Epithelial-mesenchymal transition downregulates laminin $\alpha_5$ chain and upregulates laminin $\alpha_4$ chain in oral squamous carcinoma cells

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Abstract Basement membranes maintain the epithelial phenotype and prevent invasion and metastasis. We hypothesized that expression of basement membrane laminins might be regulated by epithelial-mesenchymal transition (EMT), hallmark of cancer progression. As EMT is mediated by transcription factor Snail, we used oral squamous carcinoma cells obtained from a primary tumor (43A), from its EMT-experienced recurrence (43B) and Snail-transfected 43A cells (43A-SNA) displaying full EMT, as a model to study laminins and their receptors. Northern blotting, immunofluorescence, and immunoprecipitation showed a gradual loss of laminin-511 and its receptor Lutheran from 43A to 43B and 43A-SNA cells. In contrast, neoexpression of laminin $\alpha_4$ mRNA was found congruent with synthesis of laminin-411. Chromatin immunoprecipitation disclosed direct binding of Snail to regions upstream of laminin $\alpha_5$ and $\alpha_4$ genes. Immunofluorescence and immunoprecipitation showed a switch from hemidesmosomal integrin $\alpha_6\beta_4$ to $\alpha_6\beta_1$ and neoexpression of $\alpha_1\beta_1$ in 43A-SNA cells, and upregulation of integrin-linked kinase in both 43B and 43A-SNA cells. The cells adhered potently to laminin-511 and fibronectin, whereas adhesion to laminin-411 was minimal. In contrast, laminin-411 inhibited cell adhesion to other extracellular matrix proteins. In conclusion, EMT induces a switch from laminin-511 to laminin-411 expression, which may be directly controlled by Snail. Concomitant changes take place in laminin- and collagen-binding receptors. Laminin-411 reduces adhesion to laminin-511 and fibronectin, suggesting that tumor cells could utilize laminin-411 in their invasive behavior.

Keywords Epithelial-mesenchymal transition · Basement membrane · Laminin $\alpha_5$ chain · Laminin $\alpha_4$ chain · Oral squamous cell carcinoma · Snail

Abbreviations

BM Basement membrane
ECM Extracellular matrix
EMT Epithelial-mesenchymal transition
GAPDH Glutaraldehyde-3-phosphate-dehydrogenase
ILK Integrin-linked kinase
Lm Laminin
MAb Monoclonal antibody
Introduction

Basement membranes (BM) are sheets of extracellular matrix (ECM) generated by the cells at epithelial-mesenchymal interface. BMs underlie epithelia and endothelia and encircle certain isolated cells. Structural components of BM include laminins, type IV collagens, nidogens, and proteoglycans (Kalluri 2003). BM guards the epithelial phenotype and has classically been regarded to act as a barrier that averts carcinoma cells from invading the surrounding interstitial stroma. Therefore, breakdown of BM has been considered as a crucial step towards progression of malignancy (Bosman et al. 1992; Liotta and Kohn 2001).

Laminins are trimeric glycoproteins composed of α, β, and γ chains. To date, 15 different laminins have been recognized (Miner and Yurchenco 2004; Aumailley et al. 2005). Expression of different laminin chains is cell- and tissue-specific, and individual cells are able to produce several laminins simultaneously. Secretion of the laminin trimer and hence the formation of whole BMs, as well as the cell-BM interactions are regarded primarily as α chain-dependent (Matsui et al. 1995; Yurchenco et al. 1997). Laminin α5 chain is a component of laminins-511, -521 and -523 (Miner and Yurchenco 2004; Aumailley et al. 2005). Laminin-511 is regarded as the most widely expressed laminin found in most epithelial BMs. In contrast, laminin α4 chain, a component of laminins-411, -421 and -423, is primarily produced by cells of mesenchymal origin, e.g., muscle, adipose, and endothelial cells (Lefebvre et al. 1999; Petäjäniemi et al. 2002). Laminin α4 chain has a role in cell migration, invasion and endothelial transmigration (Síxt et al. 2001; Kha-zenzon et al. 2003; Wondimu et al. 2004). Accordingly, overexpression of laminin α4 chain appears to correlate with increasing malignancy in gliomas (Ljubimova et al. 2004). However, the roles of both laminin α4 and α5 chains still remain elusive in malignant progression of carcinomas.

Adhesion and interaction of normal and tumor cells with ECM is mediated primarily by integrins, but also by some non-integrin molecules (Hynes 2002; Miner and Yurchenco 2004). Integrins, composed of α and β subunits, not only link the ECM with cytoplasmic structures, but also transmit signals to the cell interior by activating multiple pathways with effects on proliferation, survival, and apoptosis. Furthermore, Lutheran glycoprotein is a specific receptor for α5 chain laminins (Kikkawa and Miner 2005). Integrin-linked kinase (ILK) connects integrins to the actin cytoskeleton and regulates actin polymerization. Through its kinase activity, ILK activates several signaling pathways (Oluomi et al. 2004).

Epithelial-mesenchymal transition (EMT) is an important process occurring during gastrulation and mesoderm formation in embryonic development, but it also operates in the formation, invasion, and metastasis of carcinomas (Nieto 2002; Peinado et al. 2007). EMT has a role in progression of malignancy, e.g., in oral squamous cell carcinoma (SCC) (Yanjia and Xinchun 2007). EMT leads to loss of epithelial cell polarity and cell–cell contacts, which are reflected in the reorganization of the cellular cytoskeleton and loss of E-cadherin. Snail (Batlle et al. 2000; Cano et al. 2000) and other transcription factors, e.g., ZEB-1 and ZEB-2, repress E-cadherin and their profiling has associated them with EMT in different carcinomas (Peinado et al. 2007). Much interest has been devoted to Snail, which has been shown to directly repress several epithelial genes, e.g., MUC1, cytokeratin 18, claudins, and occludin (Guaita et al. 2002; Ikenouchi et al. 2003; Ohkubo and Ozawa 2004) as well as to upregulate mesenchymal genes, such as fibronectin and vimentin (Batlle et al. 2000; Cano et al. 2000; Takkunen et al. 2006). Transcriptional regulation of these molecules occurs through the consensus DNA-binding sequence for Snail, called the E-box motif (5′-CANNTG-3′) (Mauhin et al. 1993).

As our previous studies showed that EMT downregulates epithelial laminin-332 (Takkunen et al. 2006), we hypothesized that Snail could also have an effect on expression of laminins-511 and -411 during progression of oral SCC. To test this hypothesis, we evaluated the production of laminin α5 and α4 chains and their receptors in oral SCC cells. We further studied eventual functional consequences of laminin α5 and α4 chain expression with cell adhesion studies. With a cell model presenting endogenous or exogenous EMT, we examined the direct effect of transcription factor Snail on regulation of laminin expression.

Materials and methods

Cell culture

Oral squamous cell carcinoma cell line UT-SCC-43A (43A) was derived from a primary gingival tumor of a 75-year-old Caucasian female. The tumor was staged T4N1M0. This tumor later recurred after radiation therapy and surgery and the cell line UT-SCC-43B (43B) was derived from a recurrent tumor. 43A cells were permanently transfected with full-length, hemagglutinin-tagged cDNA of murine Snail (Batlle et al. 2000), manually cloned and selected with G418 (Sigma, St. Louis, MO). 43A, 43B and Snail-transfected 43A-SNA cells have been previously characterized (Takkunen et al. 2006). The cells were cultured in RPMI 1640 medium (Sigma) with 10% fetal calf serum and antibiotics.
Indirect immunofluorescence microscopy

The cells were grown on glass coverslips and fixed in methanol at −20°C or in 4% paraformaldehyde at room temperature for 15 min. Primary antibodies (Table 1) were applied for 1 h followed by Alexa Fluor® 488 or 594 conjugates (Molecular Probes/Invitrogen, Eugene, OR) for 30 min. The specimens were studied with Leica Aristoplan microscope equipped with an epi-illuminator and appropriate filters. Confocal microscopy was carried out using a Leica TCS SP2 AOBS system (Leica Microsystems AG, Mannheim, Germany) with argon excitation line 488 nm or DPSS 561 nm and HCX PL APO CS 63×1.40 NA oil immersion objective. Image stacks were collected through the specimen using a standardized 120 nm z-sampling density. Selected image stacks were further subjected to deconvolution and restoration using theoretical point spread function and iterative maximum likelihood estimation algorithm (Scientific Volume Imaging BV, Hilversum, the Netherlands). In a subset of experiments, cells were treated with 5 μM monensin (Sigma) overnight in order to inhibit secretion of newly synthesized proteins (Tartakoff 1983).

Cell morphology and invasion assays

Cell morphology and invasion were examined with modified Boyden chambers. Falcon FluoroBlok Individual Cell Culture Inserts (BD Biosciences, San Jose, CA) with 8-μm pore sizes were coated with 5 mg/ml Matrigel (BD Biosciences) for 1 h. 5 × 10^4 cells in 350 μl culture medium were added to the upper chamber of the insert, and 900 μl culture medium was added to the lower chamber. The cells were allowed to grow and invade at 37°C for 24 h, after which the filters were fixed with 4% paraformaldehyde at room temperature for 10 min and stained with rhodamine phalloidin (Molecular Probes/Invitrogen). Filters were detached from the inserts with a scalpel, mounted on objective glasses in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and covered with cover slips. Cell growth and morphology were studied in the upper chamber and the invaded cells were detected in the lower chamber. The cells were photographed and counted with Olympus AX70 microscope (Olympus Corporation) using 10× or 20× objectives or with confocal microscope as mentioned above. The experiments were repeated at least three times. The differences between the groups were tested with a two-sided, unpaired t-test with the significance level set at α = 0.05.

Northern blot

Northern blots were performed with non-radioactive, digoxigenin-labeled cRNA probes (Roche Diagnostics, Penzberg, Germany) (Takkunen et al. 2006). Total RNA was extracted with Eurozol (Euroclone, Milan, Italy), and poly-A-RNAs were enriched with Dynabeads Oligo (dT) 25-beads (Dynal Biotech, Oslo, Norway). The RNAs were separated in denaturing 1.2% agarose gels and transferred by upward capillary transfer onto Hybond membranes (Amersham Biosciences, Uppsala, Sweden). The membranes were UV-crosslinked and hybridized with digoxigenin-labeled cRNA probes generated from linearized plasmid cDNA templates.

Table 1 Antibodies and antisera used

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Antibody</th>
<th>References</th>
</tr>
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<td>168FC10</td>
<td>Petäjäniemi et al. (2002)</td>
</tr>
<tr>
<td>Laminin α4 chain</td>
<td>Polyclonal laminin α4 chain antiserum</td>
<td>Ivivanainen et al. (1997)</td>
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<tr>
<td>Laminin α4 chain</td>
<td>3H2</td>
<td>Wondimu et al. (2004)</td>
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<td>Laminin α5 chain</td>
<td>4C7</td>
<td>Engvall et al. (1986)</td>
</tr>
<tr>
<td>Laminin β2 chain</td>
<td>S5F11</td>
<td>Wewer et al. (1997b)</td>
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<tr>
<td>Laminin γ1 chain</td>
<td>113BC7</td>
<td>Määttä et al. (2001)</td>
</tr>
<tr>
<td>Snail</td>
<td>173EC3</td>
<td>Franci et al. (2006), Takkunen et al. (2006)</td>
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<td>Serotec, Oxford, UK</td>
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<td>Yläne and Virtanen (1989)</td>
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<td>Integrin β3 subunit</td>
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<td>Integrin-linked kinase</td>
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<td>Fibronectin</td>
<td>Polyclonal fibronectin antiserum</td>
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Antibodies are monoclonal unless otherwise stated.
by in vitro-transcription. Prehybridization and hybridization with DIG Easy Hyb granules (Roche) were carried out at 68°C for 30 min and for 18 h, respectively. The probes were detected with alkaline-phosphatase-conjugated anti-digoxigenin antibody and CSPD (Roche). The blots were then exposed to Hyperfilm MP (Amersham Biosciences). For re-use of the blots, the membranes were washed twice in stripping solution (50% formamide, 5% SDS, 50 mM Tris-HCl, pH 7.2) at 80°C for 60 min, and re-probed. The following cRNA probes were used: a 1.5-kb fragment of laminin z5 chain covering nucleotides 9805-11332 (Durkin et al. 1997), and a 2.7-kb fragment of laminin z4 chain covering nucleotides 94-2808 (Kortesmaa et al. 2000). Hybridizations with antisense GAPDH probes were used to confirm the equal loading of mRNA, and hybridizations with sense cRNA probes were used as negative controls (not shown). Digoxigenin-labeled RNA molecular weight marker I (Roche) was used as a size marker.

Immunoprecipitation and Western blot

For immunoprecipitation experiments of laminin z5, β2 and z4 chains, methionine-starved 43A, 43B and 43A-SNA cells were labeled overnight with [35S]methionine (50 μCi/ml; Amersham Biosciences). For laminin γ1 chain immunoprecipitations, the cells were left unlabeled. Culture medium was collected, cleared by centrifugation, and supplemented with normal mouse serum and 0.5% Triton X-100. For integrin and Lutheran immunoprecipitations, [35S]methionine-labeled cells were scraped off with rubber policeman and solubilized in ice-cold RIPA buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.1% SDS, 1.0% Triton X-100, 1.0% deoxycholate, 5 mM EDTA, and 1 mM PMSF). The samples were then preabsorbed with uncoupled GammaBind Plus Sepharose beads (Amersham Biosciences), followed by application to GammaBind Plus Sepharose beads prebound with antibodies (Table 1), and incubated at 4°C overnight. The precipitated proteins were separated with SDS-PAGE following Laemmli’s procedure with reducing 5–8% gels. [14C]Methylated Molecular Weight Marker (Amersham Biosciences) or Molecular Weight Marker (M.W. 30,000–200,000; Sigma) were used. Immunoprecipitated bands from dried gels were detected using Hyperfilm MP (Amersham Biosciences). For Western blots, the immunoprecipitated samples were diluted in reducing Laemmli’s sample buffer and transferred onto nitrocellulose filters, which were blocked with 5% dry milk in phosphate-buffered saline. Polyclonal antiserum against laminin z4 chain (Iivanainen et al. 1997) or fibronectin (Dako, Glostrup, Denmark) was applied, and the immunoreactive bands were detected with SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) using horseradish peroxidase-coupled anti-rabbit immunoglobulins (Dako).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed with ChIP-IT Express Assay Kit (Active Motif, Carlsbad, CA) according to instructions of the manufacturer. Briefly, 43A-SNA cells were fixed with 1% formaldehyde at room temperature for 15 min to crosslink the DNA-binding proteins to DNA. The DNA was sheared into fragments with a Dounce homogenizer followed by enzymatic digestion at 37°C for 15 min. A portion of chromatin lysate was stored as input control. DNA-protein complexes were immunoprecipitated at 4°C overnight using Protein G beads with 2–6 μg of mouse IgG antibody (Dako), positive antibody against RNA polymerase II, or monoclonal antibody (MAb) 173EC3 against Snail (Francí et al. 2006; Takkunen et al. 2006). The DNA was eluted, the crosslinks were reversed at 94°C for 15 min, and proteins were removed with Proteinase K at 37°C for 1 h. Then, the DNA was used as a template for PCR.

PCR with primers designed to cover laminin z5 and z4 chain promoter regions (Tables 2 and 3) were used to determine if the DNA sequences had bound Snail. First, promoter sequences for laminin z5 (NM_005560) and z4 (NM_002290) chain genes were extracted from human genome sequence using Genomatix Gene2Promoter software (Genomatix Software, Munich, Germany). Overlapping primers covering the genomic region 3,000 bp upstream of laminin z5 and z4 transcription start sites were designed with Primer3 software (Rozen and Skaletsky 2000) and were produced by Oligomer (Helsinki, Finland). Primers for GAPDH, used to detect Input DNA, were provided by the kit manufacturer. Primers were mixed with AmpliTaq Gold DNA polymerase in PCR buffer (Applied Biosystems, Foster City, CA). PCR amplification was performed in a thermal cycler (Robocycler Gradient 40; Stratagene, La Jolla, CA) as follows: reaction mixture was denatured at 95°C for 10 min, after which 40 cycles were run with denaturation at 95°C for 1 min, annealed at 60–64°C for 1 min, extended at 72°C for 1 min, and finally extended for 20 min. The samples were fractionated through 1% agarose gels with a 100-bp DNA ladder (Invitrogen, Paisley, UK). Search for E-box (5’-CA(C/G)(C/ G)TG-3’) and Z-box motifs (5’-CAGGTG/A-3’) was performed with MatInspector software (Genomatix Software).

Quantitative cell adhesion assay

Cell adhesion experiments were performed on 96-well plates using intracellular acid phosphatase activity (Prater et al. 1991). The wells were coated with 4 μg/ml recombinant human laminin-411 (Kortesmaa et al. 2002), 4 μg/ml native human laminin -511 or 5 μg/ml fibronectin at room temperature for 1 h. Laminin-511 was purified from the
culture medium of PANC-1 pancreatic adenocarcinoma cells with immunoaffinity chromatography (Tani et al. 1999), and fibronectin was purified from outdated human plasma (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) with gelatin-Sepharose affinity chromatography (Amersham Biosciences) (Engvall and Ruoslahti 1977). The wells were post-coated with 3% bovine serum albumin (Sigma) at room temperature for 1 h. In order to prevent synthesis and secretion of endogenous proteins, the cells were preincubated with cycloheximide (10 μg/ml; Sigma) at 37°C for 1 h, after which the cells were trypsini-
ed and treated with trypsin-neutralizing solution (Promocell, Heidelberg, Germany). 2 × 10^4 43A, 43B and 43A-SNA cells in serum-free medium supplied with cyclo-
heximide were added to each well, and the plates were incubated at 37°C for 1 h. After washing in phosphate-
buffed saline, phosphatase substrate solution (5 mg/ml Phosphatase substrate in 50 mM acetate buffer, pH 5.0; Sigma, 1% Triton X-100) was added and the plates were incubated at 37°C for 1 h. The reaction was stopped with 1 M NaOH and the absorbances were measured at 450 nm. The experiments were performed in triplicate, and absorbances were expressed ±SD of three wells. The difference between the groups were tested with a two-sided, unpaired t-test with the significance level set at α = 0.05.

### Results

To study the expression of laminins and their receptors in oral SCC cells, we used a cell model with characteristics of EMT. This in vitro model comprises primary tumor cell line 43A, endogenously EMT-experienced recurrence 43B, and Snail-transfected primary tumor cell line 43A-SNA (Takkunen et al. 2006). 43A cell line displays a typical, epithelial cobblestone-like phenotype with E-cadherin at cell–cell junctions, whereas 43B cells show distinct mesenchymal characteristics, e.g., N-cadherin at cell–cell junctions, abundance of vimentin filaments and disappearance of squamous cell cytokeratins. 43B cells express E-cadherin repressors ZEB-1 and ZEB-2. 43A cells acquire a complete mesenchymal phenotype when stably transfected with Snail (43A-SNA) (Takkunen et al. 2006).

In this study, we first examined the invasion capabilities of 43A, 43B and 43A-SNA cells using modified Boyden chambers. The cells were seeded on Matrigel-coated filter
chambers and allowed to grow for 24 h. 43A cells formed large, round colonies in Matrigel, reminiscent of low-metastatic cells. 43B and especially 43A-SNA cells formed branching colonies with few cell–cell contacts (Fig. 1a–c). The cells which had invaded the matrix to the lower chamber were stained and counted. A significant increase in cell invasion was found in 43B (P < 0.001) and 43A-SNA cells (P < 0.001) when compared to 43A cells. Invasion of the

Table 3  Primers used for chromatin immunoprecipitation, covering 3,000 bp upstream of laminin α4 chain (NM_002290) sequence

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Position indicates regions upstream of laminin α4 chain gene transcription start site

Fig. 1  Cell morphology and cell invasion of 43A, 43B and 43A-SNA cells. 50,000 cells were seeded on Matrigel-coated filters and allowed to grow and invade for 24 h. Confocal images show the morphological differences between cell colonies in the upper filter chamber (a–c). 43A cells formed round colonies, whereas 43B and 43A-SNA cells displayed a mesenchymal phenotype with reduced cell–cell junctions. An example of the invaded cells is shown in the lower filter chamber after 24 h (d–f). The invasion ability of EMT-experienced 43B and 43A-SNA cells was significantly (P < 0.001) increased when compared to 43A cells. Invasion of the
matrix by 43B cells was fivefold compared to invasion by 43A cells, and invasion by 43A-SNA cells was 50-fold to that by 43A cells (Fig. 1d–g).

Laminin α5 chain expression is downregulated in EMT-experienced cells

Our previous studies showed that EMT terminated the synthesis of epithelial laminin-332 (Takkunen et al. 2006). We therefore studied the expression of laminin α5 chain, found in all epithelial BMs with Northern blots. A 12-kb transcript corresponding to laminin α5 chain mRNA was detected in 43A and 43B cells, the transcript appearing slightly weaker in 43B cells, whereas in 43A-SNA cells, no expression of laminin α5 chain mRNA was detected (Fig. 2a).

Expression of the corresponding laminin α5 chain protein was studied with immunofluorescence microscopy. The cells were treated with monensin to inhibit protein secretion so that the newly synthesized proteins accumulate in the cytoplasm. Immunoreactivity for laminin α5 chain was found in cytoplasmic vesicles in 43A and to some extent also in 43B cells, whereas no reactivity was detected in 43A-SNA cells (Fig. 2b).

Synthesis and secretion of laminin α5 chain was further studied with immunoprecipitation of [35S]methionine-labeled cells (Fig. 2c). MAb 4C7 precipitated two polypeptides of M₉, 380,000 and 390,000, corresponding to laminin α5 chain (Champliaud et al. 2000), from the culture medium of 43A cells. In addition, two polypeptides of M₉, ca. 200,000 and M₉, 220,000 were detected, corresponding to laminin γ1 and β1 chains, respectively. Also in 43B cell culture medium laminin α5, β1, and γ1 chains were found, but the bands were distinctly fainter in 43B medium than in the 43A medium. In 43A-SNA cell culture medium no polypeptides were precipitated with MAb 4C7. Immuno-
precipitations with MAb S5F11 against laminin β2 chain did not detect any polypeptides in 43A, 43B, or 43A-SNA cells, indicating that laminins-521 or -421 were not produced (Fig. 2c). These results suggest that 43A cells synthesize and secrete laminin α5 chain in form of laminin-511, whereas in 43B cells, expression of laminin α5 is decreased. 43A-SNA cells do not at all express laminin α5 mRNA and do not synthesize and secrete laminin-511.

Laminin α4 chain is upregulated in EMT

Since laminin α4 chain is produced by cells of mesenchymal origin, we investigated the expression of laminin α4 chain mRNA in EMT-experienced SCC cells. Northern blots with cRNA probe to laminin α4 chain showed no transcripts in 43A cells, whereas 6.5-kb transcripts of laminin α4 chain were found in 43B and 43A-SNA cells (Fig. 3a). Similarly, after monensin treatment, MAb 168FC10 showed no immunoreactivity for laminin α4 chain protein in cytoplasmic vesicles in 43A cells, whereas laminin α4 chain protein was found in both 43B and 43A-SNA cells (Fig. 3b).

Further, immunoprecipitation with MAb 3H2 against laminin α4 chain showed no polypeptides in 43A cell culture medium, whereas prominent bands of Mr 180,000 and 220,000 corresponding to laminin γ1 and β1 chains (Champliaud et al. 2000) were detected in 43B and 43A-SNA cells (Fig. 3c). However, because precipitates of laminin α4 chain are of the same sizes as γ1 and β1 chains, the size of laminin α4 chain could not be verified confidently. Therefore, additional Western blots were performed to detect laminin α4 chain. Immunoprecipitation of culture

Fig. 3 Expression of laminin α4 chain and laminin-411 in 43A, 43B and 43A-SNA cells. Northern blots of 43A, 43B and 43A-SNA cells (a) with an antisense probe to laminin α4 chain detected a 6.5-kb transcript in 43B and 43A-SNA cells. Laminin α4 chain mRNA was not found in 43A cells. Equal loading of mRNA was ensured with GAPDH hybridizations. Monensin treatment showed cytoplasmic accumulation of laminin α4 chain (b) in 43B and 43A-SNA cells, whereas 43A cells remained negative. Scale bar: 10 μm. Immunoprecipitation of culture medium with MAb against laminin α4 chain (c) showed bands of Mr 180,000–220,000 corresponding to laminin β1 and γ1 chains in 43B and 43A-SNA cells, whereas 43A cells were negative. C, control lane with no antibody. Immunoprecipitation of culture medium with MAb against laminin γ1 chain followed by Western blot with polyclonal antibody against laminin α4 chain (d) showed no bands in 43A cells. Instead, broad bands of Mr 180,000–220,000, indicating presence of laminin α4 chain, were detected in 43B and 43A-SNA cells. C, control lane with no antibody.
medium with MAb 113BC7 against laminin γ1 chain followed by Western blot with polyclonal antibody against laminin α4 chain showed no bands in 43A cells (Fig. 3d). Instead, broad laminin α4 chain bands of Mr ca. 180,000–220,000 (Kortesmaa et al. 2000; Sasaki et al. 2001) were seen in 43B and 43A-SNA cells. A slight size difference was found between the precipitated proteins in 43B and 43A-SNA cells, which suggests that the secreted laminin α4 chains are differently N-glycosylated or modified by glycosaminoglycans (Sasaki et al. 2001; Kortesmaa et al. 2002; Wondimu et al. 2004). Taken together, these results suggest that expression of laminin α4 chain mRNA and laminin-411 is induced upon EMT.

Snail binds directly to laminin α5 and laminin α4 promoter sequences

To determine whether downregulation of laminin α5 chain and upregulation of laminin α4 chain represents a direct effect of Snail action, we conducted chromatin immunoprecipitation (ChIP). Immunoprecipitation of 43A-SNA cells with Snail antibody followed by PCR amplification with primers (Tables 2 and 3) covering 3,000 bp upstream of laminin α5 and α4 chains were used to detect Snail-chromatin complexes. ChIP with Snail antibodies disclosed two promoter regions (−1890/−1535; −1016/−572) upstream of laminin α5 (Fig. 4a) and three regions (−2059/−1732; −1339/−1007; −873/−533) upstream of laminin α4 chain gene (Fig. 4b).

Snail binds to E-box motifs 5′-CANNTG-3′ in promoter sites (Mauhin et al. 1993). E-box motifs were present in both of the laminin α5 promoter precipitates (one CAGGTG sequence within −1890/−1535; two CAGGTG, and two CAGCTG sequences within −1016/−572), and in two of the three laminin α4 promoter precipitates (one CAGGTG sequence within −1339/−1007 and one CAGCTG sequence within −873/−533) (Fig. 4c). Within −2059/−1732 of laminin α4 promoter precipitate, a highly similar sequence, CAGGTA, also called Z-box motif, was detected. Taken together, these results suggest that Snail...
binds in vivo to specific 5′-flanking regions of laminin α5 and laminin α4 chain genes and thus may directly control their expression.

Laminin α5 chain receptor Lutheran is downregulated in EMT

To find out whether EMT also affects the laminin α5 chain-mediated signaling at the receptor level, we first studied the expression of Lutheran glycoprotein, a specific non-integrin receptor for laminin α5 chain (Kikkawa and Miner 2005). 43A cells showed a strong cell surface-confined immunofluorescence for Lutheran (Fig. 5a), whereas in 43B cells the immunoreactivity was punctate and heterogeneously distributed. No Lutheran immunoreactivity was found in 43A-SNA cells. In immunoprecipitation of RIPA-extracted cells, polyclonal antibody against Lutheran revealed a prominent Mr 85,000 band, corresponding to Lutheran (Parsons et al. 2001) (Fig. 5b). In 43B cells, only a faint band was detected, whereas in 43A-SNA cells no immunoreactive bands were found. These results suggest that during EMT, synthesis of both laminin-511 and its receptor, Lutheran, are decreased.

Integrin subunits reassemble upon EMT of oral SCC cells

We then performed immunofluorescence microscopy and integrin immunoprecipitations from RIPA-extracted cells to analyze the expression of different laminin- and collagen-binding receptors in our cell model. Integrin α6β4 binds laminin-332 at hemidesmosomes, but also acts as a receptor for laminin-511 (Kikkawa et al. 2000; Pouliot et al. 2001). Immunoreactivity for integrin α6 subunit was detected in 43A cells in a granular manner, a labeling pattern typical for hemidesmosomes (Takkunen et al. 2006) (Fig. 5a). In 43B and 43A-SNA cells, only diffuse, cell surface-confined immunoreactivity was detected. However, immunoreactivity for integrin-linked kinase (ILK) was not found in 43A cells, whereas in 43B and 43A-SNA cells prominent, elongated streaks of reactivity were detected, resembling focal adhesions.

Immunoprecipitations with MAb 102DF5 against integrin β1 subunit revealed Mr 110,000 bands in all cells associated with several z subunits (Fig. 5b; lanes 1, 6, 11). MAb AA3 against integrin β1 subunit showed in 43A cells Mr 140,000 and Mr 210,000 bands, corresponding to integrin α6 and β4 (lane 2), respectively, whereas in 43B and 43A-SNA cells no integrin subunits were detected (lanes 7, 12). MAb TS2/7 against integrin α1 subunit did not precipitate any polypeptides in 43A and 43B cells (lanes 3, 8), whereas in 43A-SNA cells, prominent bands of Mr 200000 and Mr 110,000 bands were found, corresponding to integrin α1 and β1 subunits (lane 13). MAb GoH3 against integrin α5 subunit showed that integrin α5 pairs with β4 in 43A and 43B cells (lanes 4, 9), but in 43A-SNA cells, α6 pairs with β1 (lane 14). Western blots showed no ILK bands in 43A cells, whereas clear Mr 59,000 bands, corresponding to ILK (Somasiri et al. 2001), were seen in 43B and 43A-SNA cell lysates (Fig. 5b). These results suggest that 43A and to some extent also 43B cells express integrin α6β4, whereas in 43A-SNA cells, α6 associates with β1 subunit. Integrin α6β4 has been shown to bind laminin-332 and laminin-511, whereas integrin α6β1 is among the few receptors for laminin-411 (Kortesmaa et al. 2000; Fujiwara et al. 2001). Furthermore, in 43A-SNA cells, neoexpression of collagen receptor integrin α1β1 was detected, and ILK was found in 43B and 43A-SNA cells.

Laminin-411 decreases adhesion of oral SCC cells

In order to elicit the role of different laminins in the adhesion of oral SCC cells, we performed quantitative cell adhesion assays. Wells of 96-well plates were coated with 5 μg/ml fibronectin or with 4 μg/ml laminins -511 or -411, after which the cells were allowed to adhere. Cycloheximide was applied to prevent endogenous secretion and deposition of ECM proteins. The cells adhered significantly (P < 0.001) to fibronectin and laminin-511, whereas adhesion to laminin-411 was negligible (Fig. 6a).

Since it has been shown that laminin-411 may have a role in detachment and migration of some cancer cells (Fujiwara et al. 2001; Khazenzen et al. 2003; Vainionpää et al. 2007), we studied whether coexisting laminin-411 has an effect on cell adhesion to fibronectin and laminin-511. The wells were again coated with either 5 μg/ml fibronectin or 4 μg/ml laminin-511 but together with increasing concentrations (1–20 μg/ml) of laminin-411. Adhesion of 43A, 43B and 43A-SNA cells to fibronectin was significantly (P < 0.001) inhibited already with 5 μg/ml laminin-411 (Fig. 6b). Total inhibition of adhesion of 43A and 43B cells, and 60% inhibition of 43A-SNA cells to fibronectin was gained with 20 μg/ml laminin-411. Laminin-411 also inhibited adhesion to laminin-511, but this was not as prominent as inhibition of adhesion to fibronectin (Fig. 6c). Adhesion to laminin-511 was slightly inhibited in 43A cells (P = 0.06), and significantly in 43B (P < 0.001) and 43A-SNA (P < 0.001) cells with 20 μg/ml laminin-411. These results suggest that although oral SCC cells do not adhere to laminin-411, it may have a role in the inhibition of cell adhesion to other ECM proteins.

Finally, we aimed to elucidate whether the inhibition of cell adhesion to fibronectin by laminin-411 was due to a direct interaction between the proteins. Immunoprecipitation of 43B cell culture medium with MAb 3H2 against laminin-411 followed by Western blots with polyclonal antiserum against fibronectin showed a precipitate of Mr ca.
Fig. 5 Expression of Lutheran, integrins, and integrin-linked kinase in 43A, 43B and 43A-SNA cells. A strong, cell surface-confined immunoreactivity for Lutheran (Lu), a laminin z5 chain-specific receptor, was found in 43A cells, and a punctate heterogeneous expression for Lutheran was found in 43B cells (a). 43A-SNA cells showed no reactivity for Lutheran. Immunoreactivity for integrin subunit was detected in a typical “Swiss cheese”-like manner in 43A cells, whereas in 43B and 43A-SNA cells, expression was evenly distributed to the cell surface. Immunoreactivity for integrin-linked kinase (ILK) was not detected in 43A cells, whereas in 43B and 43A-SNA cells, strong reactivity was found in nail-like structures. Scale bar: 10 μm.

Immunoprecipitation of 43A, 43B and 43A-SNA cell lysates (b) showed decreasing amounts of Mr 85,000 form of Lutheran during EMT in 43B and 43A-SNA cells. C, control lane with no antibody. Immunoprecipitation with MAb against integrin β1 subunit precipitated several z subunits in 43A, 43B and 43A-SNA cells (lanes 1, 6, 11). MAb against integrin β2 precipitated z4 and β1 subunits in 43A cells (lane 2), whereas 43B and 43A-SNA cells remained negative (lanes 7, 12). MAb against collagen receptor integrin z4 detected in 43A-SNA cells integrin z4 and β3 subunits (lane 13), which were not found in 43A or 43B cells (lanes 3, 8). MAb against integrin z4 precipitated strong bands of z4 and β1 in 43A and fainter bands in 43B cells (lanes 4, 9), whereas in 43A-SNA cells, z4 precipitated with β1 (lane 14), suggesting a change in integrin subunit association. Lanes 5, 10, 15; controls with no antibody.

Western blots showed expression of Mr 59,000 ILK in 43B and 43A-SNA cell lysates, but not in 43A cells. Actin bands indicate equal loading. Asterisk, unspecific bands.
220,000, corresponding to fibronectin (Fig. 6d). These findings suggest that laminin-411 binds to fibronectin and hinders its functions as a cell adhesion substrate.

Discussion

At the periphery of carcinomas, individual malignant cells detach from the tumor mass and act independently within the extracellular matrix of the stroma. This change in tissue architecture has been suggested to take place through EMT (Guarino et al. 2007; Peinado et al. 2007). BM proteins form the first barrier which migrating carcinoma cells must penetrate during malignant transformation, followed by invasion to interstitial stroma. Fragmented or totally absent BMs have been detected in epithelial carcinomas, including also oral SCC (Hagedorn et al. 1998; Määttä et al. 2001), and this phenomenon has been recently linked in colon carcinomas to EMT (Spaderna et al. 2006). EMT has a role also in oral SCC (Yanjia and Xinchun 2007), as microarray studies have indicated upregulation of EMT-related genes in high-risk head and neck SCC tumors (Chung et al. 2006) and in their metastases (Yang et al. 2007). Accordingly, we have detected Snail expression in invasive fronts of laryngeal SCC (Francí et al. 2006).

Recent studies attribute a more dynamic role for BM laminins in the maintenance of epithelial cell differentiation and tumorigenesis (Ziober et al. 2006). We have shown earlier that oral SCC cells lose the expression of laminin-332 chains via endogenous and also via Snail-induced EMT (Takkunen et al. 2006). In line with our results, it was recently suggested that transcription factors Snail, ZEB-1 and ZEB-2 repress laminin α5 chain expression also in vivo (Spaderna et al. 2006). In this study, we hypothesized that apart from laminin-332, EMT might regulate the expression of other laminins as well. To clarify this point, EMT was studied at several levels, namely expression, regulation and signal transduction, coupled with functional adhesion studies, using a set of oral SCC cells. Upon EMT, the cells formed branching colonies in Matrigel and were significantly more invasive than the primary tumor 43A cells. In particular, EMT induced a switch of laminin α5 chain to laminin α4 chain synthesis, probably due to binding of transcription factor Snail to specific regions upstream of both laminin α chain genes. A concomitant loss of laminin α5 chain receptor Lutheran and induction of mesenchyme-
linked integrin αβ1 and ILK were found. Laminin-332 and laminin-511 receptor integrin αβ1 was dissociated and instead integrin α6 subunit paired with β1, potentially relating to their invasion capabilities. Finally, laminin-411 inhibited adhesion of oral SCC cells to laminin-511 and fibronectin, and laminin-411 was found to interact with the fibronectin molecule.

Laminin α5 chain is crucial for development, as laminin α5 knock-out mice die at E13.5-17 with severe defects in placental vessels, neural tube, limbs, and kidney (Miner et al. 1998; Miner and Li 2000). Laminin α5 chain controls epithelial morphogenesis, for instance, in submandibular gland development (Rebustini et al. 2007). Peptides corresponding to active sites in the globular domain of laminin α5 chain inhibit tumor cell migration, tumor formation, invasion, and angiogenesis (Hibino et al. 2004), suggesting that laminin α5 chain and laminin-511 may have a protective role in tumorigenesis. Our present findings support this assumption. 43A cells expressed strongly laminin α5 chain mRNA and laminin-511 protein, whereas in cells which had undergone EMT, laminin α5 chain and laminin-511 were diminished or totally lacking. Although well-preserved laminin α5 chain expression has been reported in BMs of, e.g., renal tumors (Lohi et al. 1996) and prostate carcinoma (Brar et al. 2003), reduced laminin α5 chain expression has been described in oral SCC (Kosmehl et al. 1999), non-small cell lung carcinomas (Akashi et al. 2001), invasive areas of colorectal carcinomas (Lohi et al. 2000), and diffuse-type gastric carcinomas (Tani et al. 1996). Therefore, it may be assumed that different carcinomas behave differently upon malignant transformation in respect of laminin α5 chain expression.

Laminin α4 chain is principally produced by cells of mesenchymal origin (Lefebvre et al. 1999; Petäjäniemi et al. 2002). Laminin α4 knockout mice are viable, but present an abnormal structure of capillary BM leading to microvascular defects and hemorrhage (Thyboll et al. 2002). Laminin α4 chain has been suggested to play a role in invasion (Khazenzon et al. 2003) and migration through endothelia (Sixt et al. 2001; Wondimu et al. 2004). Studies concerning laminin α4 chain expression in malignancies have thus far focused on non-epithelial cancers. For example, increased laminin α4 expression has been found during progression of gliomas (Ljubimova et al. 2004). Recently, laminin α4 chain expression was reported in renal cell carcinomas (Vainionpää et al. 2007). Our results showed that EMT induced 43B and 43A-SNA cells to synthesize laminin α4 chain concomitantly with β1 and γ1 chains, forming laminin-411, whereas no such expression was found in 43A cells. Interestingly, in 43B cells, a simultaneous expression of laminins-511 and -411 was detected. These cells have undergone an endogenous, i.e., spontaneous EMT driven by transcription factors ZEB-1 and ZEB-2, and seem to represent a milder form of EMT (Takkunen et al. 2006).

Only limited information is available concerning transcriptional regulation of laminins in general, and laminin α4 and α5 chains in particular (Aberdam et al. 2000). Since laminins have strictly regulated expression profiles during development and in adult tissues, it is presumable that their regulation is of utmost importance. Cells losing expression of one laminin chain have been suggested to acquire other, compensatory laminin chains. For instance, knock-out of laminin α5 induces the levels of α1-, α2-, and α4-containing laminins (Miner et al. 1998). Factors controlling these events are yet unclear. Genes encoding laminin α5 and α4 chains reside at 20q13.2-q13.3 and 6q21, respectively, but their promoter elements have not been characterized. Therefore, we performed chromatin immunoprecipitations to test our hypothesis and to gain information of eventual binding of transcription factor Snail to these regions. We found that Snail bound to two chromatin regions in laminin α5 chain promoter, and to three regions in laminin α4 chain promoter. All but one of these regions contained E-boxes, which represent previously characterized consensus binding sites for Snail (Mauhin et al. 1993). The fifth region of interest, lacking E-box, contained a highly similar sequence with a single nucleotide deviation (CAGGTG → CAGGTA, also called Z-box), which has been recently shown to bind some other Snail- and EMT-related transcription factors (Spadera et al. 2006). Our results suggest that Snail binds to specific regions upstream of both laminin α5 and α4 chain sequences, suggesting that Snail may control their expression in vivo. In 43B cells, the potential regulators of laminin expression are ZEB-1 and ZEB-2 transcription factors, which also bind E-box motifs (Postigo and Dean 2000). To our knowledge, this is the first study disclosing how transcription of laminin α5 and α4 chains is regulated and suggesting that they obey a reciprocal transcription mode.

We were also interested in the expression of laminin receptors in our cell system. Lutheran is a specific receptor of α5 chain laminins (Moulson et al. 2001; Parsons et al. 2001), of which only laminin-511 was found in our cell model. We have shown before that Lutheran is expressed coaligned with laminin α5 chain in normal oral BM (Willberg et al. 2007). Here, a strong cell surface-confined immunoreactivity was evident for Lutheran in 43A cells, whereas in 43B cells, only a diffuse and faint expression was found. 43A-SNA cells completely lacked Lutheran expression. A similar decrease in Lutheran expression was detected with immunoprecipitations. Therefore, the results suggest that concurrent with decreasing expression of laminin-511, also expression of its counterpart Lutheran is diminished. Lutheran or its shorter spliceform, B-CAM, has been studied in different carcinomas (Kikkawa and
Miner 2005; Määttä et al. 2005). B-CAM has been detected in squamous cell and basal cell carcinomas of skin (Schön et al. 2000). As it was not found in malignant melanoma, an epithelial origin was suggested for B-CAM. In tumorigenesis of ovarian epithelium, polarized expression of Lutheran is lost (Määttä et al. 2005). This has been suggested to indicate that the stabilizing interactions between epithelial cells and BM may be lost as a consequence of progression of malignancy, which conclusion is clearly supported by the present findings.

Oral epithelial cells, as well as oral SCC cells, have been shown to contain integrins \( \alpha_6\beta_4, \alpha_6\beta_1, \alpha_2\beta_1, \) and \( \alpha_2\beta_4 \) (zieber et al. 2006). Distribution of integrin \( \alpha_6 \) was found in a granular pattern in 43A cells, whereas in EMT-experienced cells, its distribution was diffusely organized along the cell surface. Immunoprecipitation studies showed that integrin \( \alpha_6 \) paired with \( \beta_2 \) subunit in 43A cells and to some extent in 43B cells. However, in 43A-SNA cells, \( \alpha_6 \) subunit coprecipitated \( \beta_1 \) subunit. When pairing with \( \beta_4 \), integrin \( \alpha_6\beta_4 \) mediates the formation of hemidesmosomes, which link the intermediate filament cytoskeleton to BM laminin-332. Integrin \( \alpha_6\beta_4 \) also transmits cell adhesion to laminin-511 (Kikkawa et al. 2000). Extensive loss or changes in polarization of integrin \( \alpha_6\beta_4 \) complex have been reported in oral SCC (Downer et al. 1993; Garzino-Demo et al. 1998). On the other hand, integrin \( \alpha_6\beta_1 \), associated with focal contacts, is the main receptor for laminin-411 and promotes cell motility and tumorigenesis in breast carcinoma (Wewer et al. 1997a; Fujiwara et al. 2001). We have shown before that EMT reduces integrin \( \alpha_6\beta_4 \) complex in 43B and 43A-SNA cells (Takkunen et al. 2006). Here, we showed that integrin \( \alpha_6 \) subunit redistributed and colocalized with \( \beta_1 \) in 43A-SNA cells, corroborating previous findings on prostate carcinoma of a switch from \( \beta_3 \) to \( \beta_1 \) expression (Cress et al. 1995). We propose that a reduction of integrin \( \beta_2 \) subunit allows 43B and 43A-SNA cells to escape from hemidesmosomal contacts and use laminin-411 receptor integrin \( \alpha_6\beta_4 \) to become motile.

Our studies also showed neoexpression of integrin \( \alpha_1\beta_1 \) in 43A-SNA cells. Integrin \( \alpha_1\beta_1 \) is a predominantly mesenchymal integrin, detected, e.g., in endothelium, visceral and smooth muscle, being absent from epithelium (Miettinen et al. 1993; Gardner et al. 1996). Integrin \( \alpha_1 \) subunit activates MAPK/Ras-pathway, which has a role in induction of EMT (Pozzi et al. 1998; Peinado et al. 2007). Dysregulation of integrin \( \alpha_1 \) has been found in carcinomas, and it is upregulated in, e.g., bladder carcinoma (Liebert et al. 1994) and in several mesenchymal tumors (Miettinen et al. 1993). Altered expression of integrin \( \alpha_1 \) has been linked to cells with migratory function, as in repair of tissue injury, or in T cell invasion (Gardner et al. 1996). Therefore, neoexpression of collagen receptor integrin \( \alpha_1\beta_1 \) in oral SCC cells may indicate tendency to migration and invasion.

Integrin-linked kinase, a component of focal adhesion plaques, interacts with the cytoplasmic domains of \( \beta_1 \) and \( \beta_3 \) integrin subunits. ILK connects BM, cell adhesion molecules, integrins and growth factors to the actin cytoskeleton and to a range of signaling pathways (Oloumi et al. 2004). Overexpressed ILK promotes oncogenic transformation and increased invasion, possibly through repression of E-cadherin (Novak et al. 1998). ILK overexpression has been shown to be involved in the initiation of EMT (Somasiri et al. 2001), and interestingly, to activate Snail through GSK-3/\( \beta \) pathway (Tan et al. 2001). Our results showed neoexpression of ILK in EMT-experienced cells, where it was topologically confined to elongated streaks resembling focal adhesions. Therefore, our results extend earlier findings by showing that not only does ILK activate Snail, but also that both endogenous and Snail-induced EMT, in 43B and 43A-SNA cells, respectively, upregulate ILK. This could be a consequence of a positive feedback loop, where fibronectin, abundantly deposited in EMT, stimulates engagement of integrins, further upregulates TGF-\( \beta \) and ILK, thus maintaining EMT (Nieto 2002; Oloumi et al. 2004).

Finally, to address the potential functional consequences of laminin expression in oral SCC cells, we studied their adhesion properties to fibronectin, laminin-511, and laminin-411. Quantitative cell adhesion assay showed that all the cells adhered well to fibronectin and laminin-511. In contrast, these cells did not adhere to laminin-411. In a mouse autoimmune encephalomyelitis model, vascular BMs containing laminin-511 were impermeable to leukocytes, whereas these cells easily passed endothelial BMs containing laminin-411 (Sixt et al. 2001). Laminin-411 may participate in other processes where enhanced endothelial BMs containing laminin-411 (Sixt et al. 2001). Laminin-411 inhibited adhesion of oral SCC cells to fibronectin and laminin-511 significantly. In immunoprecipitation, laminin-411 and fibronectin coprecipitated, suggesting that they bind to each other in vivo. Recently, laminin-411 has been found to have some qualities resembling matricellular proteins (Vainionpää et al. 2007). Matricellular proteins, e.g., SPARC and tenascin-C, induce intermediate state of adhesion, or de-adhesion, and may enhance cell migration (Murphy-Ullrich 2001; Bornstein and Sage 2002). Tenascin-C coprecipitates with fibronectin and inhibits cell adhesion by preventing binding of syndecan-4 to fibronectin (Huang et al. 2001). Therefore, our results suggest that EMT-experienced cells seem to synthesize laminin-411 and escape from strong adhesion to BM or extracellular matrix, which enables them to invade surrounding structures. Laminin-411 may impair cell adhesion to fibronectin by blocking active sites in the fibronectin molecule.
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