

Regulation of fibronectin alternative splicing by a basement membrane-like extracellular matrix

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Abstract Hepatocytes are the source of plasma fibronectin (FN) which lacks the alternatively spliced EDI segment, distinctive of oncofetal FN. When hepatic or other epithelial cells are cultured on plastic, EDI inclusion is triggered. Here we report that EDI inclusion is inhibited when hepatic cells are cultured on a basement membrane-like extracellular matrix (ECM), demonstrating a new role for the ECM in the control of gene expression. The effect is duplicated by collagen IV and laminin but not by collagen I; is not observed with another alternatively spliced FN exon (EDII); and correlates with a decrease in cell proliferation, consistently with high EDI inclusion levels observed in many physiological and pathological proliferative processes. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Alternative splicing; Extracellular matrix; Fibronectin

1. Introduction

It is now estimated that more than 50% of human genes generate diversity through alternative mRNA splicing [1,2]. This process can be regulated in a cell type-, development-, and age-dependent manner. In the case of the human fibronectin (FN) gene, alternative splicing in three different regions referred to as EDII, EDI and IIICS gives rise to up to 20 different mRNAs and polypeptides in a cell-specific manner. In vivo, EDI+ FN is poorly represented in the extracellular matrix (ECM) of adult normal tissues. However, this variant is overexpressed in developing embryos [3], wound healing [4], liver fibrosis [5], ovary granulosa cell proliferation [6] and some tumors [7].

Although the roles of cis and trans-acting elements in the control of alternative splicing has been emerging [8,9], little is known about the extracellular signals and transduction pathways that control this event. Changes in alternative splicing of several pre-mRNAs upon stimulation by growth factors, cytokines, hormones and stress stimuli have been reported [10–12].

Cell-ECM interactions initiate a dynamic flow of informa-

tion that acts to regulate many fundamental processes throughout development. These include cell migration in the early embryo, morphogenesis during organ formation, and the modulation of growth, survival and differentiation of many specialized cell types [13,14]. Mechanical and biochemical connections between the ECM and the cell nucleus lead to changes in gene expression [15,16].

In the present study we report that a laminin-rich basement membrane as well as its two main protein components, laminin and type IV collagen, are able to modulate the alternative splicing of the FN EDI exon. This effect is independent from the promoter and does not influence another FN alternative exon, EDII.

2. Materials and methods

2.1. Cell culture

The human hepatoma cell line Hep3B was routinely grown in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), and penicillin/streptomycin (100 units/ml and 100 µg/ml respectively; Gibco BRL).

2.2. Transfections

Stable or transient transfections were performed using Lipofectamine reagent (Gibco/BRL) according to the manufacturer's instructions. For transient transfection assays, approximately 1.5×10^5 cells were transfected with 2 µg of plasmid DNA and 6 µl of lipofectamine in 35 mm-tissue culture dishes. After 6 h, the transfection mixture was replaced by fresh DMEM, 0.25% (v/v) FBS, with or without Matrigel treatment (see cell treatments). Co-transfection with the pCMV-β-galactosidase plasmid allowed standardization of the RNA samples by transfection efficiencies: an aliquot of the cells was used to measure β-galactosidase activity and the rest was used to prepare total RNA. Stable cell lines used here were reported previously [17].

2.3. Cell treatments

Approximately 1.5×10^4 cells/cm² were plated in DMEM supplemented with 1% (v/v) FBS. After 24 h, the medium was replaced with 0.25% (v/v) FBS-DMEM with or without the correspondent treatment: 1.5% (v/v) Matrigel, Type I or IV collagens, human plasma fibronectin (Collaborative Biomedical Products), laminin (Sigma), BSA (Promega), TGF β1 (2.5 ng/ml) (provided by Tomas Santa Coloma), EGF (50 ng/ml), HGF-SF (25 ng/ml) (Collaborative Biomedical Products). The cells were maintained for 72 h except when indicated, with a change of medium plus treatment after 48 h. Experiments were always run in duplicates.

2.4. RNA isolation and radioactive RT-PCR amplification

Total RNA purification from cultured cells and RT-PCR analysis was carried out as previously described [18].

The sets of primers used were: pSV5'j and pSV3'j for the amplification of the minigene splicing products [19].

hFN-dir4860 (5' AGCCCCGCAAGCAGCAAGCC 3') and hFN-

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Abbreviations: FN, Fibronectin; ECM, extracellular matrix; TGF β1, transforming growth factor β1; EGF, epidermal growth factor; HGF/SF, hepatocyte growth factor/scatter factor

rev5758 (5' GTAGCATCTGTACACGAG 3') for endogenous FN mRNAs containing or lacking the EDI exon.

hEDB-dir (5' GCATCTGGAGGCAAACCCTGAC 3') and hEDB-rev (5' GTCAATGCCAGTTGGGGAATCAAG 3') for endogenous FN mRNAs containing or lacking the EDII exon.

RT-PCR products were electrophoresed in 6% (w/v) polyacrylamide native gels and detected by autoradiography. Radioactivity in the bands was measured in a scintillation counter (Cerenkov method).

2.5. Cell proliferation assay

DNA synthesis was measured by [³H]-thymidine incorporation. Cells were cultured with or without Matrigel during 3 days. Either 18 or 6 h prior to the ending of the 3 day-period, 1 µl of [³H]-thymidine (1 mCi/ml) was added to each well of 24-well plates.

After the corresponding incubation time, cells were washed twice with cold PBS, incubated at 4°C with cold 5% trichloro acetic acid (TCA) during 50 min, washed once with cold PBS, and incubated O/N at room temp. with 0.5 M NaOH/0.1% (v/v) triton X-100. Cell lysates were mixed with scintillation fluid and counted in a scintillation counter.

2.6. Substrata

When indicated, 35 mm-tissue culture wells were pre-coated on ice with 0.2 ml of cold Matrigel. Matrigel was then allowed to gel at 37°C during 15 min and cells were immediately plated on top of it. For studies on the influence of cell shape, culture plates were coated with the non-adhesive substratum poly(2-hydroxyethyl methacrylate) ('polyHEMA'; Sigma) as described [20,21], at concentration of 3 mg/ml in 95% ethanol before drying.

3. Results

3.1. Basement membrane-like ECM inhibits FN EDI exon inclusion

To study the influence of the cellular microenvironment on FN alternative splicing we evaluated the effect of an exogenous extracellular matrix on FN EDI exon inclusion. Hep3B cells were cultured with or without an overlay of a laminin-rich basement membrane (Matrigel). Cells were harvested after different time points, total RNA was purified and subjected to RT-PCR with primers designed to assess EDI inclusion levels (Fig. 1A). The addition of Matrigel inhibited the inclusion of the EDI exon by 40–50% as shown in Fig. 1B. This effect was clearly detected after 3 days of treatment and persisted for at least 5 days.

As an alternative approach, Matrigel was used as a substratum and Hep 3B cells were either plated directly on tissue culture plastic or on top of a thick basement membrane gel. Despite the fact that these two ways of treatment with Matrigel induced different degrees of cell shape changes (Fig. 1C), they elicited similar results with respect to the regulation of EDI alternative splicing.

The basement membrane effect on alternative splicing was almost abolished when cells were cultured with 10% (v/v) serum but became more and more pronounced with decreasing concentrations of serum. As Hep3B cells do not survive long periods under serum deprivation, we reduced the level of serum to 0.25%, condition that kept the cells healthy for more than 72 h and allowed us to observe a significant inhibition of EDI inclusion when Matrigel was added.

We then decided to evaluate whether the basement membrane could regulate another FN alternative exon, EDII (also known as EDB or EIIIB). PCR reactions with the same cDNAs already tested for EDI inclusion but with a different pair of primers, specific for EDII splicing (Fig. 1A), revealed that the splicing pattern of the EDII exon was not modified upon addition of Matrigel (Fig. 2).

3.2. Inhibition of EDI inclusion by basement membrane does not depend on either promoter or chromatin structures

Our laboratory has demonstrated that differences in promoter structure control splice site selection [22], providing evidence for coupling between alternative splicing and transcription machineries. Based on these results we decided to investigate whether the observed basement membrane-induced inhibition of EDI inclusion was dependent on promoter architecture. We used a formerly reported system, consisting in the transfection of Hep3B cells with α -globin/fibronectin minigenes under the control of different promoters (FN, cytomegalovirus and α -globin) [22] (Fig. 3A). Total RNA from transfected cells was extracted and subjected to RT-PCR with 2 different pairs of primers. One set of primers to analyze the mRNA isoforms generated from the endogenous FN gene (Fig. 1A) and another set to analyze the isoforms generated from the minigene (Fig. 3A). In every case, the Matrigel inhibited EDI inclusion independently from the promoter that gave rise to the transcripts. Moreover, the effect was observed whether the minigene was transfected transiently or stably into the cells, suggesting that this splicing regulation does not depend on chromatin organization. Nevertheless, the effect was more pronounced (up to 60% inhibition of EDI exon inclusion) with one of the minigenes analyzed, pFNmut, stably transfected into Hep3B cells (Fig. 3B). For that reason we continued our studies with this cell clone, named 'Hep3B FN mut1'. In every experiment we evaluated EDI exon inclusion in transcripts derived from both the endogenous FN gene as well as the inserted minigene, obtaining similar results.

3.3. Regulation of EDI inclusion by basement membrane proteins

To assure that the changes observed were not due to contaminating growth factors present in the Matrigel preparation, we tested the effect of a Matrigel partially depleted of growth factors (GFR Matrigel) as well as different available growth factors (TGF β 1; EGF; HGF/SF) on FN EDI alternative splicing in Hep3B FN mut1 cells.

GFR Matrigel was able to inhibit the inclusion of EDI exon to the same extent as regular Matrigel (Fig. 3B). In addition, the three growth factors tested showed the opposite effect, that means they induced the inclusion of this exon (Fig. 3C). These results are in agreement with previous reports in other cell systems that showed that TGF β 1 as well as HGF/SF stimulates EDI inclusion [23–26]. None of the tested growth factors was able to alter the pattern of EDII exon inclusion (data not shown).

Matrigel main components are laminin (60%) and type IV collagen (30%). To determine whether any of these two proteins is able to trigger the inhibition of FN EDI inclusion, Hep3B FN mut1 cells were cultured either with purified laminin or type IV collagen during 3 days. We also tested the effect of type I collagen and fibronectin, two additional ECM proteins that are not usually associated with the basement membrane, and also of bovine serum albumin. We treated Hep3B FN mut1 cells with 150 µg/ml of these purified proteins, a dose equivalent to the amount of laminin present in the volume of Matrigel previously used. Under these conditions, both laminin and type IV collagen inhibited EDI exon inclusion similarly to Matrigel (Fig. 4A). Neither type I collagen nor bovine serum albumin elicited any effect on EDI alternative splicing (Fig. 4B). Treatment with plasma FN re-

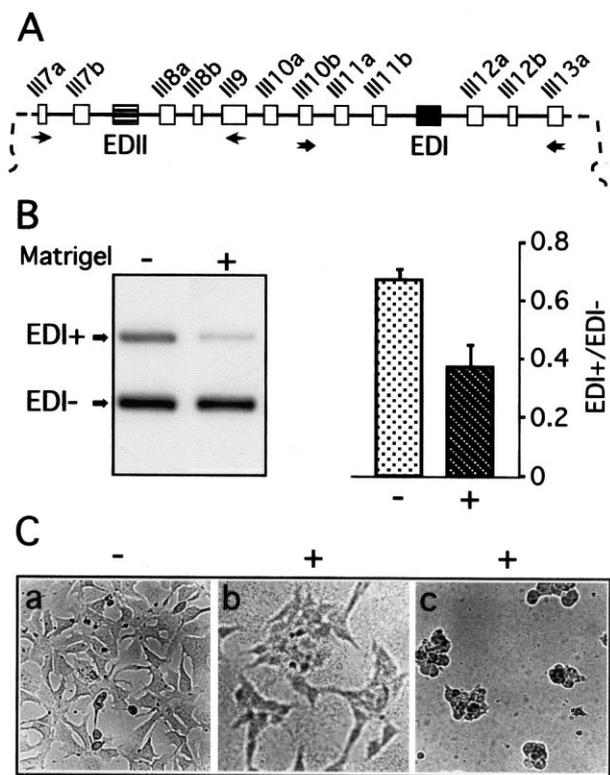


Fig. 1. Effect of a reconstituted basement membrane (Matrigel) on EDI alternative splicing. A. Fragment of the endogenous FN gene showing EDI (black box) and EDII (dashed box) alternative exons with their neighboring introns (lines) and exons (white boxes). Arrows indicate positions of the primers used for amplification of FN EDI+ and EDI- or FN EDII+ and EDII- mRNA isoforms. B. RT-PCR analysis of endogenous FN EDI+ and EDI- mRNA isoforms produced by Hep3B cells cultured in the absence (-) or presence (+) of a Matrigel overlay. Quantification of each band was carried out with a scintillation counter. EDI+/EDI- ratios shown in the histogram are the average of three independent experiments. C. Morphology of Hep3B cells cultured for three days in the absence of Matrigel (a), with a Matrigel overlay (b) or on top of a Matrigel gel (c). Magnification: 100 \times .

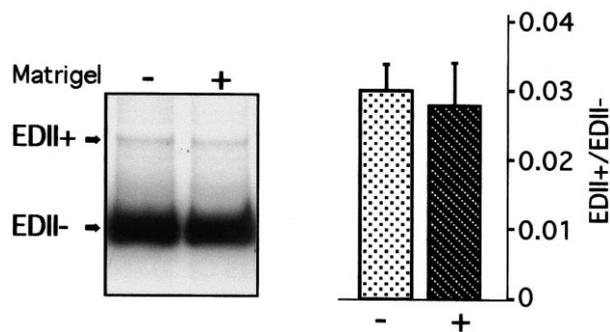


Fig. 2. Effect of Matrigel on EDII alternative splicing. RT-PCR analysis of endogenous FN EDII+ and EDII- mRNA isoforms produced by Hep3B cells cultured in the absence (-) or presence (+) of a Matrigel overlay. Quantification of each band was carried out with a scintillation counter. EDII+/EDII- ratios shown in the histogram are the average of three independent experiments.

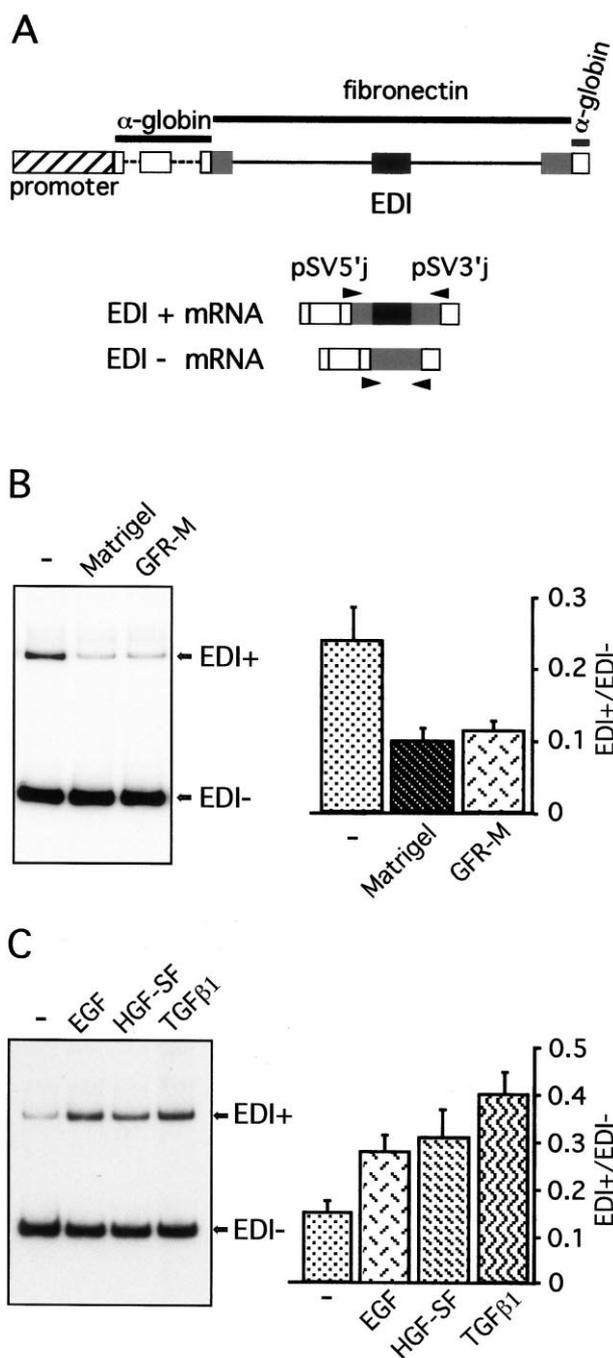


Fig. 3. Effect of Matrigel and growth factors on EDI alternative splicing in Hep3B FN mut1 cells. A. Scheme of the minigene stably transfected into Hep3B cells. The cartoon shows a promoter (dashed box) driving the expression of the minigene composed by α -globin exons (with boxes), α -globin introns (dotted lines), FN introns (black lines) and FN exons (gray and black boxes). EDI exon is shown in black. Arrows indicate positions of the primers used for amplification of FN EDI+ and EDI- mRNA isoforms generated from the transgene. B-C. RT-PCR analysis of EDI+ and EDI- mRNA isoforms derived from a stably transfected minigene (pFN mut) in Hep3B cells. B. Cells were cultured in the absence (-) or presence of regular Matrigel (Matrigel) or growth factor reduced (GFR)-Matrigel (GFR-M). C. Cells were cultured without the addition of any growth factor (-) or with EGF, HGF-SF or TGF β 1. EDI+/EDI- ratios shown in the histograms are the average of three independent experiments.

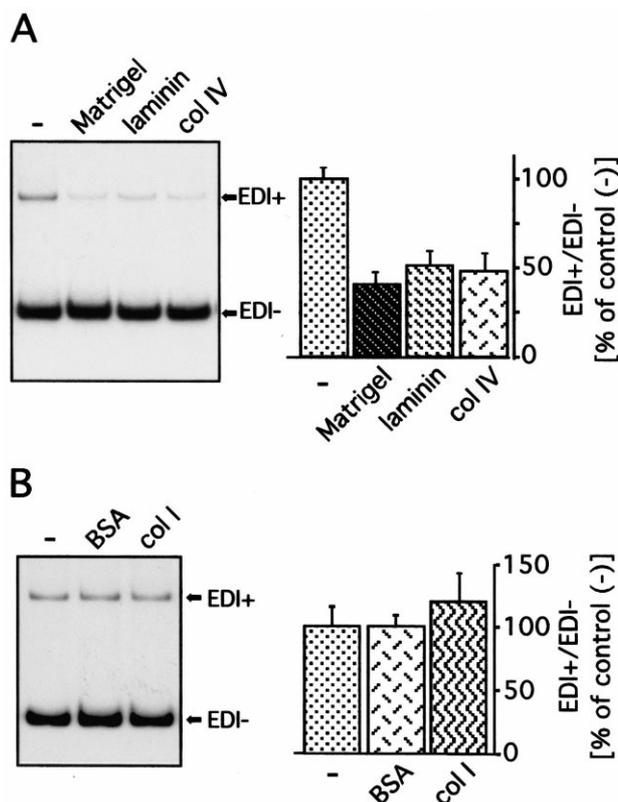


Fig. 4. Effect of purified proteins on EDI alternative splicing in Hep3B FN mut 1 cells. A. Cells were treated with Matrigel, laminin, or type IV collagen (col IV) or left untreated (-). B. Cells were treated with bovine serum albumin (BSA) or type I collagen (col I) or left untreated (-). EDI+/EDI- ratios corresponding to each treatment shown in the histograms are expressed as the percentage of the EDI+/EDI- ratio from untreated cells.

sulted in cell detachment, making hardly impossible to draw any conclusion on the effect of this protein on FN alternative splicing in this cell type.

3.4. EDI exclusion vs. proliferation

Overexpression of EDI+ FN is associated with several proliferative processes [7]. On the other hand, it has been shown that many cell types undergo growth arrest when cultured with a basement membrane [27,28]. Therefore, we asked whether the observed inhibition of EDI inclusion in Hep3B cells could correlate with an inhibition in cell-cycle progression. To answer this question, Hep3B FN mut 1 cells were cultured for 3 days with or without Matrigel and with 0.25% (v/v) serum and during the last 18 or 6 h of the experiment the percentage of cells entering the S-phase was measured by [³H]-thymidine incorporation. Fig. 5 shows a significant decrease in the fraction of cells entering S-phase when cultured in the presence of Matrigel compared to cells cultured in its absence.

4. Discussion

We show here that Matrigel, a basement membrane-like ECM, can regulate pre-mRNA splicing. As ECMs are complex three-dimensional arrays of several proteins and can also contain multiple bound growth factors, we dissected the composition of Matrigel into its main components and found that laminin and type IV collagen are able to down regulate the

inclusion of the FN EDI alternative exon into mature mRNA. Our findings agree with reports that different collagen species are able to alter promoter usage and mRNA splicing patterns of parathyroid hormone-related peptide (PTHrP) in a human breast cancer cell line [29].

It is known that regular, two-dimensional tissue culture conditions represent quite an abnormal microenvironment, not permissive for sustained tissue-specific functions [30,31]. In agreement with this concept, our finding that Hep3B cells in contact with a basement membrane not only downregulate the expression of the EDI+ FN isoform but also show a dramatic decrease in cell cycle progression correlates well with the already described expression pattern of EDI+ FN in vivo. On one hand, plasma FN, which lacks EDI, is made by hepatocytes which are lying on their basement membranes in vivo [32]. Culture of these cells on plastic triggers FN EDI inclusion. On the other hand, inclusion of EDI exon is greatly increased in physiological and pathological processes that involve cell proliferation, such as embryo development [3], wound healing [4], vascular intima thickening [33], regenerating liver [34] and ovarian follicular development [6].

When cells are placed on a reconstituted basement membrane not only signalling pathways are activated by integrin ligands but also dramatic changes in cell morphology take place [21]. Schischmanoff et al. [35] showed previously that the alternative splicing of the pre-mRNA for protein 4.1 is modulated by changes in cell shape in mammary epithelial cells. Hep3B FN mut1 cells can be forced to change their shape without the addition of Matrigel, by plating them onto an inert, non-adhesive polymer, poly(2-hydroxyethyl methacrylate) (poly-HEMA) [20]. Under these conditions, the cells aggregate in rounded clusters that remain in suspension. Hep3B FNmut1 cells plated on polyHEMA-coated dishes also displayed downregulation of EDI exon inclusion (not shown). This effect was smaller than the one exerted by the Matrigel, suggesting that the cell shape change is indeed involved in the regulation but it is not sufficient to produce the whole effect. However, we can not rule out the possibility that the change in cell shape induces the deposition of some endogenous basement membrane [28,36], which in turn leads to the downregulation of EDI exon inclusion.

The inhibitory effect of Matrigel on EDI splicing correlates

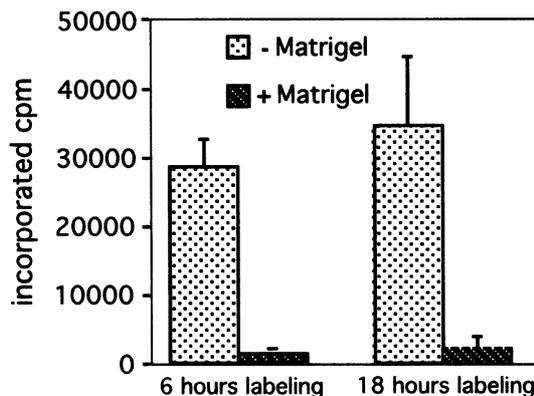


Fig. 5. Effect of Matrigel on the rate of cell-cycle progression. Cell proliferation assay of Hep3B FN mut 1 cells cultured in the absence (-Matrigel) or presence (+Matrigel) of a Matrigel overlay. The incorporation of [³H]-thymidine was carried out during the last 6 or last 18 h of the experiment.

with a decrease in cell proliferation. The different steps in mRNA processing are regulated by protein phosphorylation and dephosphorylation of splicing factors such as SR proteins.

It has been reported that the protein casein kinase I α is able to phosphorylate SR proteins and at the same time plays a role in cell cycle progression [37]. In this context, it is possible to speculate that SR proteins involved in FN alternative splicing are phosphorylated-dephosphorylated in a cell cycle-dependent manner, therefore linking proliferation with EDI exon inclusion. It has been shown that the EDI segment of FN can act as a mitogen [6], therefore it would be possible to postulate that the absence of basement membrane-dependent signals leads to an increase in EDI+ FN synthesis and secretion, which in turn triggers cellular proliferation in an autocrine or paracrine way.

Our results showing that FN EDII alternative splicing is not modulated by Matrigel neither but few growth factors that do regulate EDI are consistent with the idea that alternative splicing of EDI and EDII exons is controlled by different molecular mechanisms [17,18,38].

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