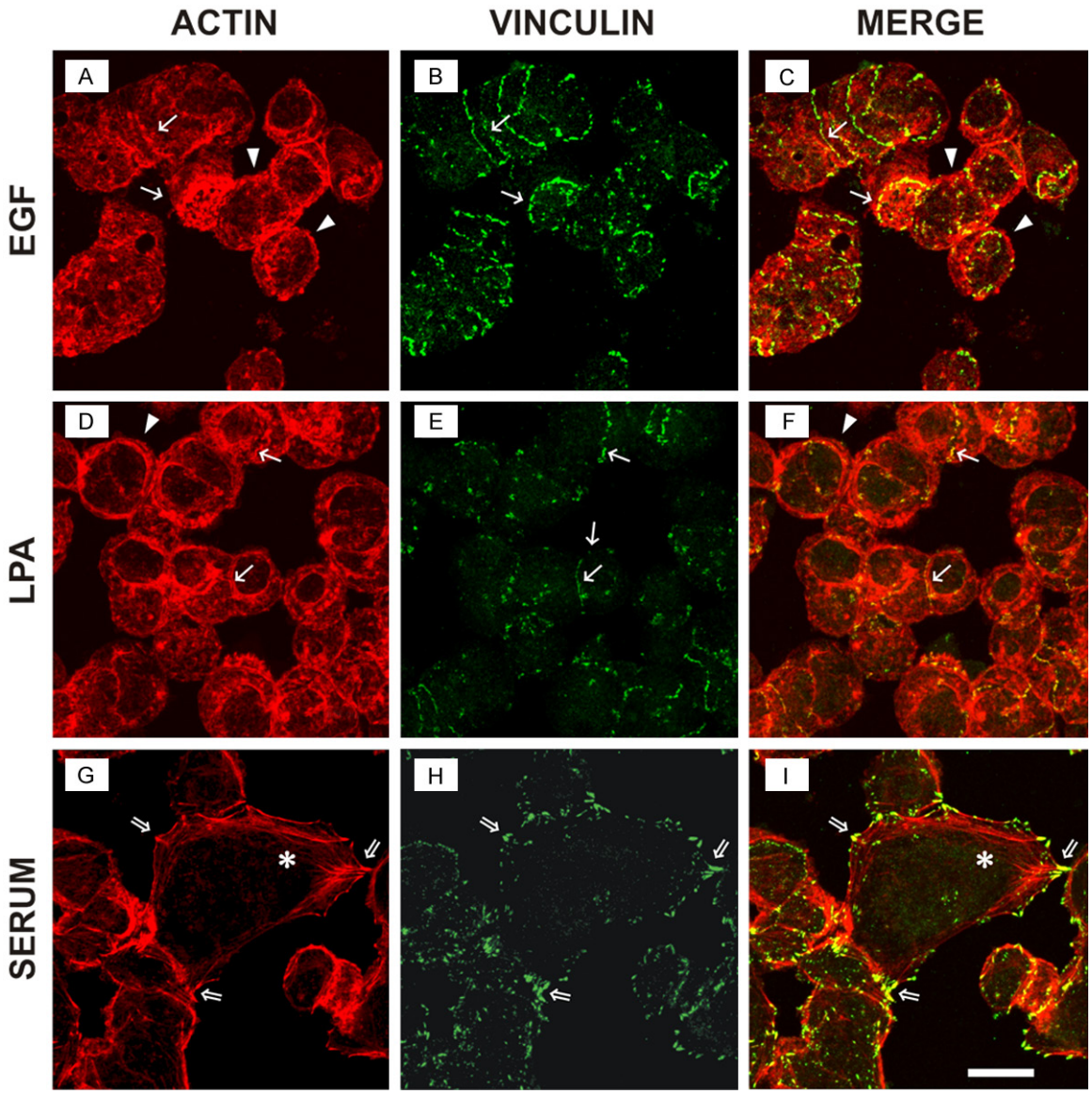

Figure 6. Actin cytoskeleton organization and vinculin distribution GH_3 cells cultured on C I/III and stimulated with EGF, LPA or serum. Representative images of cells cultured on C I/III and stimulated with 50 ng/ml EGF (A-C), 2 ng/ml LPA (D-F) or 15% horse serum and 2.5% fetal calf serum (G-I) during a 48-h culture period. Cells were fixed, and labeled with a mono-clonal anti-vinculin and stained with a FITC-conjugated secondary antibody (green). Actin fibers were stained with TRITC-phalloidin (red). Cells stimulated with EGF and LPA showed rings (arrowheads), and the cell-cell cell-cell complexes (thin arrows). Cells cultured with serum showed stress fibers (asterisk) and adhesion complexes (empty arrows). Bar = 15 μm.

RIA. The Prl concentration was determined by double-antibody RIA employing reagents using the protocol from the National Hormone & Peptide Program with intra- and inter-assay coefficients with variation of 3 and 5.3% respectively. Samples were measured in a gamma counter (Cobra II AUTO GAMMA, Packard, USA). In parallel, an MTT assay was performed in order to rectify the amount of Prl secreted for changes in cell number.

Data analysis

We analyzed data using ANOVA (two-tailed), and a Bartlett method was used to evaluate the differences in SDs in order to determine whether a parametric or non-parametric test was appropriate. Differences between groups with equal SDs were compared using a Tukey-Kramer multiple comparison test, and differences between groups with significantly differ-

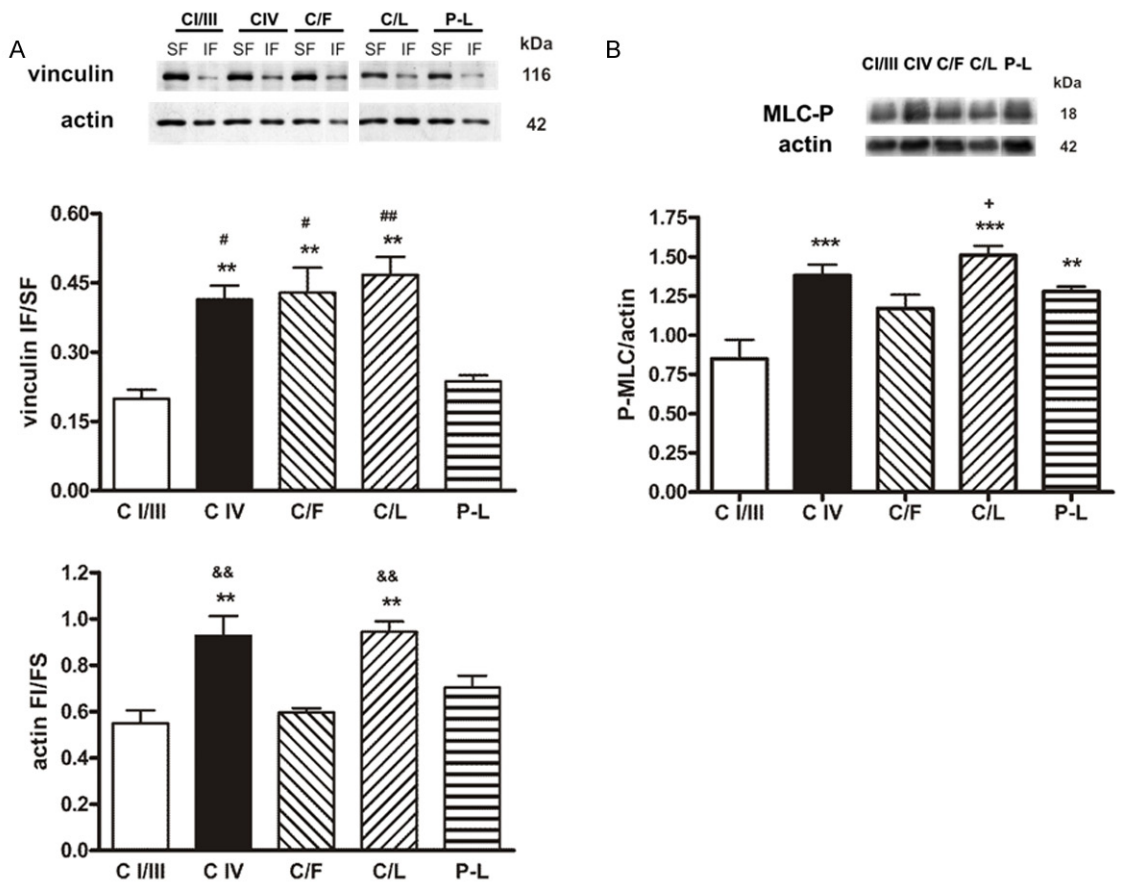

Figure 7. Actin filaments cross-linked, vinculin associated to cytoskeleton and activated MLC in GH_3 cells cultured on different ECM proteins. Cells were cultured on C I/III, C IV, C/F, C/L or P-L with DEF medium, and after 48-h were Triton extracted in order to obtain the cytoskeletal (IF) and cytosolic fractions (SF) (A), or were assayed with the trichloroacetic acid/urea method for MLC (B). The fractions were SDS-PAGE assayed and determined by immunoblot for vinculin, actin and phosphorylated-MLC with their respective antibodies, and the densitometry values were compared. (A) Data corresponded to the ratio between IF and SF of vinculin and actin present in each fraction. (B) Data of the ratio between phosphor-MLC and actin. $**p<0.01$, and $***p<0.001$ with respect to C I/III; $\#p<0.05$, and $\#\#p<0.01$ with respect to P-L; $\&p<0.05$, and $\&\&p<0.01$ with respect to C/F (ANOVA test followed by Tukey- Kramer multiple comparison test).

ent medians were analyzed using a Kruskal-Wallis test followed by a Dunn's multiple comparison test. The comparison between the DEF-C I/III group and the Ser-C I/III group was performed using an unpaired t-test and calculating the F-value in order to choose a parametric or non-parametric test. The level of significance was $p<0.05$ (GraphPadInstat 1.14, 1990, USA). We show data as the mean ± SEM of at least three independent experiments.

Results

Expression of integrin subunits in GH_3 cells

We investigated the expression of integrin sub-units in GH_3 cells, in order to know the possible integrin sub-unit combinatorial that function as receptors for the ECM proteins present in the pituitary tissue. **Figure 1A** shows immunofluorescence curves obtained for the different integrin sub-units. The cytofluorometric analysis of the α1, α2, α5, β1 and β4 integrin sub-units showed the presence of all these sub-units in GH_3 cells. It is interesting to note that GH_3 cells express high positivity of the following integrin sub-units: α2 and β4. According to this data, GH_3 cells express the receptors for the ECM proteins reported in pituitary tissue and used in this study: α1β1 and α2β1 recognize collagens types I/III and IV, α5β1 is a fibronectin receptor, and α6β4 (α6 not shown in this study, however is the partner of β4) is a laminin receptor. Taking into account that normal pituitary cells not express α2 integrin subunit (Figure S1) we

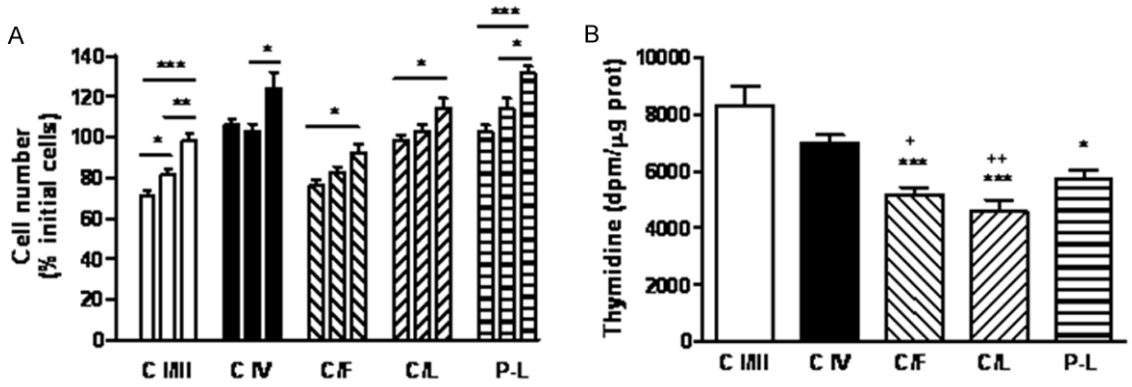

Figure 8. Cell survival and proliferation of GH_3 cells cultured on different ECM proteins. Cells were cultured on C I/III (filled bars), C IV (open bars), C/F (left dashed bars), C/L (right dashed bars) or P-L (horizontal dashed bars) with DEF medium during 24, 48 and 72-h. After the end of each culture period the amount of cells were obtained using the MTT assay. The initial amount of cells seeded were obtained after 4-h of seeding. Data on the amount of cells of each group at the three different culture periods is expressed as per cent of the amount of seeded cells (A). In other set of experiments, cells were cultured for 48-h and then assayed for Thymidine incorporation using the trichloroacetic acid method (B). In (A), statistical significance between the 3 culture periods of each group was: *p<0.05, **p<0.001, and ***p<0.001 (ANOVA test followed by Tukey-Kramer multiple comparison test). In (B), statistical significance between groups was: *p<0.05, and ***p<0.001 with respect to C I/III, +p<0.05, and ++p<0.01 with respect to C IV (Nonparametric ANOVA Kruskal-Wallis test followed by Dunn's multiple comparisons test).

stained for this integrin sub-unit GH_3 cells (**Figure 1B** and **1C**).

Effect of ECM proteins on GH_3 cell spreading, substrate adhesion and motility

Table 1 shows the cell surface area achieved by the cells after 24, 48 or 72 h of culture. Significant differences in surface area were observed for GH_3 cells cultured with DEF medium; a smaller surface area was observed in cells cultured over C I/III, C IV and C/F after 24 h. Cells on C IV after 48 h achieved a surface area significantly higher with respect to cells on C I/III and C/F. The larger surface area was observed in cells cultured over C/L at the three culture periods. Cells cultured with serum on C I/III at 24 h exhibited a similar median value, 82.0 μm^2 (67-99, 25% and 75% percentile respectively), than cells cultured with DEF medium on the same MEC protein at 24 h of culture. However, at 48 and 72 h cells cultured with serum exhibited the largest surface areas, 104 and 182 μm^2 respectively (25% and 75% percentage: 79-143.5 at 48 h and 137-244 at 72 h). Considering the different spreading capacity developed by GH_3 cells over the different ECM proteins we looked for their substrate adhesion capacity (**Figure 2**). GH_3 cells adhered to C I/III, C IV, C/F, C/L and P-L was obtained after 60 min (**Figure 2A**) and at longer periods of 24, 48 and 72 h (**Figure 2B**). At 60 min and 24 h, the least amount of cell attachment was observed in cells cultured over C I/III and C/F, followed by cells cultured over C IV and C/L. At 60 min, over P-L most of the cells were attached (93%). After 24 h, eleven per cent of cells cultured over C/L were detached. All the cells seeded on P-L were attached. At 48 h, a 15% increase in attached cells was observed in cells cultured over C IV, and no changes were observed in any of the other groups. At the final time point (72 h), a 21% increase in cell attachment was observed in the group of cells adhered to C/F. A set of images of a time lapse video were obtained from GH_3 cells cultured on different ECM proteins with DEF medium or enriched with serum. Immediately after seeding and during 4 h cells were recorded. All GH_3 cells cultured on C I/III, C IV, C/F and C/L in DEF medium looked round during the first 4 h of cultured (Data not shown). However, cells exhibited different displacement capacities and velocities. Cells cultured on C I/III or C/F shown a high motility with a high velocity, than cells over C IV and C/L. Interestingly, cells over C I/III moved longer distance, but with random movements, while cells over C/L evidenced a more uniform displacement, since the distance they traversed was obtained with less movements (**Table 2**).

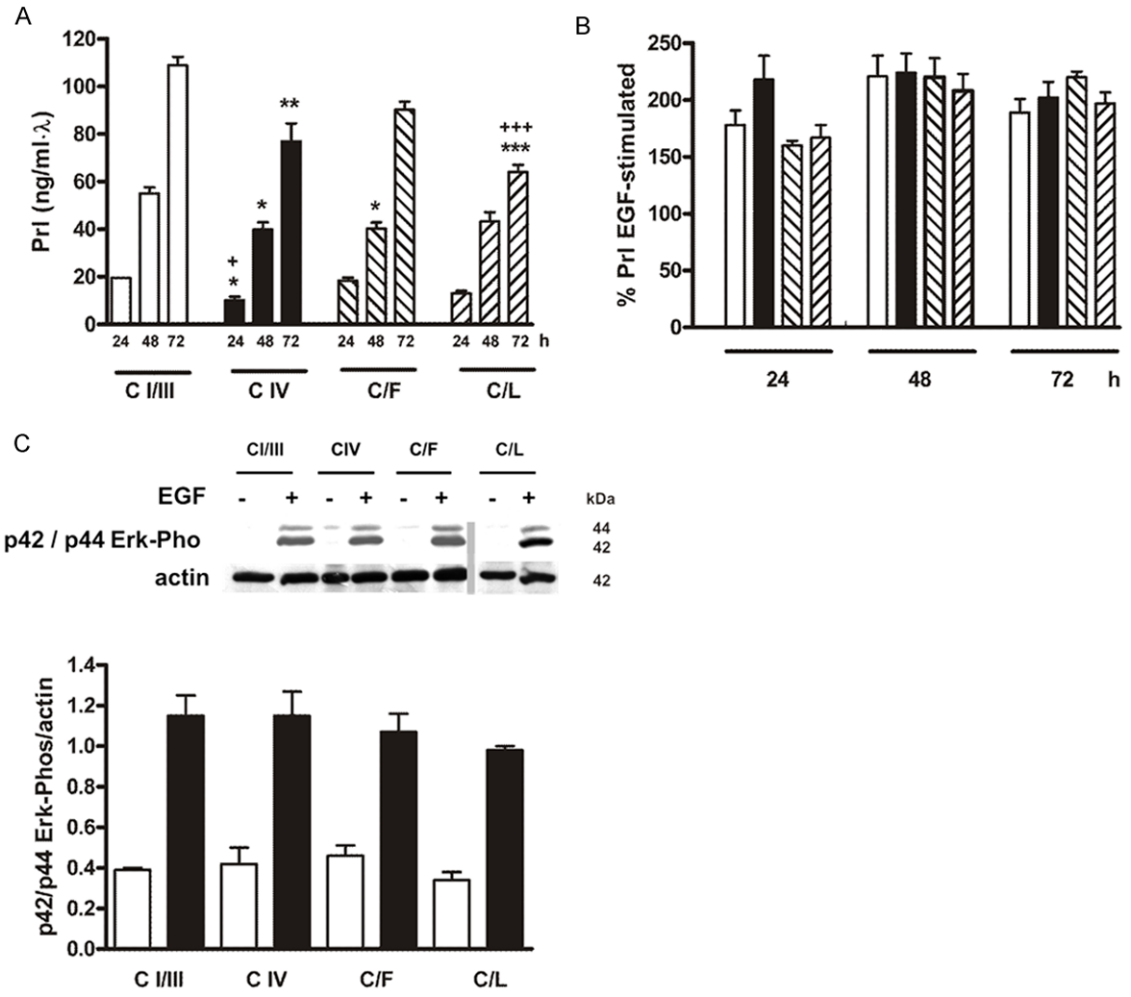

Figure 9. Prl secretion and EGF effect in GH_3 cells cultured on different ECM proteins. Cells were cultured on C I/III (open bars), C IV (filled bars), C/F (left dashed bars), or C/L (right dashed bars) with DEF medium during 24, 48 and 72-h. After the end of each period the medium was obtained for Prl quantification by RIA, and the cells were quantified using the MTT method. (A) The Prl concentration contained in the culture medium was obtained and normalized with respect to MTT data in each experimental group and each culture period. (B) Cells were stimulated with 50 ng/ml EGF throughout the culture periods and the medium was collected for Prl determination and the cell number were determined by the MTT method. Data is presented as the percentage increase in EGF stimulated Prl secretion with respect to the corresponding basal Prl secretion in each group and each culture period. (C) EGF effect in GH_3 cells cultured over different ECM proteins. EGF receptor activation was determined by the analysis of Erk activation in GH_3 cells cultured in different ECM proteins for a 48-h culture period. The cells were stimulated with 50 ng/ml EGF and after a 10 min period were lysed for further SDS-PAGE separation, and the protein levels of phosphor-Erk and actin were analyzed by immunoblotting with their respective antibodies. A densitometry analysis was performed for each group. Statistical differences between groups in basal Prl secretion were: *p<0.05, **p<0.01, and ***p<0.001 with respect to C I/III; +p<0.05, and +++p<0.001 with respect to C/F (for the 24-h period a Kruskal-Wallis nonparametric ANOVA test followed by a Dunn's multiple comparison test was performed, and for the 48 and 72-h periods an ANOVA test followed by a Tukey-Kramer multiple comparison test were performed).

Cell morphology and, F-actin and vinculin arrangement in GH_3 cells induced by ECM proteins

A variety of cell morphology patterns in GH_3 cells cultured on different ECM proteins for 48 h were observed, using conventional microscopy, and are displayed in **Figure 3**. Cells cultured on C I/III, C/F and P-L appeared rounded (**Figure 3A**, **3C** and **3E**, respectively), but cells cultured over fibronectin showed small membrane processes. Cells cultured on C IV appeared polyhedral or elongated with several membrane protrusions (**Figure 3B**). The presence of laminin induced a flattened, rounded shape or a polyhedral shape rich in membrane protrusions,

and cells cultured with serum exhibited a predominantly polyhedral shape (**Figure 3D** and **3F**, respectively). Moreover, the cells displayed important differences in cell membrane organization, visualized using a scanning electron microscope (**Figure 4**). The cell membranes of GH_3 cells cultured on C I/III appeared with small blebs, and cells cultured on the same collagen but enriched with fibronectin exhibited lamellipodia and membrane projections between cells (**Figure 4A** and **4C**, respectively). The rounded cells observed on P-L showed few blebs and thin membrane extensions, such as lamellipodia with filopodia (**Figure 4E**). Cells grown on C IV presented small, digit-form projections on planar membranes with filopodia (**Figure 4B**), while the presence of laminin induced extended lamellipodia all around the cell (a fried egg shape) with blebs and thin filopodia (**Figure 4D**). Cells cultured with FBS-enriched medium appeared as flattened cells with membrane-fine extensions that touched the neighbor cells (**Figure 4F**). GH_3 cells exhibited different F-actin organization in response to the different ECM substrates used (**Figure 5**), and this correlated with the shape observed with scanning microscopy (**Figure 4**). However, vinculin appeared diffusely distributed, with few defined arrangements at the membrane border and clearly defined arrangements at the cell-cell adhesion site. Cells cultured over C I/III exhibited discontinuous cortical actin belt and small plaques of F-actin present at the substrate adhesion level and vinculin present primarily at cell-cell interaction sites (**Figure 5A** and **5B**, respectively). A similar arrangement was observed when fibronectin was present, although well-defined rings of filaments were present (**Figure 5E**). Cells cultured over collagen type IV exhibited F-actin arranged in arcs with a continuous bundle of F-actin and small protrusions where concentrated vinculin was observed (**Figure 5C** and **5D**, respectively), while cells cultured over a laminin-enriched matrix exhibited small actin fibers all around the cell border with spots of vinculin (**Figure 5G** and **5H**, respectively). **Figure 6** shows cells grown on C I/III and stimulated with EGF and LPA exhibited a rounded shape, with actin fibers arranged very similarly to those observed in cells grown over C I/III without EGF stimulation, and the LPA to those in the C/F group (**Figure 6A** and **6C**, respectively). However, EGF cells exhibited an increase in vinculin localization at the cell-cell boundaries (**Figure 6B**). LPA induced a significant increase in the cell surface area and vinculin was arrange in spots (**Figure 6D**). When the cells grown over C I/III were cultured with a serum enriched media, they exhibited stress fibers and arcs, and the vinculin distribution resembled focal adhesions (**Figure 6E** and **6F**, respectively). We analyzed the fraction of vinculin and actin associated with the actin cytoskeleton in cells cultured over the different ECM proteins and observed that the organized actin filaments and vinculin associated to the actin cytoskeleton were significantly decreased in cells cultured over C I/III, C/F or P-L compared to the corresponding levels present in cells cultured over the other ECM proteins (**Figure 7A**); a higher fraction of vinculin associated with organized actin filaments was present in cells stimulated with FBS, 0.500 ± 0.009, actin fraction was 1.6 ± 0.3. Differences in actin filament tension were evaluated by analyzing the phosphorylation of MLC at 48 h (**Figure 7B**). The level of phosphor-MLC observed in cells cultured over C IV/L was higher than that of cells cultured over C IV, followed by those cultured over P-L and C/F; the phosphor-MLC levels were even lower in the presence of C I/III (**Figure 7B**). Cells cultured with serum displayed the highest level of phosphorylated MLC (2.3 ± 0.2; statistically different with respect to C I/III group of cells cultured with DEF medium $p<0.001$).

GH_3 cell proliferation in DEF medium induced by ECM proteins

We evaluated the capacity of ECM proteins to induce proliferation of GH_3 cells. In **Figure 8A** the number of living cells present after 24, 48, or 72 h in culture is expressed as a percentage of the initial number of cells seeded. After 24 h, a significant decrease in the number of living cells was observed in cells cultured over C I/III and C/F, but during successive periods, the cell numbers reached the level of the cell number seeded. The percentage increase in cell number at the 72 h time point for each condition was the following: 27% for C I/III, 20% for C IV, 16% for C/F and C/L, and 29% for P-L. The presence of FBS induced a 55% increase in cell number at 72 h. In another set of experiments, we measured the incorporation of thymidine (**Figure 8B**), and observed that cells cultured over C I/III exhibited a higher level of DNA synthesis and that cells cultured over C/L exhibited the lowest level of DNA synthesis.

GH_3 Prl secretion induced by ECM proteins and EGF-stimulated Prl secretion

We studied Prl secretion and the effect of EGF when cells were attached to different ECM proteins. **Figure 9A** shows the concentration of Prl secreted into the medium normalized by the number of living cells present in the culture. Lower basal levels of Prl secretion were observed during the first 24 h of culture in those cells cultured over C IV, whereas higher levels were observed in cells cultured over C I/III. Cells cultured over C I/III also secreted higher levels of Prl during the other two culture periods evaluated (23.7 ± 3.2% more than the other groups). At 72 h, the lowest Prl concentration present in the medium was observed for cells cultured over C/L. EGF-stimulated levels of Prl secretion were significantly higher in cells cultured over C I/III only during the first 24 h of culture. It is interesting to note that cells cultured over C IV showed the lowest level of basal Prl secretion during the three culture periods evaluated. Therefore, we measured differences in the percentage increase in EGF-stimulated Prl secretion with respect to basal secretion (**Figure 9B**). The mean percentage increase for all the groups, including cells cultured with serum, was 202.68 ± 7.95%, and a statistical difference ($p<0.05$) was only observed between C IV and C/F groups at 24 h (C IV: 245.15 ± 31.55% and C/F: 144.89 ± 15.20%). EGF activation of the MAPK pathway was studied by analyzing the phosphorylation of ERK 1/2, and there were no significant differences observed between any groups (**Figure 9C**).

Discussion

Taking into account the proteins present in the ECM in normal pituitary tissue, the following ECM proteins were chosen to be tested: types I/III and IV collagen, and the mixtures of C I/III with fibronectin and C IV with laminin [13, 17, 23]. Our results demonstrated that GH_3 cell cultured on these ECM proteins exhibited differences in various cell processes. A variety of cell morphologies with different types of cell membrane arrangements were observed in GH_3 cells, depending on the ECM protein used as the substrate. These differences in morphology were analyzed at the actin cytoskeleton level, and we observed a variety of actin filament structures with differences in the extent of tension, amounts of filaments involved, levels of vinculin anchored to them, and differences in cell adhesion capacity. Differences in cell shape were correlated with differences in cellular processes, such as cell adhesion, cell migration, cell secretion and cell proliferation [26]. Our data indicated that GH_3 cells responded to ECM components, changing the proliferation rate and Prl secretion. Considering that binding to different components of the ECM is known to be performed by receptors and that integrins are the most important receptor family, we decided to investigate which integrin sub-units are expressed in GH_3 cells. In a previous study, it was shown that GH_3 cells express α5β1 and α6β1 [27], and in the present study, we found that they also express α1, α2, and β4 integrin sub-units. It is interesting to note that normal rat pituitary cells do not express the α2 integrin sub-unit [23]; therefore, the expression of this sub-unit in GH_3 cells suggests that it is a result of the tumor phenotype of these cells. Considering the repertoire of integrin sub-units observed in these cells and the review of Hynes [5], integrin sub-units could assemble into heterodimers and be available as receptors for the ECM proteins tested in this study. Thus, it is important to consider that particular ECM components can bind to more than one integrin [28]. However, GH_3 cells were capable of differentiating between the various ECM proteins tested, eliciting different responses. Our results showed that GH_3 cells were spherical over C I/III and C/F, and elongated and flattened over C IV and C/L. The cell-substrate attachment of GH_3 cells differ according to the protein used as the substrate and correlates with the spreading of cells and the motility behavior: cells with a spherical shape were less adhesive and exhibited a high motility with random movements. Furthermore, the fraction of actin filaments and associated vinculin, which participates in the organized actin cytoskeleton of the cells over C I/III, correlates with an arrangement in discontinuous cortical actin and low myosin activation and small blebs at the membrane surface. As could be seen, cells on C/F were observed with a cytoskeleton organization as cells on C I/III, however more cells were attached together and a higher cytoskeletal vinculin concentration was obtained. Taking into account that vinculin is present at cell-cell and cell-substrate adhesions [29, 30]; we have to consider both vinculin cytoskeletal fractions showing that intercellular adhesion is stimulated by fibronectin. In

contrast, cells over C IV or C/L which looked more spread, showed vinculin and actin filaments arranged as focal adhesions. These cells exhibited a higher fraction of phosphorylated MLC and extended cell surface membrane. Furthermore, cells over C/L had a uniform displacement. The overall data showed that C I/III and C/F induce a behavior of random motility and low attachment, evidencing an amoeboid behavior, and C IV and C/L induces a more mesenchymal migration behavior [31]. Tumor cells can acquire: an amoeboid phenotype, in which cells have a rounded shape and exhibit a weak interaction with the substrate, or a mesenchymal phenotype exhibited by those cells with a fibroblast-like morphology and strong interaction with the substrate [31]. In a previous study, we observed different cytoskeleton organization of cultured human pituitary adenoma cells: discontinuous actin belts in rounded cells, in non-secretory invasive tumor cells, and small stress fibers in fibroblast like cells, in secretory invasive tumor cells [32]. Our data show that GH_3 cells assume either in response to MEC. The observed rounded shape and F-actin arranged in rings in cells cultured over C I/III and stimulated with EGF and LPA, corroborate the conclusion that filamentous collagens induce a specific pattern of cytoskeletal organization. Taking into account that GH_3 cells belong to a rat pituitary adenoma [33], and the acquisition of the tumor phenotype could change the expression of some proteins, thus, the finding that these cells express α2 integrin sub-unit acquires relevance. Horiguchi et al. [23], and in the present study, showed that normal rat pituitary cells not express α2 integrin suggesting that this integrin sub-unit could be engaged with the different response observed to C I/III in GH_3 cells. According to our results, the ECM dictates the way the GH_3 cell organizes its cytoskeleton and the shape that it assumes. Moreover, the proliferation and survival process is also modulated, as the present data demonstrates. The lowest level of survival after 24 h of culture was observed in cells cultured over C I/III; these cells also exhibited a decreased capacity for attachment, suggesting that cells that could not attach died. Notwithstanding, the highest DNA synthesis was observed in cells adhered to the C I/III substrate; these cultures exhibited a large increase in cell number; whereas cells adhered to the C/L substrate exhibited a lower increase in cell number and low thymidine incorporation. This data is in agreement with that of Kuchenbauer et al. [21]; they observed a decrease in laminin expression in prolactinomas and in GH_3 cells, suggesting a role for this ECM protein in tumor development. It was interesting that cells adhered to P-L had higher cell numbers without exhibiting significant changes in DNA synthesis. This result could be due to the high degree of cell attachment to the poly-lysine substrate, thereby overwhelming cell death triggers induced by detachment, as observed in the group of C I/III. The other cell process analyzed was prolactin secretion. Our results showed that the ECM also modulates the basal secretion of prolactin without significant changes in EGF cell-signaling activation. Our data showed that attachment to C I/III promote a higher Prl secretion than with the other ECM proteins. On the contrary, C IV, an ECM protein of the basal lamina, induces a low Prl secretion. Moreover, in agreement with Kuchenbauer et al. [21], we observed that the presence of laminin diminished Prl secretion; but, it was significantly after 72 h. These results suggest that basal lamina contributes to diminish the high basal rate of Prl secretion observed in lactotrope cells. One explanation could be the arrangement of the cortical belt and cellular membrane organization observed in cells on the different MEC proteins. A discontinuous belt with small blebs is observed in cells on C I/III, while C IV and C/L induced a well define bundle of cortical actin. Those membrane protrusions observed in cells on C I/III suggest that secretion is favorable in these cells. Studies of the role of cortical actin cytoskeleton showed that function as a dynamic physical barrier to regulated exocytosis [34]. Moreover, regulated exocytosis is accompanied by a focal and transient remodeling of the cortical actin network [35]. It is well know that blebs are membrane structures where a cyclic rupture and assembly of cortical F-actin is performed together with membrane protrusion [36]. With regard to the EGF response in prolactin secretion, it was similar in all groups and was correlated with the phosphorylated Erk levels. It is well known that the increase in prolactin secretion by EGF is accounted for by a rapid stimulation of prolactin gene transcription and synthesis [37]. Our data suggested that EGF receptor activity is not modified by the cytoskeletal arrangements observed in GH_3 cells, hence the intracellular

signals activated by EGF that stimulate Prl synthesis were not modified [38]. The variation of the secreted Prl by GH_3 cells attached to different ECM proteins suggests that the cytoskeletal organization regulates the exocytosis more than modulation of hormone synthesis. However, we suggest an ECM role in cell cycle progression because of the different proliferation rates observed with the different MEC proteins. It has been observed that mechanical forces performed by the structural network, e.g., the organized cytoskeleton linked to adhered complexes, can profoundly affect cell behaviors, such as growth, differentiation and apoptosis [3, 26, 39, 40]. Our results show that GH_3 cells acquired different actin cytoskeletal organization with differences in internal tension, observed as variations in phosphorylated-MLC levels, in response to specific ECM proteins. We conclude that the ECM proteins send information to the cells, which respond with a specific cytoskeletal organization that modulates specific cellular patterns of behavior. Finally, our data shows the ECM composition influence in the functionality of an endocrine cell and gives evidence of the possible ECM organization role in the pituitary as well as understand some differences observed in pituitary tumors. Our next challenge is to find the intracellular partners involved in the acquisition of different cytoskeleton patterns.

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Address correspondence to: Dr. María Eugenia Mendoza-Garrido, Department of Physiology, Biophysics and Neuroscience, CINVESTAV-IPN, Ave. IPN 2108, Col. San Pedro Zacatenco, México D.F. 07360, México. Tel: 52 (55) 5747-3800 Ext. 5122; Fax: 52 (55) 5747-3754; E-mail: erica.azorin@inin.gob.mx; piantaomx@yahoo.com.mx

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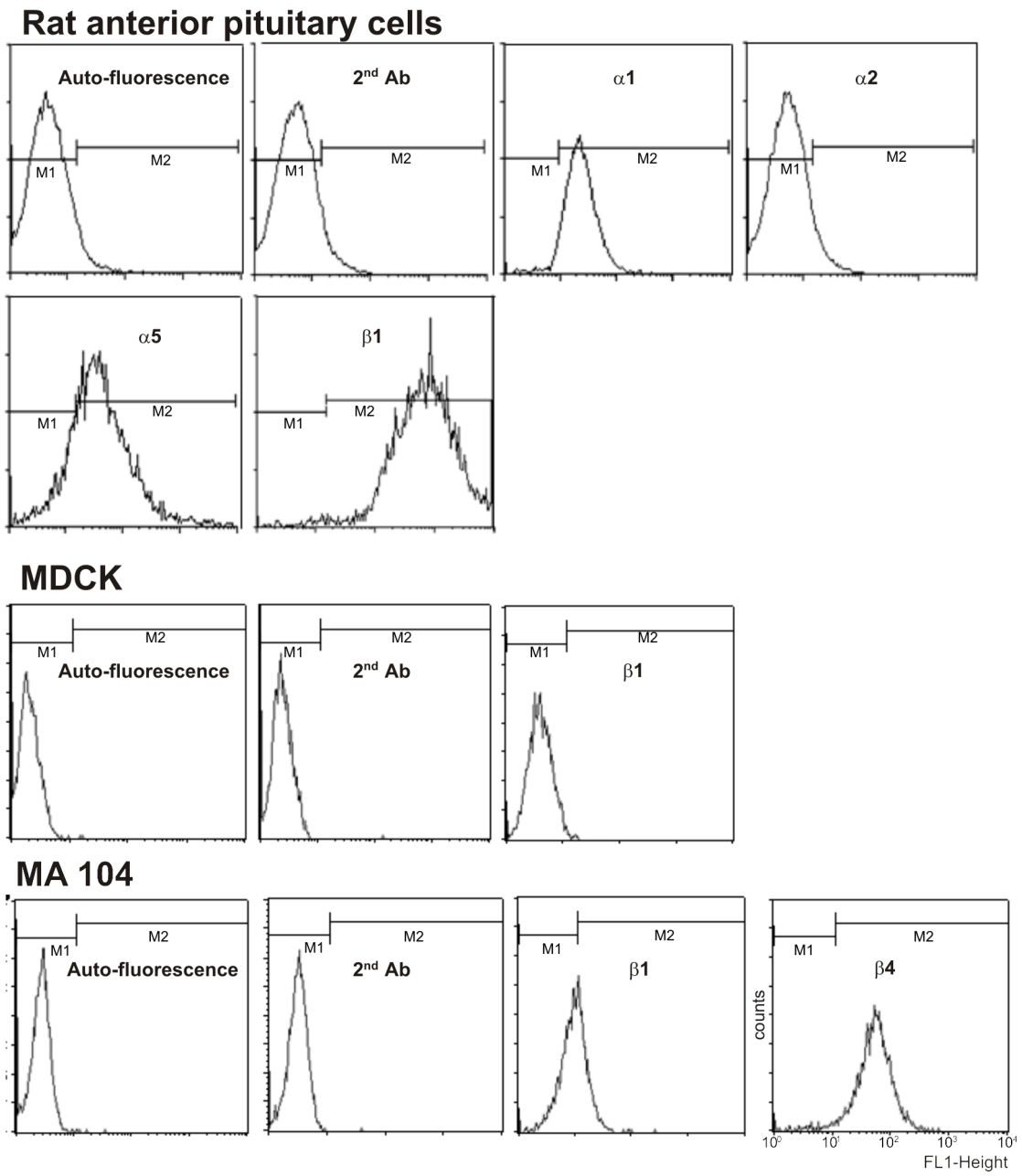

Figure S1. Integrin sub-unit expression in rat anterior pituitary cells and controls of the specificity of the antibodies used for immunofluorescence for integrin sub-units expression. Adult rat anterior pituitary cells, MDCK (j-l) and MA104 cells were assayed for immunocytochemistry for α1, α2, α5, β1 and β4, and further analyzed by flow cytometry. 10,000 cells per experimental condition were analyzed. Graphs correspond to representative single-parameter histograms obtained for each integrin subunit. Auto-fluorescence of pituitary cells, MDCK and MA104 cells and unspecific binding of secondary antibody of pituitary cells, MDCK and MA104 cells were obtained.