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Integrin-Mediated Endothelial Cell Adhesion and Activation of c-Src, EGFR and ErbB2 are Required for Endothelial-Mesenchymal Transition

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1. Introduction

The adhesion of cells to the extracellular matrix (ECM) is a critical requisite to generate cell shape, migration, proliferation, differentiation, and gene expression (Geiger & Yamada, 2011; Papusheva & Heisenberg, 2010). Beyond this role, cell-ECM adhesion promotes various intracellular signalling pathways and effectors modulating cell survival or apoptosis and tumor growth (Geiger & Yamada, 2011). Cells use a number of different cell surface receptors that mediate cell-ECM adhesion, being one of the best studied the integrins (Jean et al., 2011; Schwartz, 2010). It is known that binding of integrins to their ligands is dependent on the presence of divalent cations including Ca^{2+}, Mg^{2+}, and Mn^{2+} (Cierniewska-Cieslak et al., 2002; Cluzel et al., 2005; Leitinger et al., 2000; Luo & Springer, 2006). These heterodimeric transmembrane proteins which lack intrinsic enzymatic activity, in addition to being considered as indispensable to mediate cell-ECM adhesion, promote the assembly of cell-to-cell adhesion also promote receptor tyrosine kinase (RTK) activation triggering signalling cascades that regulate cell growth, proliferation and control cell death. Of note, growth factors that activate RTK may also modulate some of the cellular events that are mediated by integrins (Streuli & Akhtar, 2009). Now, it is clear that binding of certain integrins to their ligands as well as mechanical stimuli including shear stress, compression and tensile forces, promotes integrin clustering and that clustered integrins provoke recruitment and activation of other signalling molecules, including the RTKs, and therefore controlling cell survival, proliferation and migration (Guo & Giancotti, 2004; Ivaska and Heino, 2010; Streuli & Akhtar, 2009; Yamada & Even-Ram, 2002). Interestingly, functional cooperation between integrins and RTKs is actually considered as critical during normal vascular development and in vascular and inflammatory diseases (Eliceiri, 2001; Streuli & Akhtar, 2009) as well as in tumor progression and metastasis (Desgrosellier & Cheresh, 2010; Guo & Giancotti, 2004). In this context, studies have demonstrated that during cell-ECM adhesion some members of the epidermal growth factor (EGF) receptor family such as

EGFR/ErbB1 and ErbB2/Neu which has no known ligand, may be partially activated by association with particular integrins regulating important cellular functions including adhesion, proliferation, differentiation, survival, and migration in the absence of their ligands (Bill et al., 2004; Moro et al., 1998). These functions are mediated via upstream and downstream signalling pathways. However, the implications of the interactions between integrins and RTKs need further investigation.

Cell-ECM adhesion also involves various signalling intermediates including non-receptor tyrosine kinases (non-RTK) such as c-Src, focal adhesion kinase (FAK) and paxillin (Geiger & Yamada, 2011; Huveneers & Danen, 2010). c-Src may regulate not only increased cell growth and survival, but also it may promote cytoskeletal reorganization and decreased cell-ECM adhesion and cell-to-cell adhesion, facilitating cell spreading, migration, differentiation and transcription (Alper & Bowden, 2005; Guarino, 2010). Importantly, studies in diverse human cancer cells have revealed overexpression and /or overactivation of EGFRs and c-Src in the absence of ligands (Alper, & Bowden, 2005; Donepudi & Resh, 2008; Guarino, 2010; Marcotte et al., 2009). Also, increasing evidence indicates that in the absence of growth factors the activation of c-Src and tyrosine phosphorylation at 416-419 residues occurs by direct interaction with the cytoplasmic tail of integrins and that activated c-Src phosphorylates EGFR at Tyr845 residue and ErbB2 at Tyr877 residue (Bill et al., 2004; Cabodi et al., 2004; Desgrosellier & Cheresh, 2010; Guarino, 2010; Guo et al., 2006; Kim et al., 2005; Marcotte et al., 2009; Moro et al., 2002; Streuli & Akhtar, 2009). Interestingly, these patterns of phosphorylation in response to adhesion are different from those triggered by the binding of EGF (Bill et al., 2004; Cabodi et al., 2004; Moro et al., 2002). However, mechanisms involved in c-Src, EGFR and ErbB2 interactions as well as their implications have not being completely understood.

Growth factors can also indirectly influence integrin function by disrupting cell-to-cell contacts modulating cell-ECM interaction and therefore facilitating cell migration and invasion. Outstandingly, overstimulation signalling of growth factors or aberrant activation tyrosine kinases that lead to loss of epithelial apico-basal polarity and cell-to-cell contacts have been consistently reported during the epithelial mesenchymal transition (EMT) (Baum et al., 2008; Guarino, 2007). Nevertheless, the molecular, cellular, and mechanical aspects by which EGF and their receptors exert these actions remain to be elucidated. In the context of endothelial cells, there are reports showing that disruption of endothelial cell-cell contacts or adherens junctions (AJs) and changes in the reorganization of microtubules (MTs) and actin cytoskeleton may occur in response to mechanical injury, shear stress and / or cross talk of a variety of growth factors including transforming growth factor-β (TGFβ), insulin-like growth factor II (IGFII), and fibroblast growth factor-2 (FGF-2), and that such events are necessary in the transition of endothelial cells to a mesenchymal phenotype (EndoMT) (Arciniegas et al., 2007). However, how these factors and intracellular signals induce the endothelial transformation is still matter of debate (Arciniegas & Candelle, 2008). Recent evidence from studies on EGF and their receptors indicate that ErbB2, which has no known ligand, may form heterodimer with EGFR in response to ligands including EGF and TGF-α and that this heterodimer is essential during cardiovascular development and angiogenesis (Camenisch et al., 2002; Dreux et al., 2006; Fuller et al., 2008; Mukherjee et al., 2006; Negro et al., 2004). Moreover, expression of EGF, TGF-α and heparin binding- EGF (HB-EGF) as well as their respective receptors EGFR and ErbB2, have been detected in the intimal thickening

and medial smooth muscle cells (SMCs) of atherosclerotic lesions, whereas little or no presence of these molecules is observed in the cells of healthy vessels (Dreux et al., 2006). EndoMT is not only recognized as a phenomenon that occurs during cardiac fibrosis and intimal thickening formation observed in atherosclerosis and restenosis but also in heart and vascular development (Arciniegas et al., 2000; Mironov et al., 2005), pulmonary arterial hypertension (Arciniegas et al., 2007; Morrell et al., 2009; Sakao et al., 2010), cardiac (Goumans et al., 2008) and kidney fibrosis (Zeisberg et al., 2008), hyperthrophic scarring (Xi-Qiao et al., 2009), diabetic nephropathy (Li & Bertram, 2010), and during cancer progression (Potenta et al., 2008). However, there are no studies about the specific role of EGF signalling pathway in the EndoMT process.

In view of the above observations, in this study we examined the presence, organization, and spatial distribution of integrin β_3 in monolayers of primary embryonic aortic endothelial cells adhered to fibronectin (FN) and maintained in the presence of Ca^{2+} and in the absence of growth factors, considering that integrin $\alpha_v\beta_3$ is a receptor widely expressed on the apical surface of endothelial cells that binds proteins that are normally present in plasma, such as FN, vitronectin, fibrinogen and vWf (Bombeli et al., 1998; Soldi et al., 1999). Given that activation of c-Src, EGFR and ErbB2 may occur by association with integrins (Arias-Salgado et al., 2003; Bill et al., 2004; Cabodi et al., 2004; Desgrosellier & Cheresh, 2010; Ivaska & Heino, 2009; Huveneers & Danen, 2010; Streuli & Akhtar, 2009), we also investigated whether the activated forms of these molecules were present in these monolayers. Since some soluble growth factors can also indirectly influence integrin function by disrupting cell-to-cell contacts modulating cell-ECM interaction and facilitating cell migration and invasion events that are considered necessary in the progression of EndoMT (Arciniegas et al., 2007; Arciniegas & Candelle, 2008), we also investigated whether EGF and TGF-α as well as their activated receptors participate in the EndoMT process and if they were present in chicken embryo aortic wall during embryonic days 12-14 (days E12-E14) (stages 38 and 40) when intimal thickening is apparent and endothelial transformation occurs.

2. Materials and methods

2.1 Embryonic aortic explants

Fertilized chicken eggs (White leghorn) were obtained from local hatchery (Granja Avicola Agropollito, CA, Paracotos, Estado Miranda) and incubated at 37ºC and 60% humidity for 10-11 days (stages 36 and 37 of development). Embryos were staged according to Hamburger & Hamilton (1992). Aortae were dissected in Hank`s balanced salt solution without Ca^{2+} and Mg^{2+} (Sigma-Aldrich, St. Louis, MO) at 37ºC. For simplicity, this buffer is subsequently referred to as HBSS. Aortic segments, approximately 8mm² in surface area, were isolated (distal to the aortic arches) and opened along longitudinal axis. Explants were rinsed in HBSS and left in the same buffer 5-10 min before to initiate the assays.

2.1.1 Endothelial cell adhesion assays

To determine the effects of calcium (Ca^{2+}) on endothelial cell adhesion, 35mm Petri dishes (Nunclon, Delta, IL) were coated with HBSS containing plasma fibronectin (pFN) (25-50µg/ml) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and incubated at 37ºC for 2 hr in a humidified atmosphere consisiting of 5% CO_2, and 95% air. The dishes were then rinsed

twice with HBSS, blocked with 2% bovine serum albumin (BSA) (Sigma-Aldrich) in HBSS for 1 hr at 37ºC with 5% CO_2 and finally washed three times with the same buffer. After that, 300µl of HBSS containing 0.025% BSA (Sigma-Aldrich), 100µg/ml streptomycin, and 100U/ml penicillin (GIBCO, Invitrogen, Carlsbad, CA) were added to some dishes and incubated at 37ºC with 5% CO_2. Other dishes were incubated with 300µl of HBSS supplemented with either 2mM $CaCl_2$ (Ca^{2+}) (Sigma-Aldrich) or 2mM EGTA (Sigma-Aldrich) and incubated at 37ºC with 5% CO_2 for 1 hr. Aortic explants were placed with the endothelial apical surface down on coated dishes and allowed to adhere for 4 hr at 37ºC with 5% CO_2 . At the end of this time, 1ml of the corresponding HBSS was gently added to each dish. One hour later, the adhered explants were removed with the aid of a thin needle, leaving a monolayer of retracted endothelial cells that exhibited zones denuded of cells or wounds.

In order to emphasize the possible role of Ca^{2+} in the endothelial cell adhesion, some aortic explants were incubated on pFN-coated dishes containing either medium 199 containing 0.025% BSA (Sigma-Aldrich), 100µg/ml streptomycin, 100U/ml penicillin (GIBCO) (subsequently referred to as serum-free M199) (SFM199) or SFM199 containing 2mM EGTA (Sigma-Aldrich) and maintained as before.

Monolayers were examined with an inverted microscope (IX70 Olympus, Olympus America Inc., Melville, NY). Images were captured using an image editing capture and processing software program (Image Pro Plus, Media Cybernetics, Silver Spring, MD). Four independent experiments were performed and each one included at least 15 dishes.

2.1.2 Cell cultures

Cell cultures were initiated when the monolayers of endothelial cells adhered to the surface of the coated dishes were rinsed five times with HBSS and incubated with SFM199 for 2 hr at 37ºC with 5% CO_2 . Images were captured using the Image Pro Plus software program.

2.1.3 Effects of EGF and TGF-α on embryonic endothelial cells

Epidermal growth factor (EGF) and transforming growth factor-alpha (TGF-α) have been reported to stimulate cell migration, differentiation, and proliferation in presence or absence of serum (Ackland et al., 2003; Ellis et al., 2007). To determine whether EGF or TGF-α stimulates endothelial cell separation, detachment, and migration, the medium of some monolayers that had been maintained in SFM199 for 2 hr was switched to SFM199 supplemented with either recombinant human EGF (100ηg/ml) or recombinant human TGF-α (5-10ηg/ml) (R&D Systems, Inc., Minneapolis, MN) and low amount of chicken serum (ChS) (0.1%) (Sigma-Aldrich), using 10mM acetic acid as vehicle, and incubated at 37ºC with 5% CO_2 for an additional 6-8 hr period. During this period the images were captured each 2 hr using the Image Pro Plus software program.

2.1.4 Immunofluorescence

Fixed and permeabilized cells were processed for immunofluorescence as previously described (Arciniegas et al., 2005) using the following antibodies: a mouse monoclonal antibody (mab) anti-human integrin β_3 (GPIIIa, CD61) and a mab anti-chicken integrin β_1

(Millipore Chemicon Corporation, Billerica, CA), a mouse mab raised against full length β-catenin of chicken origin (clone 6F9) (Santa Cruz Biotechnology), a mouse mab raised against aminoacids 8-349 of mouse p120-catenin (clone 6H11) (Santa Cruz Biotechnology), a mouse mab raised against native chick brain microtubules (clone DM1A) (Santa Cruz Biotechnology), a mouse mab raised against a phospho-peptide corresponding to amino acids 416-422 of human c-Src (clone 9A6) (Santa Cruz Biotechnology), and a rabbit polyclonal antibody (pab) phospho-FAK (Tyr397) (Sigma-Aldrich). Negative controls were performed by omitting the primary antibody incubation step or by using non immune serum in place of primary antibody. Immunofluorescence images were captured on an inverted microscope confocal laser scanning microscope (CLSM) (Eclipse TE-300 Nikon) (Nikon Instruments Inc., Melville, NY) equipped with a Nikon objective Plan-Apo BC x60, 1.2 wi coupled to a C1-LU2 unit Argon cooled air (488 ηm) laser. This laser unit was controlled by a D-eclipse C1 interface. Other immunofluorescence images were captured on a IX81 Olympus inverted microscope with the Fluo-View confocal laser scanning configuration (CLSM) (Olympus America). Fluorescence intensity was measured by using a processing software program (FV10.ASW version 02.01.01.04, Olympus America).

2.1.5 Immunoperoxidase

Fixed cells were processed for immunoperoxidase as described previously (Arciniegas et al., 2005) using a rabbit pab raised against a short amino acid sequence containing phosphorylated Tyr845 of EGFR of human origin, and a rabbit pab raised against a short amino acid sequence containing phosphorylated Tyr877 of ErbB2/Neu of human origin (Santa Cruz Biotechnology). The images were captured using the Image Pro Plus software program.

2.1.6 Flow cytometry analysis of phospho-EGFR (Tyr845) and phospho-ErbB2 (Tyr877) expression in embryonic endothelial cells

We evaluated by flow cytometry the expression of phospho-EGFR (Tyr845) and phospho-ErbB2 (Tyr877) in monolayers of endothelial cells that were maintained in SFM199 for 2 hr and monolayers whose medium was switched to SFM199 supplemented with either rhEGF (100ηg/ml) or rhTGF-α (5-10ηg/ml) (R&D Systems) and low-ChS (0.1%) (Sigma-Aldrich) and incubated for an additional 6-8 period. Briefly, supernatants from cultures were removed, and the cells were harvested and incubated in permeabilizing buffer. After incubation, the cells were resuspended in PBS-0.1% BSA containing a rabbit pab anti-human phospho-EGFR (Tyr845) or a rabbit pab anti-human phospho-ErbB2 (Tyr877) (Santa Cruz Biotechnology). Negative control staining reactions were performed by incubating the cells with nonimmune rabbit Ig. After two washes with PBS, the cells were fixed with 1% paraformaldehyde in PBS. Data collection and analysis of the fluorescence intensities were carried out using a FACScalibur (Becton-Dickinson, San Jose, CA). Ten thousand events were acquired (excluding cell debris) and analyzed using the CELLQuest software program.

2.1.7 Tissue extraction

The aortae were dissected from 12-and 14-day-old embryos (stages 38 and 40 of development). The excised aortae were placed in HBSS (Sigma-Aldrich), and fixed for 20

min at room temperature with 4% formaldehyde prepared from paraformaldehyde (Sigma-Aldrich) in PBS. The aortae were dehydrated in graded ethanol and embedded in paraffin. Paraffin sections (5µm thick) were mounted on silanized slides (Dako North America, Inc., Carpinteria, CA). A total of nine aortae for each stage obtained from four different lots of fertilized chicken eggs, were processed.

2.1.8 Immunoperoxidase

For histological detection of EGF, TGF-α, EGFR and ErbB2/Neu the following antibodies were used on deparaffinized sections: a neutralizing antibody anti-human EGF and anti-TGF-α, both produced in goat (R&D Systems), a rabbit pab raised against a peptide mapping at the C-terminus of EGFR of human origin, and a rabbit pab raised against a peptide mapping at C-terminus of Neu of human origin (Santa Cruz Biotechnology). Negative controls were produced by the use of purified normal serum or PBS in place of primary antibody. The images were captured using the Image pro Plus software program.

3. Results

In order to determine the effect of Ca^{2+} on adhesion of embryonic endothelial cells to FN aortic explants from days E10 and E11 (stages 36 and 37) were placed with the endothelial apical surface down on dishes coated with pFN and containing HBSS alone or HBSS supplemented with 2mM Ca^{2+} and incubated for 5 hours.

Once the explants were removed, monolayers of endothelial cells that were transiently mechanically altered during the explant removal and therefore exhibited zones denuded of cells or wounds, were found adhered to the surface of the pFN-coated dishes that contained HBSS supplemented with Ca^{2+} (Fig. 1b), but not on the dishes that contained HBSS alone (Fig. 1a).

To emphasize the possible role of Ca^{2+} in the endothelial cell adhesion, aortic explants were incubated on pFN-coated dishes containing SFM199 or SFM199 containing 2mM EGTA to chelate extracellular Ca^{2+}. Like other culture medium, M199 is known for containing Ca^{2+} and relatively low levels of Mg^{2+}. When the explants were removed, monolayers of retracted endothelial cells exhibiting zones that were denuded of cells or wounds were found adhered to the surface of the pFN-coated dishes that contained SFM199 (Fig. 1c), but not on dishes that contained SFM199 and EGTA (Fig. 1d). When the endothelial cell monolayers were maintained in the presence SFM199 for 2-3 hr and examined, we observed that they adopted a contact inhibited cobblestone-like appearance of polygonal cells. Under this culture condition neither migrating cells from the wounded edges nor spreading, separating, detaching nor migrating cells from edges of the monolayer were observed (Figs. 2a, b). However, when, after this interval, the medium of some cultures was switched to SFM199 containing EGF or TGF-α and low-ChS and maintained for an additional 6-8 hr period, the polygonal cells from along wounds edges appeared to move toward each other extending lamellipodia into denuded area, whereas the cells located at the marginal edges of the monolayer appear to lose its cobblestone appearance, spreading, separating, detaching and migrating toward cell-free areas (Figs. 2c,d).

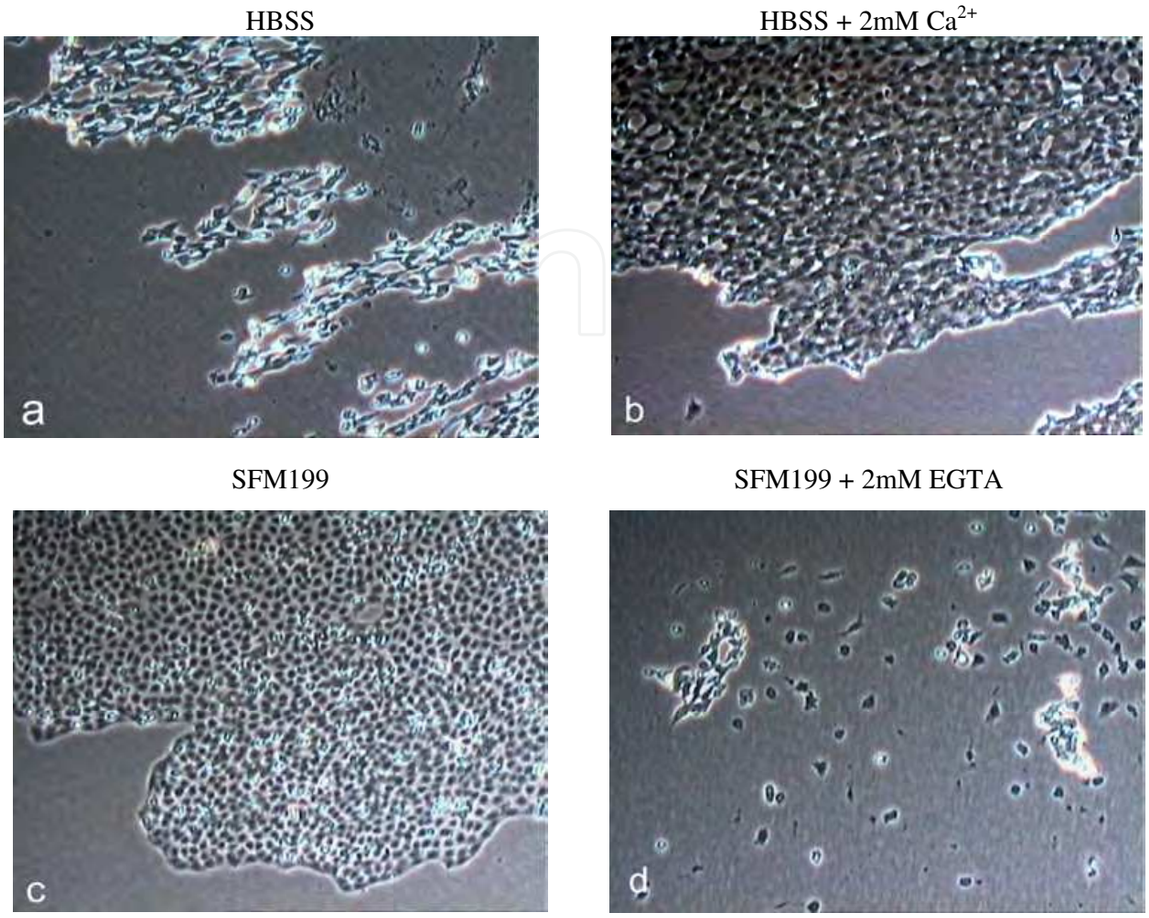

Fig. 1. Phase-contrast images captured when the aortic explants that had been incubated on pFN-coated dishes containing a) HBSS alone, b) HBSS supplemented with 2mM Ca^{2+}, c) SFM199 alone, and d) SFM199 supplemented with 2mM EGTA were removed after 5 hr. Note that only when the explants were incubated with HBSS supplemented with Ca^{2+} and with SFM199 alone monolayers of endothelial cells were found adhered to the surface of the dish after removal. Scale bar: 150μm.

Immunofluorescence analyzed by confocal microscopy determined that in the SFM199 condition in which the endothelial cells displayed a cobblestone appearance and no spreading, separating, detaching, migrating cells were observed, β-catenin and p120 catenin, proteins considered as regulators of VE-cadherin function, were localized at cell-cell contacts or AJs with MTs emanating from the microtubule organizing center (MTOC), organized radially and some of them oriented perpendicular to the cell edge probably interacting with sites of cell-cell contacts (Akhmanova et al., 2009) (Figs. 3a-c). Whereas in the EGF or TGF-α and low-ChS condition where spreading, separating, detaching and migrating cells are observed, the distribution of β-catenin and p120-catenin was disrupted at the cell-cell junctions and the organization of MTs was altered (Figs. 3d-f). Specifically, MTs were found oriented parallel to the long axis of the cell and the MTOC on the side of the cell facing the leading edge (Fig. 3f). Interestingly, β-catenin was also detected in the nucleus of

some separating, detaching and migrating cells (Fig. 3d). This location indicates that translocation of β-catenin from plasma membranes to the nucleus may also occur in response to EGF or TGF-α and low-ChS**.** These observations indicate that the presence of Ca^{2+} in the absence of growth factors would represent an important requirement to promote the adhesion and the eventual organization of explanted embryonic endothelial cells into monolayers forming AJs, whereas the addition of EGF or TGF-α and ChS may lead to the loss of cell-cell contacts, detachment, and cell migration; in other words, to the loss of endothelial cell polarity.

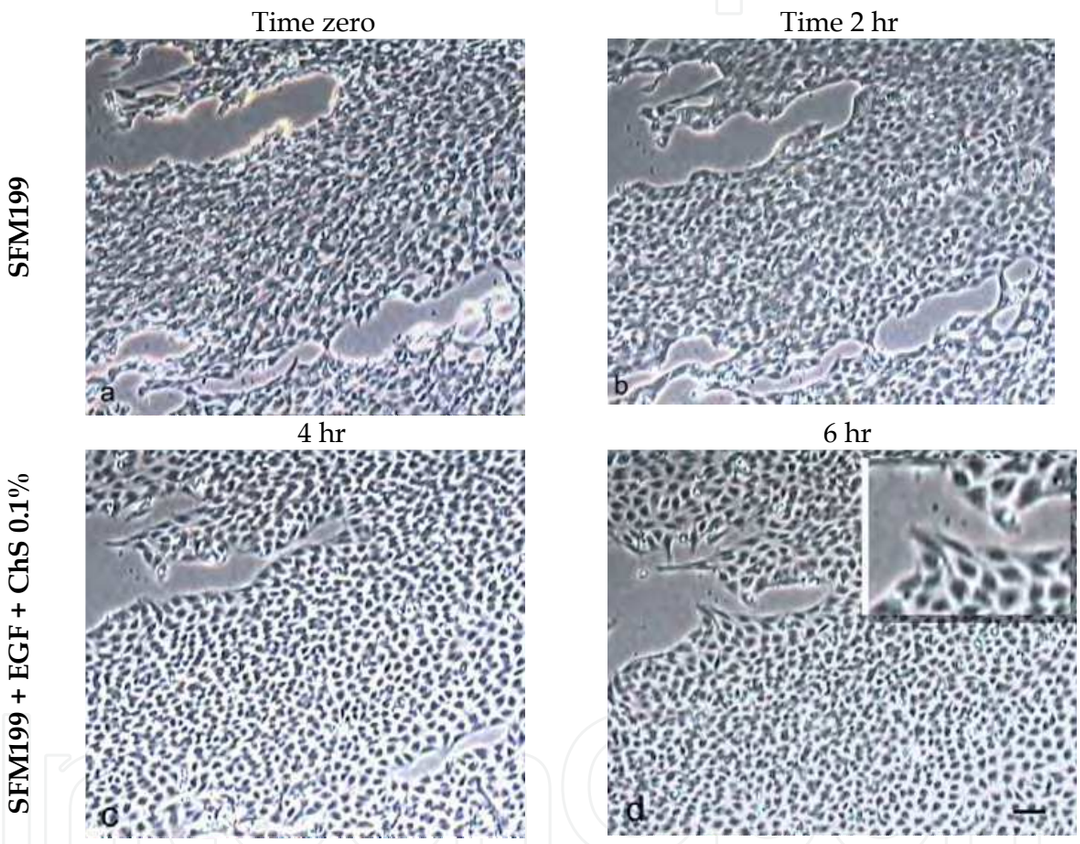

Fig. 2. Serie of phase-contrast images of a monolayer of endothelial cells adhered to the surface of the pFN-coated dish maintained in SFM199 and switched to SFM199 supplemented with EGF and low-ChS. a) time zero, shows a monolayer of retracted endothelial exhibiting zones denuded of cells or wounds after removing the explant. b) Time 2hr. Cells adopted a cobblestone appearance where neither spreading, separating, detaching nor migrating cells from the edges are observed. c) Time 4hr. cell spreading, separating detaching and migration are observed when the medium of the same culture was switched and maintained for additional 2hr (4hr). d) Time 6hr. Cells from the wound moving toward each other extending lamellipodia into denuded area. Detail of "d" showing migrating cells. Scale bar: 150µm; inset 40µm.

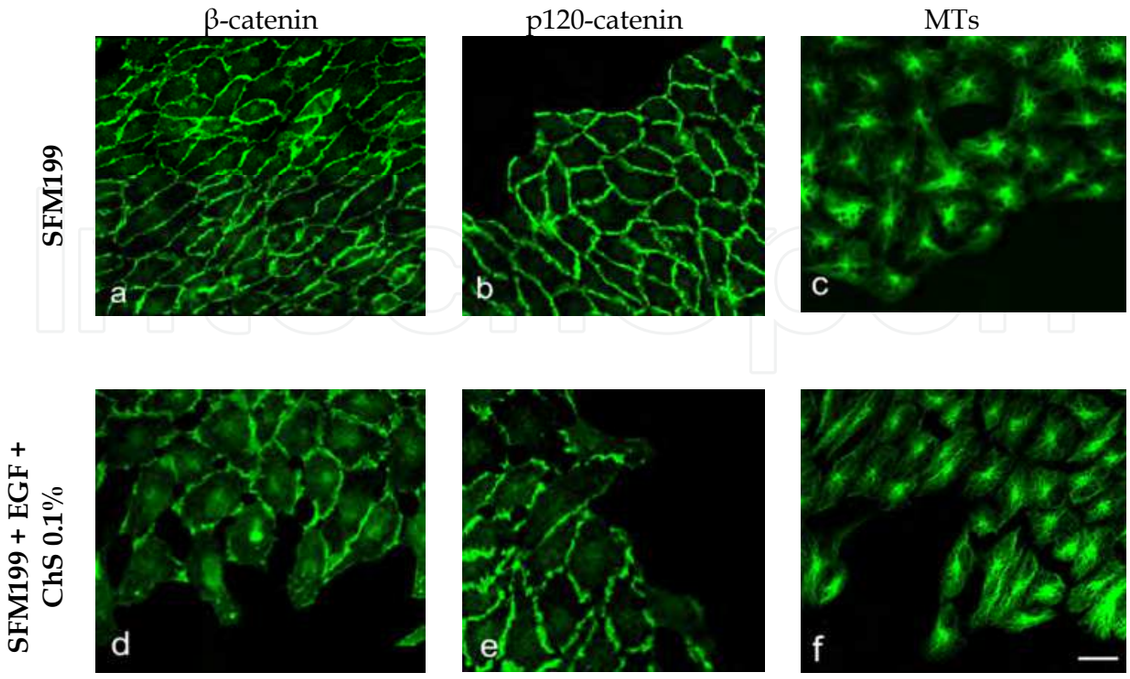

Fig. 3. Representative CLSM fluorescence images of β-catenin, p120-catenin, and MTs localization in monolayers of endothelial cells adhered to pFN.
(a-c) Immunolocalization after 2hr in culture in SFM199. (d-f) Immunolocalization in monolayers whose medium was switched to medium containing EGF and low-ChS and maintained for additional 4hr (6hr). In this condition where spreading, separating, detaching and migrating cells are observed, the distribution of β- and p120-catenins is disrupted in the cell-cell junctions and the organization of MTs is altered. Note that β-catenin is also detected in the nucleus and cytoplasm of some cells.
a), b), c), f) Scale bar = 30μm; d), e) scale bar = 25μm.

3.1 Endothelial cell adhesion to FN in the presence of Ca^{2+} and in the absence of serum or growth factors is mediated by integrin β_3, and addition of EGF or TGF-α produces changes in the distribution and organization of integrin β_3

As the cell-ECM adhesion in the presence of divalent cations such as Ca^{2+} and Mg^{2+} is actually correlated with the activation, organization and spatial distribution of particular integrins in normal and tumor cells (Cierniewska-Cieslak et al., 2002; Cluzel et al., 2005; Leitinger et al., 2000; Luo & Springer, 2006) and as integrin $\alpha_v\beta_3$ is a receptor widely expressed on the apical surface of endothelial cells that binds proteins that are normally present in plasma, such as pFN, vitronectin, fibrinogen and vWf (Bombeli et al., 1998; Soldi et al., 1999), we then examined the presence, organization and distribution of integrin β_3 by immunofluorescence staining in monolayers of retracted endothelial cells that were found adhered to pFN after removal of the explants and incubated with SFM199 for 2-3 hr as well as in those whose medium was switched to medium containing EGF or TGF-α and low-ChS and maintained for an additional 6-8 hr period.

Examination of the monolayers maintained in SFM199 revealed that integrin β_3 , in addition to be localized on the apical surface, also appeared distributed delineating the margins of the cells that had adopted a cobblestone appearance (Fig. 4a). When the medium was switched to medium containing EGF or TGF-α and low-ChS the location and distribution of integrin β_3 appeared altered (Fig. 4b). Specifically, this receptor was found delineating the margin of some cells and organized into linear streaks and a punctuate pattern typical of focal adhesions in the leading edge of the cells that were spreading, separating, detaching and migrating (Fig. 4b).

These observations suggest that the adhesion to Fn and eventual organization of embryonic endothelial cells in the presence of Ca^{2+} and in the absence of serum or growth factors may be mediated by integrin β_3 and that the presence of EGF or TGF-α and low-ChS would produce changes in the spatial distribution and organization of this receptor.

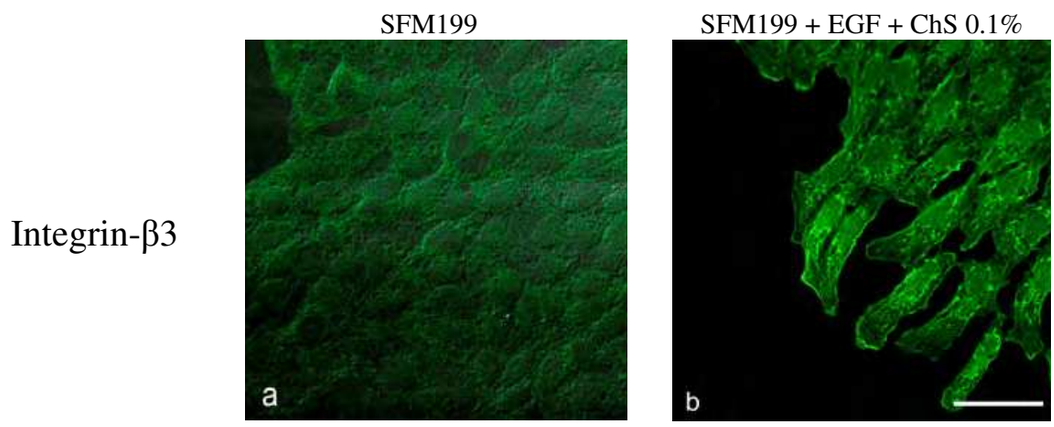

Fig. 4. a) Overlay of transmission and green fluorescence images of integrin β_3 in a monolayer of endothelial cells adhered to pFN after 2 hr in culture in the presence of SFM199. Integrin β_3 is seen on the apical surface and delineating the margin of many cells that displayed a cobblestone appearance. b) CLSM fluorescence image of integrin β_3 in a monolayer whose medium was switched to medium containing EGF and low-ChS. Integrin β_3 is seen delineating the margin of some cells and organized into linear streaks and a punctuate pattern at the leading edge of the migrating cells. Scale bar: 50µm.

3.1.1 Immunolocalization of integrin β_1 and phospho-FAK in vitro

Focal adhesions are considered not only as structural and dynamics links between the ECM and the actin cytoskeleton controlling cell shape, spreading, and migration but also as sites for signal transduction through integrins such as integrin β_1 and β_3, and adaptor proteins such as FAK and paxillin that are present in these sites (Geiger & Yamada, 2011; Gu et al., 2011; Mitra et al., 2005). We therefore examined the presence and organization and distribution of integrin β_1 and FAK when the medium was switched to medium containing EGF and low-ChS. Immunolocalization with anti-integrin β_1 and anti-phospho-FAK (Tyr397) revealed that both proteins appeared delineating the margin of some cells and organized into linear streaks typical of focal adhesions at leading edge of the cells that were separating, detaching and migrating (Figs. 5a,b).

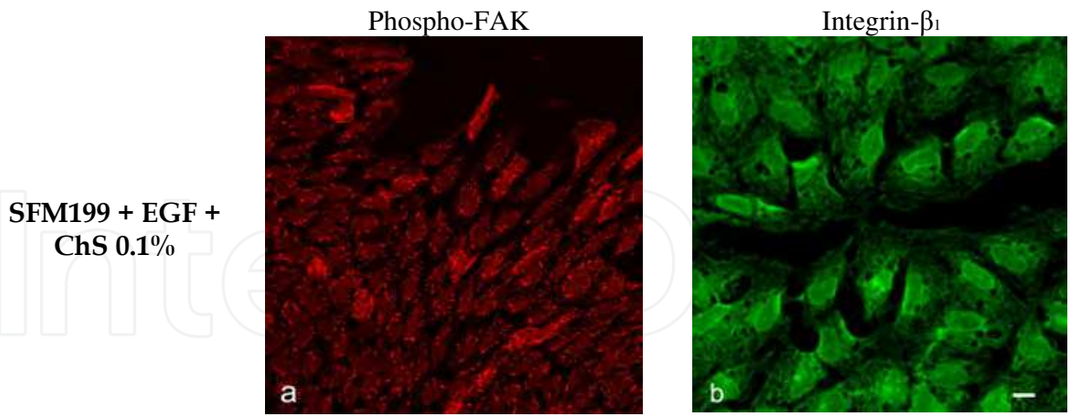

Fig. 5. Representative CLSM fluorescence images of integrin β_1 and phospho-FAK in monolayers of endothelial cells adhered to pFN after 8 hr in culture in the presence of EGF and low-ChS.
a) Integrin β_1 and b) phospho-FAK are seen delineating the margin of some cells and organized into arrays typical of focal adhesions at the leading of the migrating cells. a) Scale bar: 5µm; b) scale bar 10µm.

3.1.2 Partial activation of EGFR/ErbB1 and ErbB2/Neu mediated by integrin β_3 is increased by addition of EGF or TGF-α and low-ChS

Increasing evidence suggest that the adhesion mediated by integrins can promote the recruitment and partial activation of certain RTKs including EGFR and ErbB2/Neu in the absence of serum or growth factors after cell-matrix adhesion (Desgrosellier & Cheresh, 2010; Ivaska & Heino, 2010; Streuli & Akhtar, 2009; Yamada & Even-Ram,2002); therefore, we also investigated whether activated EGFR and ErbB2 were present in monolayers of embryonic endothelial cells adhered to pFN-coated dishes that were incubated in SFM199 for 2-3 hr and switched to SFM199 supplemented with EGF or TGF-α and low-ChS and maintained for an additional 6-8 hr period. Under these conditions the expression of both activated EGFR and ErbB2 was investigated by immunoperoxidase staining using anti-phospho-EGFR (Tyr845) and anti-phospho-ErbB2 (Tyr877). In the SFM199 condition, phospho-EGFR and phospho-ErbB2 staining was mostly localized in sites of cell-cell contacts. In addition, perinuclear and nuclear staining for these receptors suggestive of activation, internalization and nuclear translocation was observed in some cells of the monolayer that had adopted a cobblestone appearance (Figs. 6a,b). Remarkably, in the EGF or TGF-α and low-ChS condition, strong perinuclear and nuclear staining for phospho-EGFR was detected in many cells of the monolayer, as well as in many spreading, separating, detaching and migrating cells; whereas in the cell-cell contacts the staining appears disrupted (Fig. 6c). For phospho-ErbB2, less intense nuclear staining was found in many endothelial cells of the monolayer and spreading, separating, detaching and migrating cells. In this condition the staining for ErbB2 in the sites of cell-cell contacts also appeared disrupted (Fig. 6d). No immunolabeling was observed when a non immune serum was used as negative control and when the primary antibody was omitted (not shown).

Phospho-EGFR (Tyr845) and phospho-ErbB2 (Tyr877) expression was confirmed by flow cytometry in some cultures (Fig. 7). This technique revealed a relatively elevated expression of phospho-EGFR and phospho-ErbB2 in the presence of EGF or TGF-α and low-ChS, in contrast to that detected in SFM199.

Taken together, these observations would suggest that the partial phosphorylation or activation of EGFR and ErbB2 mediated by integrin β_3 can be increased by addition of EGF or TGF-α and low-ChS.

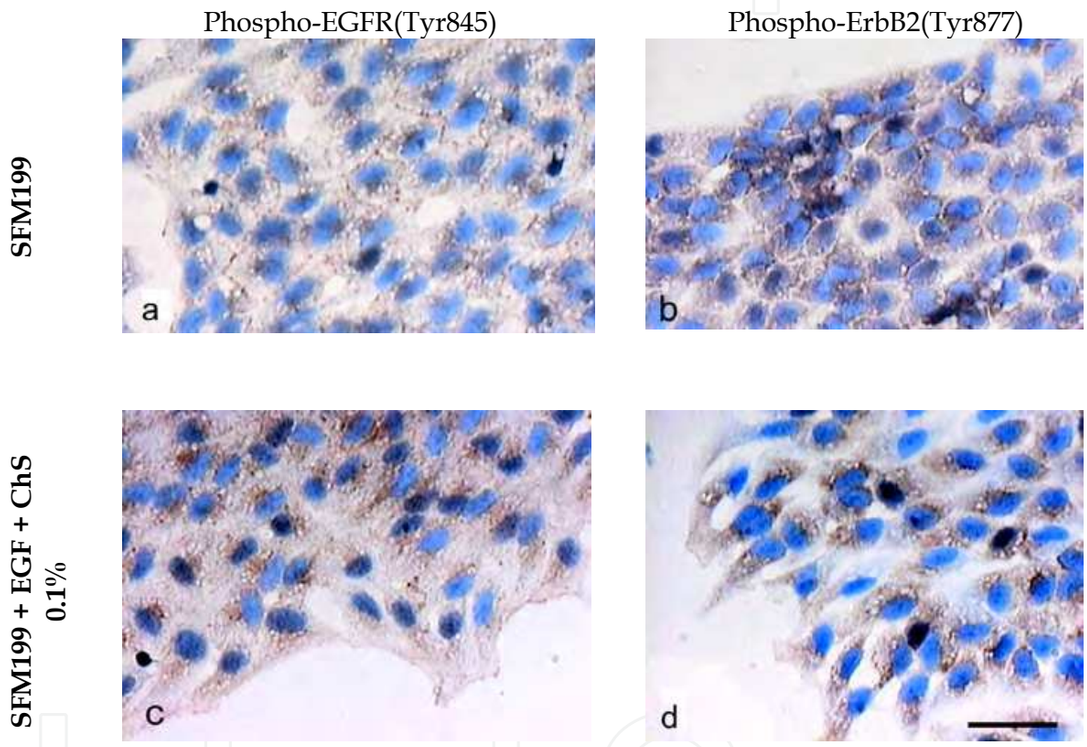

Fig. 6. (a,b) Immunolocalization of phospho-EGFR (Tyr845) and phospho-ErbB2 (Tyr877) in monolayers of endothelial cells adhered to pFN after 2hr in culture in the presence of SFM199. Staining for both receptors is detected in sites of cell-cell contacts as well as in the nucleus and perinuclear region of some cells of the monolayers. (c,d) Immunolocalization of phospho-EGFR (Tyr845) and phospho-ErbB2 (Tyr877) in monolayers whose medium were switched to medium containing EGF and low-ChS and maintained for additional 4hr (6hr). Strong nuclear and perinuclear staining for phospho-EGFR (Tyr845) is observed in many separating, detaching and migrating cells whereas in the cell-cell contacts the staining appears disrupted. For phospho-ErbB2 less intense nuclear staining is detected in many cells. Note that the staining at the sites of cell-cell contacts also appears disrupted.
Scale bar: 50μm.

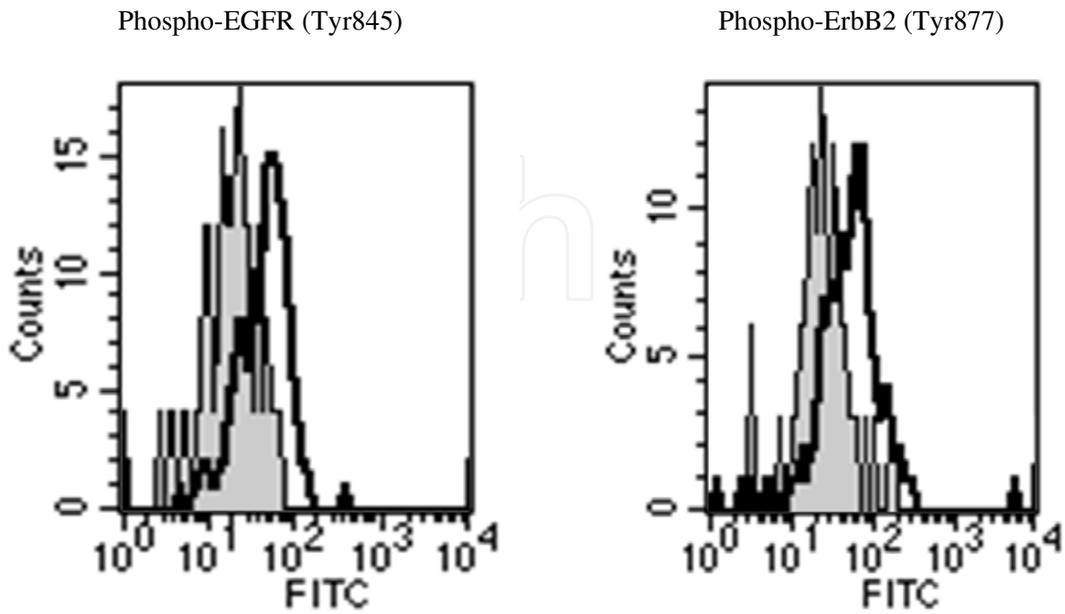

Fig. 7. Effect of TGF-α on phospho-EGFR (Tyr845) and phospho-ErbB2 (Tyr877) expression

Expression of phospho-EGFR (Tyr845) and phospho-ErbB2 (Tyr877) analyzed by flow cytometry in embryonic endothelial cells that were maintained for 2hr in SFM199, and in embryonic endothelial cells where the medium was switched to SFM199 supplemented with TGF-α and low-ChS and cultured for additional 4hr (6hr). Negative controls were stained with the respective isotype. Histograms show expression (after substracting the background) of phospho-EGFR (Tyr845) and phospho-ErbB2 (Tyr877) by SFM199 (black profile) or TGF-α treated (opened histograms) embryonic endothelial cells. Results are representative of at least three independent experiments, all of which had similar results.

3.1.3 In vitro phospho-c-Src (Tyr416) immunolocalization

Recent in vitro studies have indicated that after the adhesion of cells to the ECM, the cytoplasmic tail of integrin β_3 can directly interact with c-Src promoting its activation and that activated c-Src in turn phosphorylates ErbB2 at Tyr877 residue (Marcotte et al., 2009); therefore, we also investigated whether the activated c-Src was present in monolayers of endothelial cells when they were maintained in SFM199 and when they were switched to medium containing EGF and low-ChS. Immunolocalization with anti-phospho-c-Src (Tyr416 in chicken) showed that in the SFM199 condition this non-RTK was also localized at the cell-cell contacts or AJs (Fig. 8a) whereas in the EGF and low-ChS condition, phospho-c-Src appeared aligned with MTs and organized into linear streaks typical of focal adhesion complexes at the leading edge as well as in the nucleus of some cells that were separating, detaching and migrating (Fig. 8b).

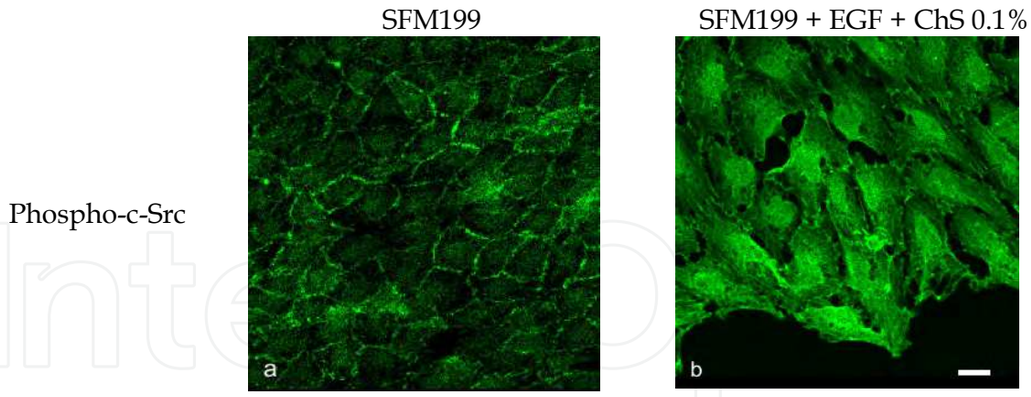

Fig. 8. a) CLSM fluorescence image of phospho-c-Src (Tyr416) in a monolayer of endothelial cells adhered to pFN after 2hr in culture in the presence of SFM199. Phospho-c-Src is seen in the cell-cell contacts. b) CLSM fluorescence image of phospho-c-Src (Tyr416) in a monolayer whose medium was switched to medium containing EGF and low-ChS. Phospho-c-Src (Tyr416) appears aligned with MTs and organized into linear streaks at the leading edge of separating, detaching and migrating cells. Note that this non-RTK is also detected in the nucleus of some cells. Scale bar: 15µm.

3.1.4 In vivo EGF, TGF-α, EGFR, and ErbB2 immunolocalization

In view of the above in vitro findings, we evaluated EGF, TGF-α, EGFR and ErbB2/Neu by immunoperoxidase staining in the aortic wall at days E12-E14 (stages 38-40) of development when intimal thickening is apparent and EndoMT occurs (Arciniegas et al., 2000).

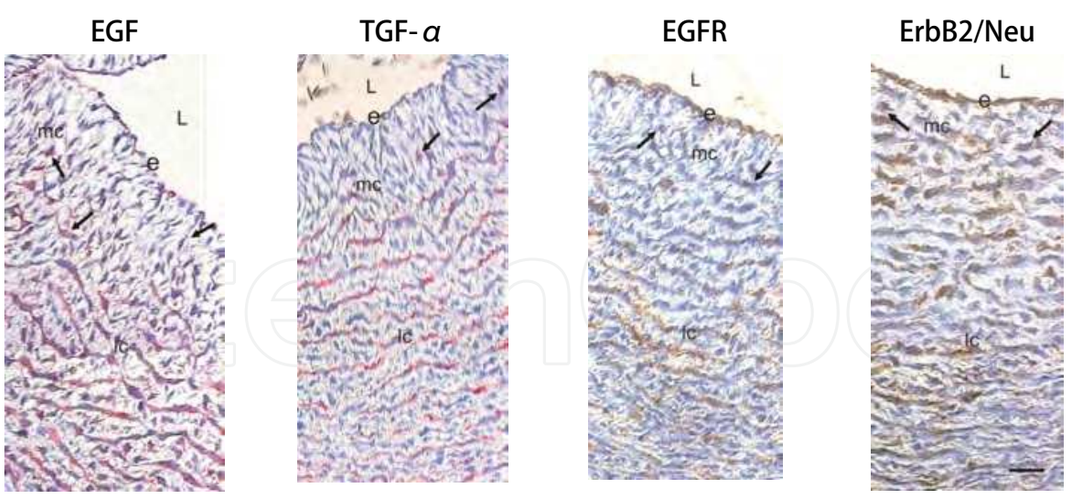

Fig. 9. Immunolocalization of EGF, TGF-α, EGFR, and ErbB2/Neu in deparaffinized sections of chicken embryo aorta at day E14 of development.
Immunoreactivity for EGF is observed in the endothelial cells (e) and some mesenchymal cells (mc) and in cells of the lamellar layers (lc). For TGF-α, a less immunoreactivity is observed in the endothelial cells (e) and some mesenchymal cells (mc) and cells of the lamellar layers (lc). Strong ErbB2/Neu and moderate EGFR immunoreactivities are detected in most endothelial cells (e) and mesenchymal cells (mc) and lamellar cells (lc). L, lumen. Scale bar = 125µm.

At days E12-E14, the aortic wall is composed by the endothelium, which limits the vessel lumen, and radially oriented mesenchymal cells originating from the endothelium that constitute the intimal thickening. At these stages it is possible to distinguish cells organized in circular lamellar and interlamellar layers. The immunoreactivity observed at these stages for EGF was more intense compared to TGF-α. Even so, both immunoreactivities were detected at the endothelial cells and in some mesenchymal cells of the intimal thickening as well as in those arranged in lamellar layers (Fig. 9). Immunoperoxidase staining also revealed strong ErbB2/Neu and moderate EGFR immunoreactivities in most endothelial and mesenchymal cells and those arranged in lamellar layers (Figs. 9). No immunoreactivity was observed when the primary antibody was omitted or replaced by non immune serum in control sections (not shown).

4. Discussion

This study provides evidence that allows us to suggest that the adhesion to pFN of explanted embryonic endothelial cells is mediated by integrin β_3 and promoted by Ca^{2+} and that the organization of these cells into monolayers of polarized cells, when they were maintained in the absence of serum or growth factors, was established by adhesion to FN and by formation of endothelial cell-cell contacts or AJs.The latter involving presence of β-catenin and p120-catenin, redistribution of integrin β_3 and importantly, reorganization of MTs with the possible participation of some tyrosine phosphatases and tyrosine kinases (Dejana et al, 2009). These cellular events are considered as relevant for the maintenance of endothelial cell shape and apico-basal polarity in vitro and during embryonic development (Bryant & Mostov, 2008; Dejana et al, 2008; 2009; Iruela-Arispe & Davis, 2009). Remarkably, the adhesion to pFN and organization of the endothelial cells into monolayers of polarized cells involving formation of AJs and reorganization of MTs in the absence of serum or growth factors was accompanied by tyrosine phosphorylation in the kinase domain of EGFR and ErbB2 and the nuclear translocation of both receptors. In fact, phosphorylated EGFR and ErbB2 were detected at sites of cell-cell contacts and in the nucleus of some cells of the monolayers by using anti-phospho EGFR (Tyr845) and anti-phospho ErbB2 (Tyr877). In this respect, phosphorylated EGFR has been detected during the formation of cell-cell contacts in monolayers of epithelial cells deprived of serum and maintained in presence of Ca^{2+}, suggesting a ligand independent activation of EGFR induced by its interaction with E-cadherin or with components of the AJs (Erez et al., 2005; Pece & Gutkind, 2000; Takahashi et al., 1997). It is noteworthy that basolateral localization of EGFR and ErbB2 along with components of the AJs has been consistently reported in polarized epithelial cells (Borg et al., 2000; Feigin & Muthuswamy, 2009; Hoschuetzky et al., 1994; Shelly et al., 2003; Tanos & Rodriguez-Boulan et al., 2008). Also, studies in epithelial cancer cells have indicated that the formation of AJs mediated by E-cadherin induces activation of EGFR in the absence of ligand leading to elevation of anti-apoptotic members of Bcl-2 family of regulatory proteins to protect cells from the apoptosis (Shen & Kramer, 2004).

Activated EGFR and ErbB2 have also been observed in the nucleus of human fetal endothelial cells (Bueter et al., 2006) and in normal and tumor epithelial cells suggesting that these receptors may regulate gene activation and transcription and possibly other nuclear events (Giri et al., 2005; Wang et al., 2010). In this sense, it is known that binding of integrins to their ligands in the absence of serum or growth factors can promote cell survival by increasing Bcl-2 transcription (Matter & Ruoslahti, 2001; Zhang et al., 1995) and that

clustering and occupancy of integrins in the absence of growth factors can also provoke recruitment and partial activation of several RTKs including members of the EGFR family (Desgrosellier & Cheresh, 2010; Eliceiri, 2001; Huveneers & Danen, 2010; Ivaska & Heino, 2010; ; Streuli & Akhtar, 2009; Yamada & Even-Ram, 2002**).** For instance, in epithelial cell lines, and human endothelial cells line ECV304, adhesion to FN mediated by integrin β_1 in the absence of serum or growth factors produces a rapid phosphorylation of EGFR at Tyr845, 1068, 1086, and 1173 residues that results critical for their survival (Bill et al., 2004; Cabodi et al., 2004; Moro et al., 2002). These levels of phosphorylation are lower than that observed with EGF. Importantly, phosphorylation of Tyr845 residue in the kinase domain of EGFR has been shown requires c-Src tyrosine kinase which is activated after integrin-mediated cell adhesion and autophosphorylated at Tyr419 residue of its kinase domain (Tyr416 in chicken) (Bill et al., 2004; Cabodi et al., 2004). Others have found that phosphorylation of Tyr877 residue in the kinase domain of ErbB2 is dependent on activated and phosphorylated c-Src (Tyr419) (Marcotte et al., 2009; Rivas et al., 2010; Xu et al., 2007). Some of them suggesting that activated and phosphorylated c-Src specifically associates with ErbB2, but not with other EGFR family members (Kim et al., 2005**;** Marcotte et al., 2009; Muthuswamy & Muller, 1995).

In view of above observations, we also investigated the presence and distribution of c-Src in the absence of serum or growth factors. Of note, c-Src phosphorylated at Tyr416 residue was localized at sites of cell-cell contacts or AJs displaying a pattern similar to that observed for β-catenin and p120-catenin and integrin β_3. To this respect, there is evidence showing that activated c-Src participates during the cell-ECM adhesion and formation of cell-cell adhesions where it might phosphorylate β-catenin and p120-catenin regulating certain signalling pathways that are considered as essentials for cell survival (Guarino, 2010; Schlessinger, 2000). Of interest, studies have shown that adhesion of osteoclast precursors to FN or vitronectin induces c-Src and MTs association and that this association affects the polarity of osteoclasts (Abu-Amer et al., 1997**).** Also, studies have revealed reciprocal interactions between c-Src and EGFR in the absence of growth factors (Donepudi & Resh, 2008). One of them, suggesting that autonomus c-Src activation may cause activation of EGFR, mimicking the effects of low concentration of EGF on EGFR activation, redistribution, and signalling (de Diesbach et al., 2010). In this context, previous studies have shown that clustering of integrin β_3 by ligands such as FN can result in the recruitment and activation of c-Src, inducing phosphorylation of Tyr416 or 419 residues in its kinase domain (Arias-Salgado et al., 2003; Desgrosellier et al., 2009; Guarino, 2010; Huveneers & Danen, 2010) and that activated and phosphorylated c-Src in turn phosphorylates ErbB2 at Tyr877 residue (Marcotte et al., 2009). Interestingly, these patterns of phosphorylation are different from those triggered by the binding of EGF.

Thus, we believe that**,** in the absence of serum or growth factors, the adhesion of explanted embryonic endothelial cells to pFN, mediated by activation and clustering of integrin β_3, promotes the activation of c-Src which is found associated with MTs and that activated c-Src contributes to the formation of endothelial cell-cell contacts or Ajs by phosphorylation of β-catenin and p120-catenin. This process would be accompanied by the EGFR and ErbB2 phosphorylation at the Tyr845 and Tyr877 residues respectively, leading to the partial activation and nuclear translocation of both receptors with consequent regulation of Bcl-2 proteins expression to protect cells from the apoptosis induced by serum-deprivation**.**

In this study, we found that the closure of the wounds (re-endothelialization) was almost complete and that endothelial cell spreading, separation, detachment and migration also took place at the marginal edges of the monolayers when the medium of some cultures was switched to medium containing EGF or TGF-α and low amount of ChS (0.1%). These observations are consistent with the possibility that EGF or TGF-α may be interacting with those EGFRs that remain exposed or accessible to ligand in the lateral cell borders after removing the explant. EGFR then would heterodimerize with ErbB2 and being internalized which results in the disruption of endothelial cell-cell contacts by remodelling of the MTs and alterations in the distribution of integrin β_3, β-catenin and p120-catenin, and therefore mediating the reparation of the wounds and the migration of the cells toward cell-free areas. This view is reinforced by previous reports suggesting that mechanical damage of monolayers of epithelial cells allows to ErbB2, which localizes on the basolateral surface, to interact with its ligand (heregulin) normally secreted to the apical surface leading to proliferation, migration and repair of the wound (Mostov & Zegers, 2003; Tanos & Rodríguez-Boulan, 2008; Vermeer et al., 2003). Notably, in this study we also found changes in the distribution and organization of phospho-c-Src (Tyr416) upon EGF or TGF-α and low-ChS addition. We believe that such changes may be associated with remodelling of MTs and alterations in the distribution of integrins β_3 and β_1 and β-catenin and p120-catenin during the disruption of endothelial cell-cell contacts. Consistent with this, studies in EGF-stimulated epithelial cells have provided evidence that MTs remodelling induces activation of c-Src which then phosphorylates β-catenin and p120-catenin, leading to the disruption of AJs, cell-ECM adhesion promoting cell migration and invasion (Guarino, 2010). Also of significance, cytoskeleton remodelling and loss of cell-cell contacts have been correlated with elevated expression of activated c-Src in tumor cells and during the EMT process (Alper & Bowden, 2005; Guarino, 2010). In relation to the nuclear localization of phospho c-Src (Tyr416) observed during EGF stimulation, emerging evidence suggest that nuclear localization of Src-family tyrosine kinases, including c-Src, upon growth factor stimulation regulates not only tyrosine phosphorylation of nuclear proteins, but also the structure of chromatine (Takahashi et al., 2009). In this study, stimulation with EGF or TGF-α also showed the localization of phospho-FAK (Tyr397), integrins β_3 and β_1 at focal adhesion when endothelial cell spreading, separation, detachment, and migration occurred. In this context, previous studies have shown that clustering of integrins β_1, β_3, and β_5 by ligands such as FN or alterations in the cytoskeleton, can result in the recruitment and autophosphorylation of FAK at Tyr397 residue and that activated FAK promotes Src binding and activation to increase FAK activity leading to the formation an activated FAK-Src signalling complex (Ilić et al., 2004; Mitra et al., 2005). Same studies propose that this activated complex affect not only the assembly and disassembly of focal adhesions, but also promotes cell migration through regulation of cytoskeleton and integrin recycling and the disruption of adherens junctions (Mitra et al., 2005). Interestingly, integrin recruitment, internalization by macropinocytosis and redistribution, have been observed during cell migration induced by growth factors (Gu et al., 2011).Thus, we believe that the endothelial cell detachment involving assembly and disassembly of focal adhesions and the migration observed upon EGF or TGF-α and low-ChS addition would be related with integrins β_3 and β_1 recruitment, internalization and redistribution, and with the activation of FAK and c-Src during EGFR and ErbB2 endocytosis. It is known that EGF or TGF-α acts by binding to EGFR leading to the receptor homo-or hetero-dimerization, activation of its receptor tyrosine kinase cytosolic domain and autophosphorylation on tyrosine residues initiating various important signal transduction pathways that could collaborate in the cell adhesion,

migration, proliferation, differentiation and survival (Carpenter & Liao 2009; Olayioye et al., 2000). Upon binding of EGF, receptors clustering and endocytosis take place followed by recycling back of the ligand-complex to the cell surface or degradation. After internalization, nuclear translocation of the receptors may also occur to regulate gene expression (Giri et al., 2005; Lemmon, 2009; Roepstorff et al., 2008; Wang et al., 2010; Yarden & Sliwkowski, 2001). Of particular interest, stimulation of EGFR endocytosis accompanied by remodelling of MTs after addition of EGF has been reported in HeLa cells (Kharchenko et al., 2007).

In this study, phosphorylation, internalization, and nuclear translocation of EGFR and ErbB2 was also observed upon EGF or TGF-α and low-ChS addition. In this condition, flow cytometry analysis revealed an increased expression of phosphorylated EGFR and ErbB2, in contrast with that detected in the SFM condition or absence of growth factors. Consistent with this, previous studies have shown that treatment of endothelial ECV304 cells with EGF, increases the levels of phosphorylation of EGFR induced by adhesion to FN and mediated by integrin β_1 (Cabodi et al., 2004**).** Of note, a recent work by Odintsova´s laboratory has reported that the integrin-mediated epithelial cell adhesion potentiates the phosphorylation of EGFR and ErbB2 induced by EGF or TGF-α facilitating receptor homo-and hetero-dimerization of these receptors and that this process could be accompanied by actin cytoskeleton organization (Alexi et al., 2011). Accordingly, our observations suggest that the dimerization, activation and phosphorylation of EGFR and ErbB2, initiated by integrin β_3, would be increased by addition of EGF or TGF-α, and that the internalization of these receptors could be accompanied by MTs remodelling which induces the activation of c-Src and phosphorylation of β-catenin and p120-catenin to promote loss of endothelial cell polarity and disruption of cell-cell contacts, facilitating cell spreading, separation, detachment, and migration, cellular events considered as essentials in the progression of EndoMT. Interestingly, overexpression and/or activation of EGFRs have been well documented to affect epithelial polarization and cell-cell contacts and leads to EMT of normal cells (Feigin & Muthuswamy, 2009). However, the precise mechanisms by which EGFRs deregulate normal epithelial architecture to promote EMT are incompletely understood (Feigin & Muthuswamy, 2009)

In addition to these findings, this study also provides in vivo evidence that EGF, TGF-α as well as their receptors EGFR and ErbB2 were present in those stages of development (days E12-E14) where the intimal thickening are clearly evident and EndoMT is an active process. These data are interesting if we consider that in vivo expression of EGF, TGF-α as well as their respective receptors have been detected in the intimal thickening and medial smooth muscle cells of atherosclerotic lesions (Dreux et al., 2006), and that expression of activated ErbB2 and ErbB3 has been demonstrated during heart cushion development (Camenish et al., 2002), suggesting an important contribution for these receptors in the initiation of atherosclerosis and in the development of cardiac valves, respectively.

Collectively, our findings suggest that integrin-mediated endothelial cell adhesion and activation and translocation of c-Src, EGFR and ErbB2 as well as the presence of their ligands are required for EndoMT.

5. Acknowledgment

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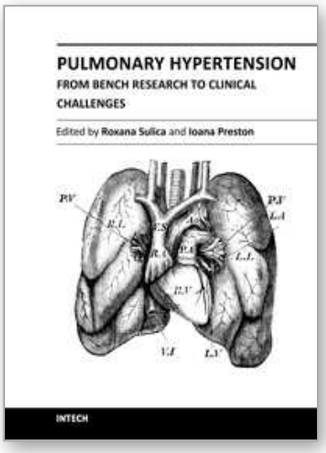

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The textbook "Pulmonary Hypertension - From Bench Research to Clinical Challenges" addresses the following topics: structure and function of the normal pulmonary vasculature; disregulated cellular pathways seen in experimental and human pulmonary hypertension; clinical aspects of pulmonary hypertension in general; presentation of several specific forms of pulmonary hypertension, and management of pulmonary hypertension in special circumstances. The textbook is unique in that it combines pulmonary and cardiac physiology and pathophysiology with clinical aspects of the disease. First two sections are reserved for the basic knowledge and the recent discoveries related to structure and cellular function of the pulmonary vasculature. The chapters also describe disregulated pathways known to be affected in pulmonary hypertension. A special section deals with the effects of hypoxia on the pulmonary vasculature and the myocardium. Other three sections introduce the methods of evaluating pulmonary hypertension to the reader. The chapters present several forms of pulmonary hypertension which are particularly challenging in clinical practice (such as pulmonary arterial hypertension associated with systemic sclerosis), and lastly, they address special considerations regarding management of pulmonary hypertension in certain clinical scenarios such as pulmonary hypertension in the critically ill.

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