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Tenascin-C inhibits extracellular matrix-dependent gene expression in mammary epithelial cells

Localization of active regions using recombinant tenascin fragments

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SUMMARY

The physiological role of tenascin in vivo has remained obscure. Although tenascin is regulated in a stage and tissue-dependent manner, knock-out mice appear normal. When tenascin expression was examined in the normal adult mouse mammary gland, little or none was present during lactation, when epithelial cells actively synthesize and secrete milk proteins in an extracellular matrix/lactogenic hormone-dependent manner. In contrast, tenascin was prominently expressed during involution, a stage characterized by the degradation of the extracellular matrix and the subsequent loss of milk production. Studies with mammary cell lines indicated that tenascin expression was high on plastic, but was suppressed in the presence of the laminin-rich, Engelbreth-Holm-Swarm (EHS) tumour biomatrix. When exogenous tenascin was added together with EHS to mammary epithelial cells, β-casein protein synthesis and steady-state mRNA levels were inhibited in a concentration-dependent manner. Moreover, this inhibition by tenascin could be segregated from its effects on cell morphology. Using two β-casein promoter constructs attached to the chloramphenicol acetyltransferase reporter gene we showed that tenascin selectively suppressed extracellular matrix/prolactin-dependent transcription of the β-casein gene in three-dimensional cultures. Finally, we mapped the active regions within the fibronectin type III repeat region of the tenascin molecule that are capable of inhibiting β-casein protein synthesis. Our data are consistent with a model where both the loss of a laminin-rich basement membrane by extracellular matrix-degrading enzymes and the induction of tenascin contribute to the loss of tissue-specific gene expression and thus the involuting process.

Key words: β-casein, extracellular matrix, mammary gland, tenascin

INTRODUCTION

Differentiation of the mammary epithelium begins during pregnancy when the ductal epithelium proliferates to form a polarized alveolar epithelium which, following parturition, actively synthesizes and secretes milk proteins into an apically located lumenal space (Neville and Daniel, 1987). This fully differentiated state requires the continued presence of an intact basement membrane and lactogenic hormones (Wicha et al., 1980; Knight and Peaker, 1982; Sympson et al., 1994). Similarly, in tissue culture milk protein synthesis requires the cooperative action of basement membranes and lactogenic hormones (Emerman and Pitelka, 1977; Lee et al., 1985; Streuli et al., 1991). When mouse mammary cells are cultured on the laminin-rich, Engelbreth-Holm-Swarm (EHS) matrix, they aggregate to form multicellular, alveolar-like structures which secrete milk into a central lumen (Barcellos-Hoff et al., 1989). The production of β-casein, one of the more abundant milk proteins, is regulated by interactions with laminin (Streuli et al., 1992), through the β_1 integrin receptor sub-unit (Streuli et al., 1991), and may also occur in the absence of cell-cell interactions (Streuli et al., 1991). Furthermore, the regulation of β-casein by the extracellular matrix (ECM) occurs at the transcriptional level via the activation of an ECM-responsive enhancer element (Schmidhauser et al., 1990, 1992).

Whereas an intact basement membrane leads to the establishment and maintenance of the lactational phenotype, the loss of milk protein expression and tissue restructuring that occurs during involution arises from alterations in the existing ECM. Studies from one of our laboratories (Talhouk et al., 1991, 1992; Sympson et al., 1994) indicate that during involution, the activity of ECM-degrading proteinases relative to their inhibitors increases: this shift in gene expression correlates with the dissolution of the basement membrane and tissue restructuring, resulting in regression of alveoli and the loss of milk protein expression (Talhouk et al., 1991). The proof that the balance between ECM-degrading enzymes and their inhibitors is crucial for involution comes from two sets of experiments: (1) by artificially increasing the levels of the proteinase inhibitor TIMP during involution, this process can be

delayed (Talhouk et al., 1992); and (2) transgenic mice that inappropriately express stromelysin-1 during pregnancy and lactation degrade the basement membrane and involute prematurely (Sympson et al., 1994).

In addition to the dissolution of the basement membrane by ECM-degrading proteinases, it is likely that other factors contribute to the loss of mammary gland function observed during involution. Given the profound effects that ECM has on cell behaviour during tissue remodelling (Alexander and Werb, 1991; Whitby and Ferguson, 1991), we asked whether qualitative changes in the ECM during involution might also accompany and contribute to this process.

TN is one ECM component which is prominent during tissue restructuring in a number of normal and pathological processes including embryonic development (Chiquet and Fambrough, 1984; Erickson and Taylor, 1987; Hoffman et al., 1988; Bartsch et al., 1992), epithelial-mesenchymal interactions (Ekblom and Aufderheide, 1989), wound healing (Mackie et al., 1988; Whitby et al., 1991) and cancer (Chiquet-Ehrismann et al., 1986; Koukoulis et al., 1991). We therefore examined the pattern of TN expression in the mouse mammary gland during lactation, when the tissue structure is stable, and during involution, when active tissue restructuring occurs. In parallel we examined the regulation of TN expression in tissue culture with or without addition of a basement membrane. These data showed that the expression of TN is inversely related to ECM-induced expression of milk protein genes. We also show that mammary epithelial cells treated with intact TN are incapable of expressing β-casein. This inhibition occurred within alveolar-like structures that appear to be polarized, and to have an intact actin-based cytoskeleton. Furthermore, the inhibition by TN occurs at the transcriptional level. Finally, we have identified two active regions within the fibronectin type III repeats of TN that are able to inhibit β-casein protein synthesis.

MATERIALS AND METHODS

Cell culture

Parent CID-9 cells (Schmidhauser et al., 1990) and their clonal derivatives, SCp2 and SCg6 (Desprez et al., 1993), were routinely grown as described previously (Desprez et al., 1993).

The Engelbreth Holm Swarm (EHS) tumour matrix was passaged and prepared as described previously (Kleinman et al., 1986; Taub et al, 1990). Purified chicken tenascin-C (a generous gift from Dr R. Chiquet-Ehrismann, Friedrich Miescher Institut, Basel) and recombinant tenascin fragments were prepared as described (Chiquet-Ehrismann et al., 1986; Aukhil et al., 1993), whereas purified human tenascin-C was purchased from Chemicon International, Temecula, CA.

Cells were plated at 5×10^4 cells per cm^2 on tissue culture plastic or with EHS in the presence of 2% FCS, insulin (5 μg/ml), hydrocortisone (Sigma; 1 μg/ml) and ovine prolactin (National Institutes of Health; 3 μg/ml). Twenty four hours after plating, the medium was changed to serum-free DMEM/F12 containing the three lactogenic hormones.

For two-dimensional culture assays, cells were plated on plastic; EHS tumour matrix was diluted to 1% (v/v) in chilled DMEM/F12 and added to the cells as an ECM overlay (Desprez et al., 1993; Streuli et al., unpublished data), either with or without intact chicken or human TN (1.0-2.5 μg/ml), or TN fragments (1.0-25.0 μg/ml). For three-dimensional cultures, cells were plated on top of EHS (Streuli et al., 1991) and then treated with 1.0-5.0 μg/ml of intact human TN or TN fragments (25 μg/ml) on the third day of culture.

All assays were performed following 6 days in culture.

Recombinant TN-C fragments

The cloning, expression, and purification of these proteins have been described previously (Aukhil et al., 1993; Joshi et al., 1993). Briefly, TN-C fragments were produced in a bacterial expression system. These fragments were expressed directly and were purified by $(NH_4)_2SO_4$ precipitation, gel filtration, and ion-exchange chromatography. Seven human TN-C segments were used for the present study (see Fig. 8A). Each of the segments began and ended precisely at the boundaries of the FN type III domains.

Immunocytochemistry

Mammary tissue from CD-1 mice was collected following 9 days of lactation, and 2 days of involution. Tissues and three-dimensional cultures were fixed in 2% paraformaldehyde, infused with 18 and 30% sucrose before embedding in Tissue-Tek OCT compound (Miles Sc. Div., Elkhart, IN), and frozen in an ethanol/dry ice bath. Sections of 5 μm were cut with a Leitz cryotome onto gelatin-coated slides.

For SCp2 and SCg6 cocultures, cells were plated at 5×10^3/cm^2 on tissue culture plastic, cultured for 6 days as described above and fixed with 2% paraformaldehyde.

To detect TN, tissue sections and cells were quenched with 0.1 M glycine. Non-specific binding sites were blocked with PBS containing 1% bovine serum albumin (BSA). All washes were performed for 1 hour at room temperature in PBS containing 1% BSA. Sections were incubated with a rabbit polyclonal antiserum to mouse TN (pK7) (a gift from Dr M. Schachner and U. Dorries, Swiss Federal Institute of Technology, Zurich). TN protein was visualized by indirect immunofluorescence using species-specific antisera and FITC conjugates (Zymed laboratories), or Texas Red conjugates (Amersham Corp.). Nuclei were counterstained with 0.5 μg/ml DAPI (4,6-diamino-2-phenylinodole) (Sigma Chemical Co., St Louis, MO).

For indirect immunofluorescence of three-dimensional SCp2 cultures, 5 μm frozen sections were stained with either a mouse monclonal antibody raised against rat β-casein (a gift from Dr C. Kaetzel, Case Western Reserve University), or a rat monoclonal antibody to E-cadherin (ECCD-2) (a gift from Dr M. Takeichi, Kyota University). Proteins were visualized indirectly with a species-specific Texas Red- or FITC-labelled biotin/streptavidin complex (Amersham Corp). Filamentous actin was visualized with rhodamine-phalloidin (Molecular Probes, Eugene, OR). Nuclei were counterstained with 0.5 μg/ml DAPI.

Northern blot analysis

Total RNA was isolated from cells according to the method of Chomcyznski and Sacchi (1987). A 10 or 20 μg sample of total RNA was run on 1% agarose/formaldehyde gels using standard methods. Hybridizations were performed with ^{32}P-labelled random primed probes prepared from 800 bp of mouse TN cDNA and 540 bp of β-casein cDNA (a gift from Dr J. Rosen, Baylor College, Houston, TX).

Protein analysis

For immunoprecipitation of milk proteins and TN, cells were pulse-labelled with [^{35}S]methionine for 3 hours, lysed, sonicated and then cleared by centrifugation. Total cpm were normalized by TCA precipitation and equal cpm (1×10^6 cpm/sample) were immunoprecipitated using either polyclonal rabbit antisera raised against mouse milk (Lee et al., 1984), or polyclonal antisera to mouse TN. Immune complexes were precipitated with Protein A-Sepharose (Sigma Chemical Co.), washed and resuspended in 2× SDS-PAGE sample buffer. Proteins were separated on 13% (for milk) and 5% (for TN) SDS/polyacrylamide gels, fixed, dried and exposed to Kodak X-Omat film for 3-5 days at −70°C.

CAT assays

CID-9 cells were stably transfected by calcium phosphate precipitation (Gorman, 1986) with 30 μg of plasmid DNA and 3 μg of SV2 neo as a selectable marker. G418-resistant colonies were pooled and CAT expression was assessed.

For CAT assays, duplicate plates of cells were cultured either on plastic, or on EHS, with and without 2.5 μg/ml of overlayed human TN. Cells were harvested using dispase (Collaborative Research, Bedford, MA), lysed, and the quantity of protein in each lysate was quantified using the Bradford assay (Bio-Rad, Richmond, CA). To measure CAT enzyme activity, 10 μg of cell extract was incubated with [^{14}C]chloramphenicol (Sigma Chemical Co.) and acetyl coenzyme, and then extracted with ethyl acetate prior to separation using thin layer chromatography. The levels of acetylated chloramphenicol were quantified from autoradiographs.

RESULTS

Developmental regulation of tenascin in the adult mouse mammary gland

Tissue sections from mammary glands of CD-1 mice, removed at 9 days of lactation and 2 days of involution, were immunostained using a polyclonal antibody against mouse TN. No TN was detected in the vicinity of established milk-producing alveoli in 9 day lactating glands (Fig. 1a and b). In contrast, TN was abundantly expressed in 2 day involuting mammary glands in the myoepithelial/sub-basement membrane region of the mammary gland, but not in the alveolar epithelium (Fig. 1c and d).

Tenascin synthesis is suppressed by an intact ECM

The absence of TN in the vicinity of basement membranes surrounding alveolar structures in the lactating gland suggested that expression of TN, like other ECM molecules, may be suppressed by an intact basement membrane, and upregulated in its absence (Streuli and Bissell, 1990). To directly test this hypothesis, we evaluated TN steady state mRNA levels in the mouse mammary epithelial cell strain CID-9, cultured on tissue culture plastic or on exogenous EHS matrices. Northern blot analysis showed that the 7 kb TN mRNA was highly expressed on plastic, but was absent on EHS (Fig. 2A), indicating that an intact ECM could suppress TN expression.

The CID-9 strain is a heterogeneous population comprising at least two distinct cell types including the epithelial subclone, SCp2, and the 'myoepithelial-like' SCg6 sub-population (Desprez et al., 1993). Given the localization of TN in the involuting mammary gland in vivo, we asked whether TN expression was restricted to this SCg6 population. SCp2 and SCg6 cells were co-cultured on tissue culture plastic and immunostained with TN antisera. The SCg6 cells, which are distinguished by their large flattened nuclei, were positive for TN, whereas the smaller SCp2 epithelial cells were negative (Fig. 2B). Immunoprecipitation of TN protein from SCg6 cells cultured on plastic indicated that two TN isoforms with apparent molecular masses of 190 kDa and 230 kDa were produced by these cells. Synthesis of both isoforms, however, decreased when cells were overlayed with EHS (Fig. 2C). Northern blot analysis of TN mRNA in SCg6 cells cultured on plastic or with EHS indicated that, as in the CID-9 population, TN gene expression is suppressed in the presence of an intact ECM (Fig. 2D).

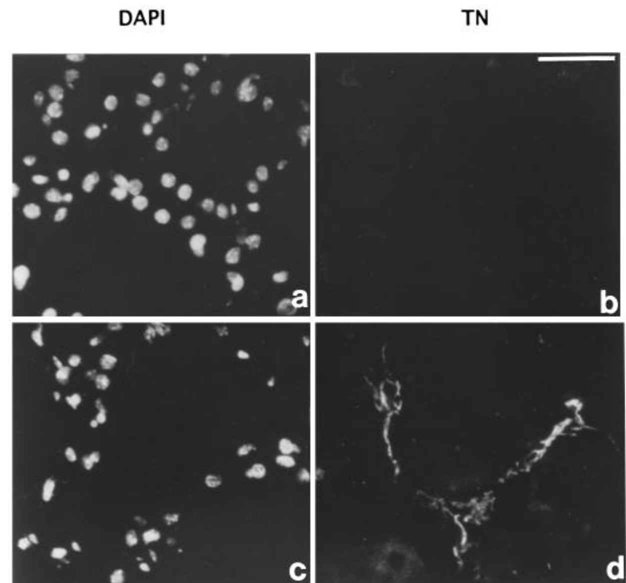


Fig. 1. Tenascin is expressed in the involuting mammary gland. Transverse frozen tissue sections from 9 day lactating (a and b), and 2 day involuting mammary gland (c and d), were stained with DAPI (left panel), and a polyclonal antiserum (pK7) to TN (right panel). No TN was detected in the sub-basement region of milk-producing alveoli in lactating tissue (b). In contrast, positive staining for TN was observed in cells surrounding the alveolar epithelium of 2 day involuting tissue (d). Bar, 50 μm.

Milk protein synthesis is inhibited by exogenous tenascin in the presence of a laminin-rich ECM

Having established that TN is down-regulated in the presence of ECM, and is not expressed by SCp2 cells, which produce milk proteins, it appeared that the presence of TN may be incompatible with the ability to produce milk proteins. To directly determine if TN could indeed interfere with the expression of milk proteins, we added exogenous TN along with EHS to SCp2 cells and assessed the effect on the synthesis of milk proteins. Immunoprecipitation of cell lysates with a broad-spectrum milk antibody after 6 days revealed that in cells cultured on plastic, little or no milk protein was expressed, whereas those overlayed with EHS synthesized high levels of milk proteins (Fig. 3A). In contrast, cells overlayed with EHS plus exogenous human (data not shown), or chicken TN (1.0-2.5 μg/ml, 4-10 nm), showed a modest decrease in the synthesis of α1-casein, transferrin and lactoferrin, and a dramatic, concentration-dependent decrease in α2- and β-casein. Addition of EHS with 2.5 μg/ml BSA had no effect on milk protein synthesis (data not shown).

When cultured on plastic, SCp2 cells formed a squamous monolayer (Fig. 3B,a); addition of EHS induced greater than 90% of SCp2 cells to aggregate and form cell clusters (Fig. 3B,b). In the presence of EHS and TN, however, the ability of the SCp2 cells to aggregate and form clusters was reduced to less than 30% with 1.0 μg/ml of TN, and to less than 10% with 2.5 μg/ml of TN (Fig. 3B,c and d). Under these conditions, the cells more closely resembled those cultured on plastic alone.

These data suggested that TN may inhibit milk protein expression by preventing cell rounding and clustering.

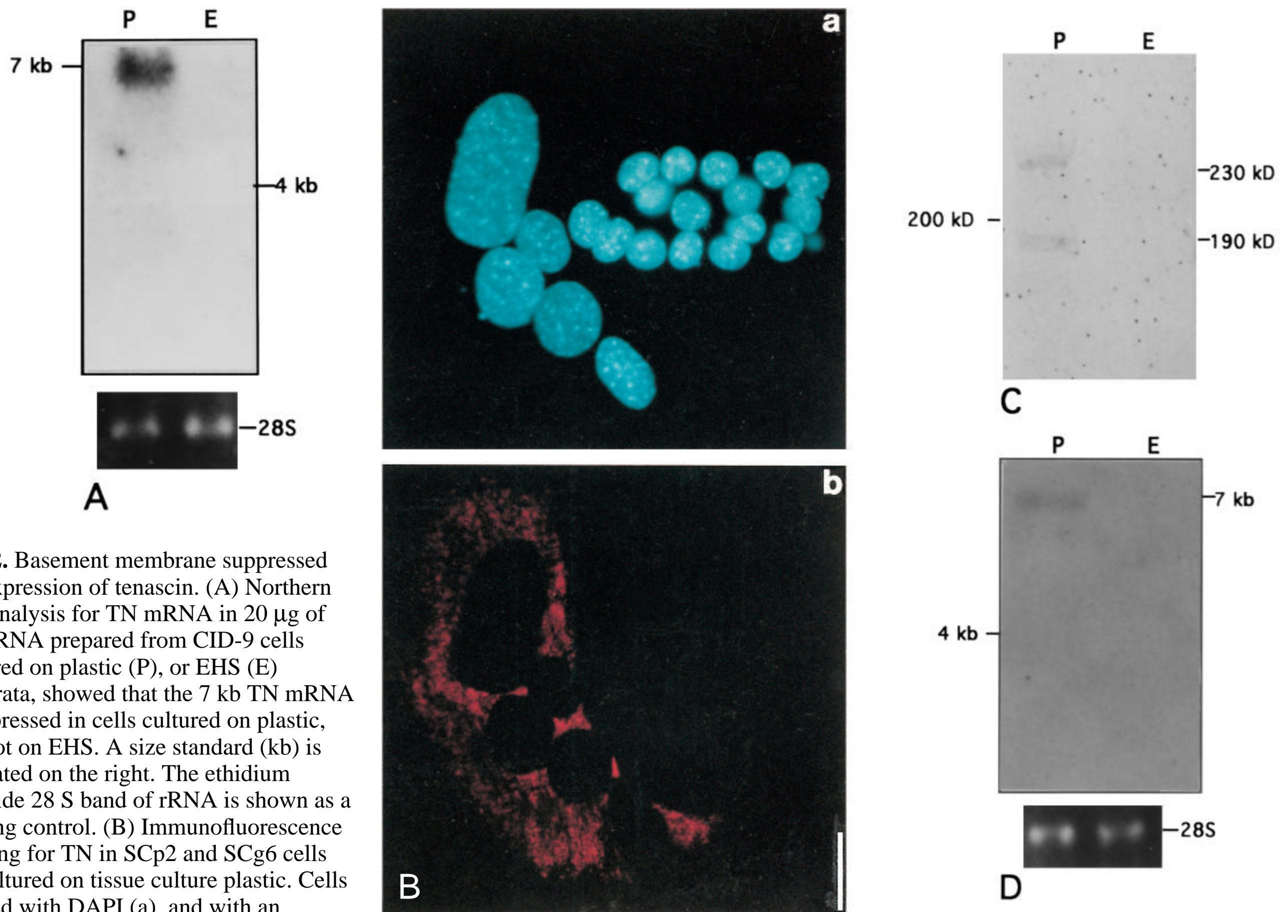


Fig. 2. Basement membrane suppressed the expression of tenascin. (A) Northern blot analysis for TN mRNA in 20 μg of total RNA prepared from CID-9 cells cultured on plastic (P), or EHS (E) substrata, showed that the 7 kb TN mRNA is expressed in cells cultured on plastic, but not on EHS. A size standard (kb) is indicated on the right. The ethidium bromide 28 S band of rRNA is shown as a loading control. (B) Immunofluorescence staining for TN in SCp2 and SCg6 cells co-cultured on tissue culture plastic. Cells stained with DAPI (a), and with an antiserum specific for mouse TN (pk7) (b). SCg6 cells, which possess large nuclei, express TN, whereas the SCp2 cell line, which possess small nuclei, are negative. Bar, 20 μm. (C) Immunoprecipitation of TN from SCg6 cells cultured either on plastic (P), or with overlayed EHS (E). SDS-PAGE of labelled cell lysates immunoprecipitated with a TN polyclonal antiserum (TN2) reveals that two TN isoforms, of apparent molecular mass 190 kDa and 230 kDa, are expressed on tissue culture plastic, but not in the presence of EHS. A size standard (kDa) is indicated on the left. (D) Northern blot analysis for TN mRNA in 10 μg of total RNA prepared from SCg6 cells cultured on tissue culture plastic (P), or with overlayed EHS (E). The 7 kb TN mRNA is expressed on tissue culture plastic, but not in the presence of EHS. A size standard (kb) is indicated on the left.

Tenascin inhibits β-casein expression in three-dimensional cultures

In order to further examine the relationship between TN's ability to modulate cell morphology and inhibit β-casein synthesis, we cultured SCp2 cells on top of EHS matrices, which induces cells to undergo complex morphological changes resulting in organized three-dimensional spheres which are functionally analogous to alveoli in vivo (Barcellos-Hoff et al., 1989). Three days later, cells were treated with 1.0 μg/ml (4 nM) or 5.0 μg/ml (20 nM) of intact TN or BSA for three additional days. As was observed with the two-dimensional cultures, treatment with TN resulted in a concentration-dependent reduction in β-casein synthesis whereas BSA had no effect (Fig. 4A).

In contrast to the two-dimensional cultures, this inhibition of β-casein expression occurred in the absence of gross morphological alterations, with TN-treated cells maintaining well-organized epithelial structures surrounding a central lumen (Fig. 4B). DAPI nuclear staining showed basally localized nuclei in both control (Fig. 5a) and TN-treated (Fig. 5b) cells on EHS matrices. No differences in the immunolocalization of E-cadherin were noted between controls (Fig. 5c) and cultures treated with TN (Fig. 5d). In addition, the pattern of actin staining was identical in both control (Fig. 5e) and TN-treated cells (Fig. 5f).

Tenascin inhibits ECM-dependent transcription of β-casein

Despite the differences in TN's ability to alter cell morphology in two-dimensional versus three-dimensional cell culture, intact TN was able to inhibit β-casein synthesis. This indicated that TN may selectively interfere with β-casein gene expression independently of its effects on cell morphology.

To establish whether the inhibition of β-casein synthesis was occurring at the level of mRNA, we compared steady state levels of β-casein mRNA in SCp2 and CID-9 cells cultured on plastic and on EHS, with and without TN. As expected, cells cultured on plastic had little or no β-casein mRNA, whereas on EHS high levels of β-casein mRNA were observed. In contrast, SCp2 and CID-9 cells cultured on EHS and overlayed with TN expressed substantially lower levels of β-casein mRNA (Fig. 6).

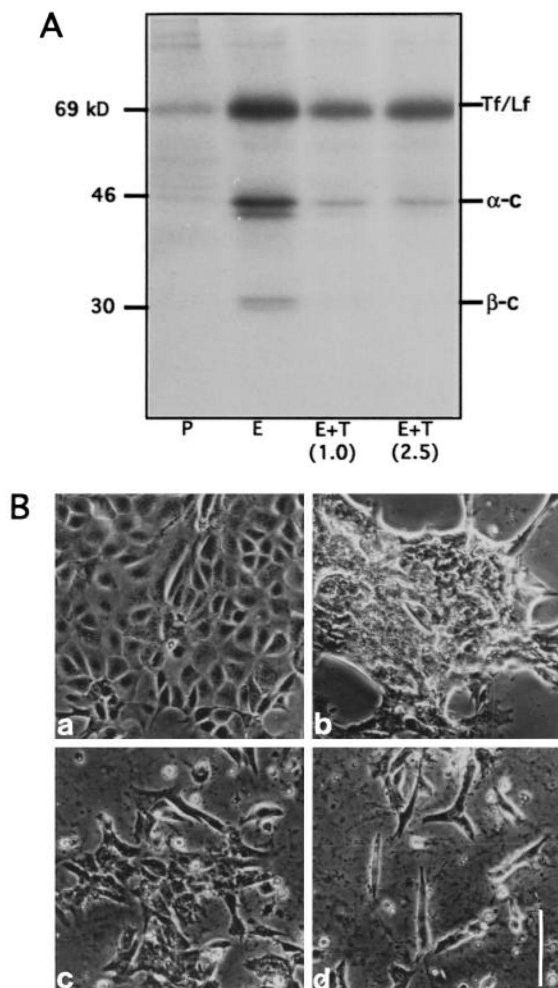
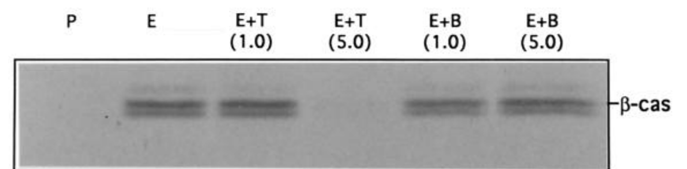


Fig. 3. Exogenous tenascin suppresses milk protein expression in cultured mammary epithelial cells. (A) Immunoprecipitation of milk proteins from SCp2 cells cultured on plastic (P), or overlayed with EHS (E), or with EHS plus intact chicken TN at 1.0 μg/ml (E+T (1.0)), or 2.5 μg/ml (E+T (2.5)). Milk protein synthesis was analyzed by SDS-PAGE following immunoprecipitation of labelled cell lysates using a polyclonal antiserum directed against total milk protein. The expression of α-2 (α-c, lower band) and β-caseins (β-c) was abolished in a concentration-dependent manner following addition of chicken TN. In contrast, the levels of α-1 casein (α-c, upper band), transferrin (Tf) and lactoferrin (Lf) were modestly reduced by TN, and were still expressed. Size standards (kDa) are indicated on the left. (B) Phase-contrast photomicroscopy reveals that SCp2 cells form a squamous monolayer on plastic (a). Cells overlayed with EHS aggregate to form cell clusters (b). Cluster formation is reduced in cells co-treated with EHS and TN (1.0 or 2.5 μg/ml) (c and d). Bar, 75 μm.



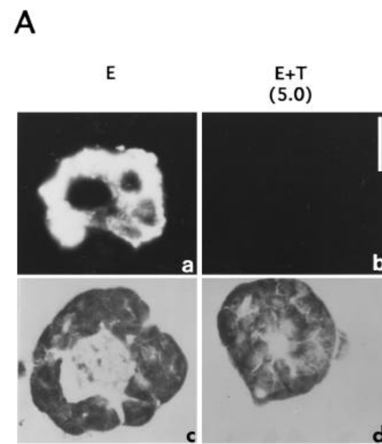


Fig. 4. Inhibition of β-casein by tenascin in three-dimensional cultures. (A) SCp2 cells were cultured either on tissue culture plastic (P), or on EHS (E), or with 1.0 μg/ml or 5.0 μg/ml of intact human TN (E+T (1.0) and E+T (5.0)), or BSA (E+B (1.0) and E+B (5.0)). Immunoprecipitation of milk proteins in labelled cell lysates shows reduced β-casein synthesis (β-cas) with 5.0 μg/ml of TN. (B) Cryosections of SCp2 cells cultured on EHS matrices, with and without 5.0 μg/ml of human TN, were examined by immunofluorescence (a,b) and phase-contrast (c,d) photomicroscopy. Immunofluorescence with a rabbit polyclonal anti-mouse antibody shows no β-casein in cells cultured with TN (b). Haemotoxylin and eosin staining revealed organized, multicellular structures with a central lumen (d). Bar, 45 μm.

Transcriptional activity of the β-casein gene is dependent upon the presence of ECM and prolactin (Schmidhauser et al., 1990, 1992). To determine whether the reduction in β-casein mRNA was a result of inhibition of transcription, we analyzed the activity of the bovine β-casein promoter linked to a CAT reporter gene stably transfected in CID-9 cells. This construct, bβcas-3815+42/CAT, contains 3.8 kb of the 5′ flanking region of the β-casein promoter, including BCE-1, an ECM-responsive enhancer element, linked to a CAT reporter gene (Schmidhauser et al., 1990). As a control, we transfected cells with a construct consisting of the Rous Sarcoma virus (RSV) promoter linked to CAT, whose activity is not modulated in the presence or absence of ECM.

Treatment of bβcas-3815+42/CAT-transfected CID-9 cells on EHS with 2.5 μg/ml of TN resulted in greater than 70% reduction in CAT activity, compared to transfected cells on EHS alone (Fig. 7). In contrast, addition of 2.5 μg/ml of fibronectin to cells preplated on EHS had no effect on the CAT activity of bβcas-3815+42/CAT-transfected CID-9 cells (data not shown). Also, addition of TN in RSV/CAT transfected cells had little effect on relative CAT activity (Fig. 7). Together, these data indicate that TN selectively inhibits transcription of the β-casein promoter.

Next we wanted to establish whether inhibition of β-casein gene transcription by TN was arising through activation of potential negative regulatory elements within the 3.8 kb promoter, or by interfering with the positive ECM-driven activity of the BCE-1 enhancer. Accordingly, we transfected cells with BCE-1/ER-1, a construct containing the isolated 161 bp BCE-l enhancer element linked to a 121 bp bovine β-casein promoter fragment and a CAT reporter gene (Schmidhauser et al., 1992) (Fig. 7). Treatment with 2.5 μg/ml TN resulted in a greater than 80% decrease in relative CAT activity when compared to cells treated with EHS alone (Fig. 7).

Specific domains of tenascin inhibit milk protein synthesis

Since TN is a large, multifunctional ECM glycoprotein (Erickson, 1993), we wanted to determine whether a particular domain(s) was responsible for down-regulating β-casein expression. Accordingly, we made use of a series of recombi-

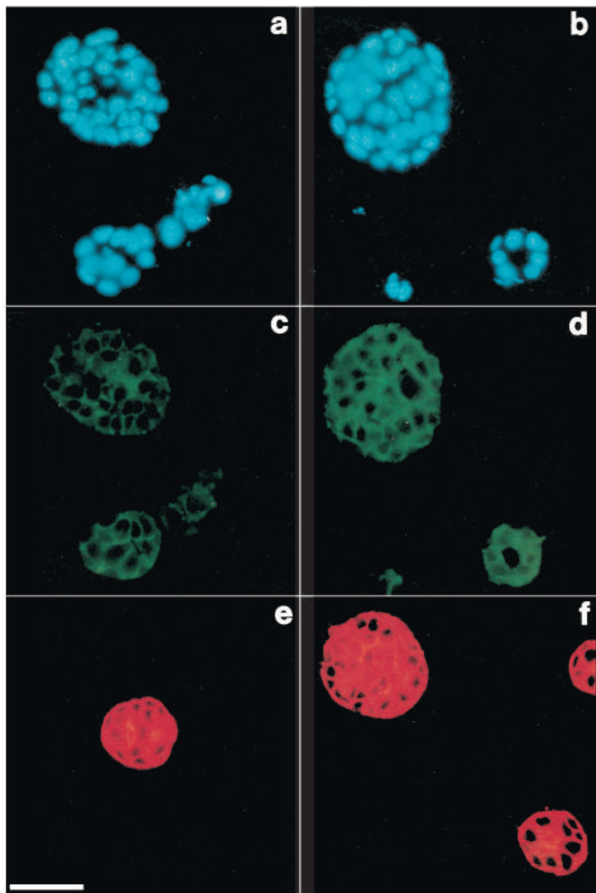


Fig. 5. Tenascin does not alter cell-cell interaction and the actin cytoskeleton in three-dimensional cultures. Cryosections of SCp2 cells cultured on EHS (E) (a,c and e), or on EHS plus 5.0 μg/ml of human TN (E+T (5.0)) (b,d and f) were examined by fluorescence photomicroscopy. Nuclear morphology of cells was revealed by DAPI staining (a,b). The distribution of E-cadherin was visualized with a mAb (ECCD-2) (c,d). Rhodamine-phalloidin was used to identify the distribution of actin (e,f). No differences in polarity, adhesion or cytoskeletal morphology were noted between control and TN-treated cells. Bar, 75 μm.

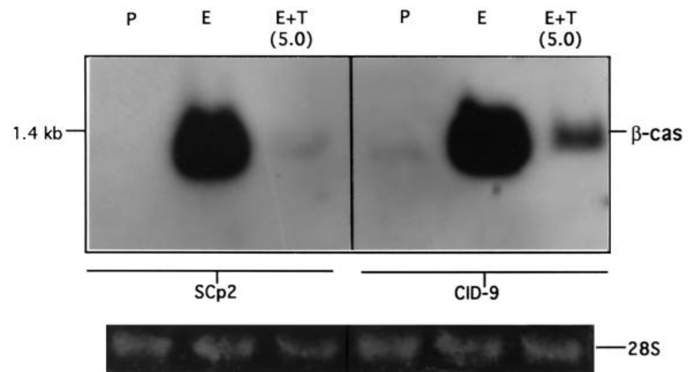


Fig. 6. Tenascin suppresses β-casein synthesis at the mRNA level. Northern blot analysis for β-casein (β-cas) in 20 μg of total RNA from SCp2 and CID-9 cells cultured either on plastic (P), on EHS (E), or on EHS with 5.0 μg/ml of human TN (E+T (5.0)). The 1.4 kb β-casein mRNA is reduced in both cell types following treatment with TN. The ethidium bromide 28 S band of total rRNA is shown as a loading control.

nant TN protein fragments, which were designed to span different and/or overlapping segments of the distal portion of the human TN-C molecule (Aukhil et al., 1993; and Fig. 8A).

TN fragments (1.0, 5.0, and 25.0 μg/ml) were added to SCp2 cells, which were overlayed with EHS, and β-casein protein synthesis was assessed after 6 days by immunoprecipitation. Two fragments, Tnfn6-8 (35, 175 and 877 nM) and Tnfbg (38, 191 and 956 nM), had no effect on milk protein synthesis when compared to cells overlayed with EHS alone. However, three fragments, Tnfn1-5 (496 nM), TnfnA-D (72 and 360 nM) and Tnfnall (168 nM), each inhibited β-casein protein synthesis in a concentration-dependent manner (Fig. 8B). Treatment of three-dimensional cultures with 25 μg/ml of Tnfn1-5 (496 nM), TnfnA-D (360 nM) and Tnfnall (168 nM) resulted in the complete inhibition of β-casein synthesis (Fig. 8C).

To further dissect which region of the TNfn1-5 fragment inhibits β-casein synthesis, we treated three-dimensional cultures with 25 μg/ml of two additional peptides derived from this fragment (Fig. 8A). The first peptide, Tnfn1-3 (815 nM), inhibited β-casein protein synthesis, whereas the second peptide, Tnfn3-5 (837 nM), had no effect (Fig. 8C).

DISCUSSION

Remarkably little is known about the nature and function of ECM components that are expressed in the mammary gland at the onset of involution. A number of previous studies have described the expression of TN in the mammary gland during normal development and in neoplastic tissue (Chiquet-Ehrismann et al., 1986; Inaguma et al., 1983; Borsi et al., 1992; Shoji et al., 1992; Lightner et al., 1994), yet its localization and putative function in the involuting mouse mammary gland have not been established. We show here that TN is expressed at high levels in the sub-basement membrane region of early involuting tissue, but not in the lactating gland. Thus TN expression is either induced by the complex events initiated at the onset of involution, or its expression is suppressed by other factors such as lactogenic hormones or basement membrane, which are present during lactation.

Our tissue culture studies indeed demonstrated that TN expression is suppressed by an intact LN-rich basement membrane. The mechanism whereby the basement membrane regulates TN synthesis was not investigated. However, TGF-β, which is a known inducer of TN (Pearson et al., 1988; Chiquet-Ehrismann et al., 1989), is also suppressed in mammary epithelial cells cultured on EHS matrix (Streuli et al., 1993). Thus a reasonable hypothesis is that EHS suppresses TN by down-regulating TGF-β.

In two-dimensional mammary epithelial cell cultures, intact TN reduces cellular clustering. Previous reports have also demonstrated that intact TN, as well as some of its domains, can modulate cell morphology (Spring et al., 1989; Lightner and Erickson, 1990; Prieto et al., 1992). For example, the TnfnA-D domain can perturb the organization of focal adhesions in endothelial cells (Murphy-Ullrich et al., 1991), as well as inhibiting adhesion of uterine epithelial cells to Matrigel (Julian et al., 1994). The Tnfbg and Tnfn1-5 domains have been shown to mediate cell adhesion

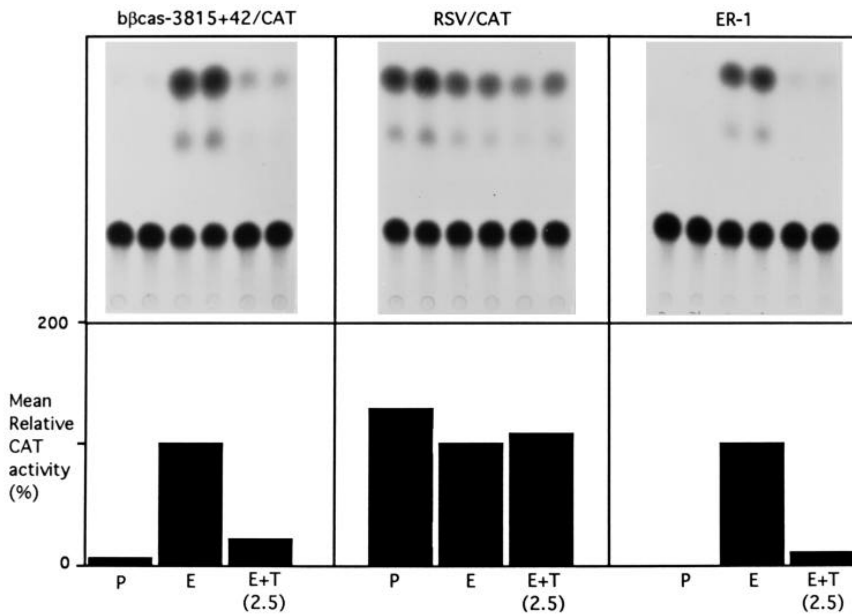
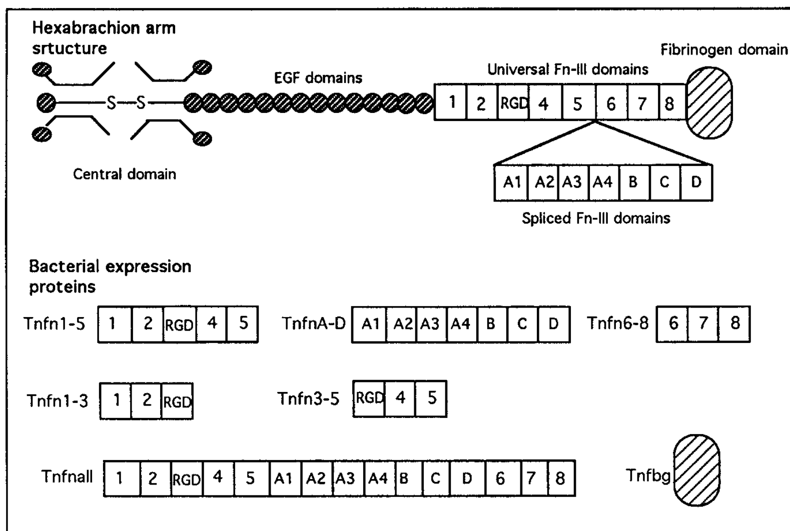


Fig. 7. Tenascin interferes with the function of the ECM/prolactin-dependent enhancer. CID-9 cells were stably transfected with the bβcas-3815+42/CAT, RSV/CAT and BCE-1/ER-1 constructs. bβcas-3815+42/CAT contained 3.8 kb of the 5′ flanking region of the bovine β-casein promoter, which includes the ECM/prolactin-responsive 161 bp BCE-1 element. This 3.8 kb sequence was linked to an inactive 121 bp 5′ flanking β-casein promoter sequence and to a CAT reporter gene (Schmidhauser et al., 1990). As a control, 560 bp of the RSV promoter and its TATA box were linked to the CAT reporter gene. BCE-1/ER-1 contains the isolated 161 bp BCE-1 element linked to the 121 bp 5′ flanking region and a CAT reporter gene (Schmidhauser et al., 1992). CAT assays were performed on duplicate plates of CID-9 cells cultured either on plastic (P), on EHS (E), or on EHS plus 2.5 μg/ml of human TN (E+T (2.5)). The upper panel shows autoradiographs, while the lower panel shows densitometric analysis of relative CAT activity. TN reduced the relative CAT activity in CID-9 cells transfected with β-casein promoter constructs.



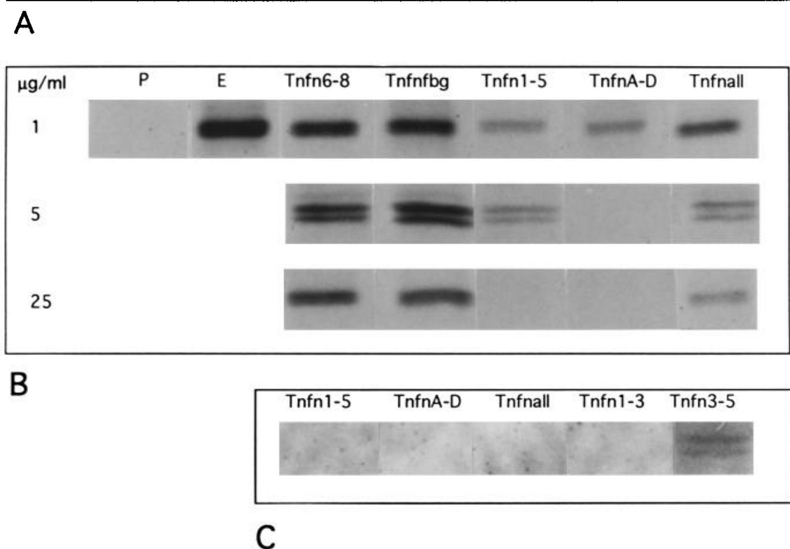


Fig. 8. Specific domains within the fibronectin type III repeat region inhibit β-casein protein expression. (A) Schematic model of the TN hexabrachion arm shown together with bacterial expression proteins (Aukhil et al., 1993). The amino-terminal end of the TN molecule is cysteine-rich, which allows individual TN polypeptide chains to associate via disulphide bonding into hexamers. Each arm of the TN molecule comprises 14 epidermal growth factor (EGF)-like repeats. This is followed by a variable number (8-15) of FN type III repeats. The universal FN type III domains are numbered 1-5 and 6-8, whereas the alternatively spliced domains are represented by letters. The first four alternatively spliced domains of human tenascin are closely related and are designated A1-A4, whereas the remaining alternatively spliced domains are designated B-D. The carboxy terminus of TN shares homology with the β-and γ-chains of fibrinogen (reviewed by Erickson, 1993). (B) SCp2 cells were cultured on plastic (P), or overlayed with EHS (E), or EHS plus the recombinant TN fragments (Tnfn6-8, Tnfnfbg, Tnfn1-5, TnfnA-D and Tnfnall), provided at 1.0, 5.0 or 25.0 μg/ml. β-Casein protein synthesis was analyzed by immunoprecipitation of labelled cell lysates using a polyclonal antiserum directed against total milk protein. Tnfn6-8 and Tnfbg had no effect on β-casein protein synthesis. In contrast, Tnfn1-5, TnfnA-D and Tnfnall, each inhibited β-casein protein synthesis in a dose-dependent manner. (C) SCp2 cells were cultured in the presence of EHS with the recombinant TN fragments (Tnfn1-5, TnfnA-D, Tnfnall, Tnfn1-3 and Tnfn3-5) provided at 25.0 μg/ml. β-casein protein synthesis was analyzed by immunoprecipitation as described above. As with two-dimensional cultures, Tnfn1-5, TnfnA-D, Tnfnall and Tnfn1-3 each inhibited β-casein synthesis, whereas Tnfn3-5 had no effect.

(Joshi et al., 1993), whereas Tnfn6-8 can inhibit cell spreading on fibronectin (Chiquet et al., 1991). In addition, it has been shown that the Tnfn1-5, TnfnA-D and Tnfn6-8 fragments can suppress branching morphogenesis in rat lung (Young et al., 1994). Ample evidence from our laboratory (Chen and Bissell, 1989; Lin and Bissell, 1993; Roskelley et al., 1994), and from others (Pienta and Coffey, 1992; Ingber, 1993), demonstrates that tissue architecture and cell morphology can profoundly influence gene expression and differentiation. However, we showed that three-dimensional mammary cell cultures treated with intact TN, down-regulate β-casein within cell structures which are indistinguishable from untreated controls in terms of size, lumen formation, E-cadherin distribution and actin morphology. Thus, by comparing the two-dimensional and three-dimensional tissue culture assays, we have demonstrated that intact TN is able to directly influence gene expression independently of its gross effects on cell morphology.

The ability of TN to regulate gene expression in the absence of an overt cell shape change has been reported in another cell system. Fibroblasts plated on a mixed substratum of FN and TN upregulate synthesis of collagenase, stromelysin, the 92 kDa gelatinase, and c-fos (Tremble et al., 1994).

Our data using the BCE-1 enhancer element indicate that TN selectively suppresses β-casein gene transcription by interfering with the positive signals generated by ECM and prolactin. Thus, it is possible that TN inhibition of β-casein synthesis may arise through disruption of cell-ECM interactions via the induction of ECM-degrading proteinases.

Recent studies in our laboratory (Roskelley et al., 1994) have identified two components of the ECM signalling pathway involved in β-casein protein synthesis. The first is physical and involves cellular rounding and clustering, whereas the second is biochemical and involves increases in tyrosine phosphorylation and integrin clustering. Therefore, a possibility exists that TN competes at the cell surface with ECM components that are essential for second-stage biochemical signalling, rather than for receptors which mediate changes in cell morphology.

Also, given that TN and LN share a common $\alpha_2\beta_1$ integrin receptor (Sriramarao et al., 1993; Hynes, 1992), and that both ECM components bind to heparin (Aukhil et al., 1993; Sung et al., 1993), a possibility exists that TN modifies and/or prevents LN-based biochemical signalling by competing for both integrin and proteoglycan receptors.

Alternatively, it is feasible that TN directly generates its own intracellular pathway which is distinct from LN-based signalling. Possible candidates for this pathway include the $\alpha_v\beta_3$ integrin (Sriramarao et al., 1993), or the recently characterized high-affinity annexin II TN-C (TnfnA-D) receptor (Chung and Erickson, 1994).

Finally, we also have demonstrated that the FN type III repeat region of TN harbours specific domains which can inhibit β-casein expression. First we showed that Tnfn1-5, TnfnA-D and Tnfnall fragments, like native TN-C, each suppresses β-casein protein synthesis, albeit to different degrees on a molar basis. Given that the Tnfnall fragment is composed of both active (Tnfn1-5 and TnfnA-D) and inactive (Tnfn6-8) domains, it is likely that the inhibitory effect observed using this fragment is attributable to the combined action of the two inhibitory domains. We have also identified the Tnfn1-3 fragment as a putative active domain within the Tnfn1-5 fragment. Thus, at least two distinct regions of the TN molecule may play an essential role in regulating mammary cell function. Determining how each domain inhibits β-casein expression has not yet been explored, but will be the focus of future studies.

During the final preparation of this manuscript, another study demonstrated that intact TN expression can inhibit the differentiated function of HC11 mammary cells (Chammas et al., 1994). Our data support and extend these studies by providing a physiological relevance and by exploring the cellular and molecular mechanisms involved. In the study by Chammas et al. (1994), it was shown that TN assembly in the extracellular space was suppressed in cells treated with lactogenic hormones. This observation is noteworthy in the light of our findings in vivo, because it suggests that a decline in the levels of of lactogenic hormones, which occurs during involution, may promote TN assembly, which in turn could inhibit β-casein synthesis.

In conclusion, our results are consistent with a model where both the loss of the LN-rich basement membrane by ECM-degrading proteinases, and the induction of TN synthesis contribute to the involuting process. Further analysis of the effects TN on mammary epithelial cell behaviour may lead to a better understanding of how this multifunctional ECM molecule regulates gene expression and cell differentiation in developing and remodelling tissues.

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