Original Research

Role of myocyte enhancing factor 2B in epithelial myofibroblast transition of human gingival keratinocytes

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Abstract

It has recently emerged that the myogenic contribution of the epithelial mesenchymal transition plays a role in neoplastic invasion and metastasis. Myocyte enhancing factor 2B (MEF2B) is the only MEF2 isoform expressed during early embryonic development, and is herein proposed to transactivate the downstream target proteins of the epithelial myofibroblast transition (EMyT). We have previously generated eight preneoplastic cell lines with spindle and cobblestone morphology from human gingival mucosal keratinocytes immortalized by E6/E7 of human papillomavirus type 16. Spindle cells formed tubulogenic morphogenesis on Matrigel and exhibited contractility, anchorage-independent growth and invasiveness to a greater extent than cobblestone cells. Expression of MEF2B mRNA and myofibroblast proteins was higher in spindle cells compared with cobblestone cells. Epidermal growth factor (EGF) treatment of cobblestone cells also induced expression of these genes. Knockdown of MEF2B in a cobblestone cell line abolished EGF-induced upregulation of MEF2, vimentin and non-muscle caldesmon proteins, but enhanced basal expression of mesenchymal vimentin and fibronectin. Differential regulation of intermediate filaments revealed an unrecognized role of MEF2B in myogenic transformation of the epithelial to a myofibroblast phenotype, which occurs as epithelioid variants in some soft tissue sarcomas.

Keywords: immortalized epithelial cells, preneoplastic progression, tumor invasion, epithelial myofibroblast transition, myogenic enhancing factor 2

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Introduction

During tumor progression, malignant cells exploit critical developmental and tissue remodeling programs, often promoting a plastic phenotype referred to as an epithelial mesenchymal transition (EMT).[1] Neoplastic cells express a different set of cytoskeleton-associated proteins, especially intermediate filaments (IFs), to reflect their morphological and functional differentiation. Carcinomas contain keratin and sarcomas of muscle origin contain desmin. Vimentin, which is primarily found in mesenchymal cells, may coexist with other IFs in other tumors. The overlapped expression of IFs associated with EMT and transdifferentiation has been recognized.[2,3] An epithelial myofibroblast transition (EMyT) has been defined to indicate a myogenic form of EMT.[4] Many studies indeed have demonstrated a myogenic contribution in EMyT by overexpression of a myogenesis master protein MyoD to a variety of differentiated cell lines and fibroblast cell lines[5] as well as skin keratinotcytes.[6] In line with this notion, epithelial marker keratins are co-expressed with myogenic genes in liposarcoma,[7] myxofibrosarcoma,[8] malignant human breast tissue,[9] as well as myofibroblast lesions of skin and soft tissue tumors[10] including rhabdomyosarcomas.[11] Among soft tissue tumors, tumors of the oral cavity and gingiva in myofibroma,[12] leiomyosarcomas[13] and rhabdomyosarcoma,[14] are frequently presented with myogenic contribution. These tumors involve spindle cell formation. However, the knowledge on contribution of transcription factors other than MyoD in EMyT has been very limited.

In addition to MyoD family proteins such as MyoD and myogenin, the other family of myogenic transcription factors consists of myocyte enhancing factor 2 (MEF2) which belongs to the superfamily of MADS (MCM1, agamous, deficiens, serum response factor [SRF])-box transcription factors. Notably, SRF and its transcriptional

co-activator myocardin-related transcription factor have been shown to regulate smooth muscle actin (α-SMA) expression[4] as well as cytoskeletal dynamics and metastasis.[15] MEF2 has been induced by MyoD in non-muscle cells,[16] and it can indirectly be regulated by myogenin.[17] These data imply a possible role of MEF2 in myogenic activation of EMyT and the myogenic phenotype[5,6] which is supported by its prevalence in nuclei of sarcoma cells,[18] and fibroblastic stromal cells of elastofibroma.[19] MEF2 consists of four members: MEF 2A, 2B, 2C and 2D. Among MEF2 members, MEF2B is herein proposed to be a candidate in myogenesis of myofibroblast-involved tumors because it is expressed in embryonic carcinoma cells,[20] fibroblast cell lines[21] and in skeletal muscle of early mouse embryo.[21,22]

We had previously generated spindle and cobblestone preneoplastic cell lines from human papillomavirus type 16 (HPV16) E6/E7-immortalized human gingival mucosal keratinocytes (GM16).[23,24] Those cell lines represented model cells to investigate possible roles of MEF2B in EMyT-related myogenic gene expression. Since soft tissue tumors are sensitive toward epidermal growth factor (EGF),[25] we performed EGF treatment of cobblestone keratinocytes to induce EMyT. Here we showed that spindle cells were more invasive than cobblestone cells concomitantly with increased expression of MEF2B mRNA as well as myofibroblast proteins, namely vimentin, α-SMA and low-molecular-weight non-muscle caldesmon (l-CaD). EGF treatment of cobblestone cells induced expression of myofibroblast proteins, morphological changes toward motile elongated cells, and increased MEF2B mRNA and MEF2 protein. MEF2B knockdown experiments revealed that MEF2B regulated myofibroblast proteins differently. Our study indicates a unique role for MEF2B in EGF-induced plasticity and EMyT of human gingival keratinocytes.

Materials and methods

Reagents and cell cultures

EGF was obtained from Sigma (Deisenhofen, Germany). Cobblestone cell lines with spindle shape (FIB and NuB1) and with epithelium-like shape (EPI, NuB2 and FuB1) were generated from the same parental GM16 cells,[23,24] and were cultured in Dulbecco's modified Eagle's medium (DMEM) (PAA, Cölbe, Germany) containing 4.5 g/L glucose, 10% fetal calf serum (FCS), and penicillin and streptomycin.

Matrigel tubulogenicity, contractility, anchorage-independent growth and invasion assays

Cellular tubulogenicity was tested by the detection of differentiation-independent growth and tubule morphology of cells cultured on Matrigel. Matrigel basement membrane matrix (BD Bioscience, Heidelberg, Germany) of 100 μL was used to coat 24-well plates. Cells were plated at 2.5×10^4 in 0.5 mL serum-free DMEM and were photographed after three days. A cellular contractility assay[26,27] was performed by mixing 140 μL 20mg/mL collagen type I solution with 3×10^5 cells in 1 mL DMEM containing 30 mmol/L HEPES at pH 7.0. A mixture of cells and collagen (500 μL) was allowed to gel for one hour in a 24-well plate in an incubator, and 500 μL DMEM was added on top of the gels. After two days in culture, collagen gels were released from the culture dish to allow gel contraction. Gels were photographed and gel diameter was measured.

Using the procedure previously reported,[23] an anchorage-independent growth assay was carried out by plating cells on semi-solid agar. Phase-contrast pictures at $10\times$ magnification were taken after two weeks in culture. The total number of colonies was an average of the number of colonies counted from six to nine pictures randomly taken with a microscope.

For invasion assays, a BD BioCoat Matrigel Invasion Chamber system (BD Bioscience) was used in a 24-well format with inserts containing 8-μm-pore membranes. Cells at 2.5×10^4 in 0.5 mL serum-free DMEM were added onto the upper compartment with 1 mL DMEM containing 10% FCS in the lower compartment. After four days, cells that invaded through membranes were fixed with methanol and stained with 1% toluidine blue in 1% Borax. The total number of invading cells was an average of cells counted from four pictures randomly taken with a light Olympus microscope (Hamburg, Germany). The reproducibility of all functional assays was obtained from at least two independent experiments.

Fractionation

NuB2 cells at $2–3 \times 10^6$ cells were seeded in 10-cm dishes overnight. Cells were treated with EGF (100 ng/mL) in serum-free DMEM for 24 h. Cellular fractionation was performed by using a Subcellular Proteoextraction kit from Calbiochem (Darmstadt, Germany). Cytosol, nuclear and cytoskeletal fractions were used at 7.5 μg protein for Western blot analyses.

Western blotting

Cell lysates were prepared using lysis buffer (50 mmol/L Tris.HCl, 1% Triton X-100, 40 mmol/L β-glyceraldehyde, 100 mmol/L NaCl, 50 mmol/L NaF, 2 mmol/L ethylenediaminetetraacetic acid, 200 μmol/L sodium orthovanadate and 0.2 mmol/L phenylmethanesulfonylfluoride) containing protease inhibitor cocktails (Calbiochem, Schwalbach, Germany). Lysates were separated and electrotransfered onto membranes. Blots were incubated with an antibody overnight at 4°C. Primary antibodies with the indicated clone in parentheses included vimentin (RV202), α-SMA (C04018), fibronectin (F1), caldesmon (EP789Y) and Ser^{789}-phospho-caldesmon (E89) from Epitomics/Biomol (Hamburg, Germany). Anti-MEF2 antibody was obtained from Santa Cruz Biotechnology (Heidelberg, Germany). The blots were incubated with secondary anti-rabbit or anti-mouse antibodies. Blots were re-probed with β-actin or β-tubulin antibody. Millipore ECL reagent (Schwalbach, Germany) was used for detection.

MEF2B siRNA

For knockdown of MEF2B, NuB2 cells were plated for 24 h prior to transfection. Cells were transfected in serum-free

DMEM with 50 nmol/L control (siRNA negative control #SR-CL000-005 from Eurogentec, Seraing, Belgium) or MEF2B siRNAs (predesigned Silencer select siRNA #S8650 from Ambion, Darmstadt, Germany) using Eurogentec ICAFectin™ 442 transfection reagent for 18 h. Cells were treated with EGF (100 ng/mL) for the indicated time period and then harvested for Western blot and reverse transcription polymerase chain reaction (RT-PCR) analyses.

RT-PCR

Total RNA was prepared from treated or transfected cells using an RNeasy Mini kit (Qiagen, Hilden, Germany). cDNA was synthesized from 2 μg RNA using a First Strand cDNA synthesis kit (Fermentas, St Leon-Rot, Germany). mRNA expression was analyzed in quadruplets by realtime PCR using the Applied Biosystems TaqMan gene expression assays with TaqMan® Universal PCR Master Mix (Applied Biosystems, Darmstadt, Germany) and run on an Applied Biosystem 7500 realtime PCR machine by using assay-on-Demand MEF2B (Hs01021281_m1) and MYH9 (Hs01066381_m1) TaqMan® primers. The expression level of targets was calculated using the $\Delta - C_t$ transformation method and showed as a ratio after having been normalized to the housekeeping gene GAPDH (Hs99999905_m1). PCR results were obtained from at least two independent experiments.

Results

Expression of IF proteins in model spindle and cobblestone cell lines

As typical cobblestone keratinocytes, GM16 cells normally proliferate well in keratinocyte growth medium.[23] GM16 cells were the precursor of the total eight cell lines which were resistant to calcium/serum-induced differentiation and were capable of proliferating in standard DMEM. EPI and FIB were generated on chronic exposure to ethanol.[23] NuB1, FuB1 and NuB2 were among those generated as a result of transfection with oncogenic promoter Nox1 (gp91*phox* homolog NAPH oxidase 1).[24] FIB and NuB1 cells exhibited spindle-elongated and fibroblast-like morphology (Figure 1a, top). Similar to the parental GM16,[23] EPI, NuB2 and FuB1 cells were cobblestone with epithelium-like morphology (Figure 1a, bottom). Compared with cobblestone cells (with [c] in figures), spindle cells (with [s] in figures) consistently showed elevated expression of vimentin and α-SMA (Figure 1b), whereas expression of another IF fibronectin was absent in all cell lines (data not shown). For a class of myogenic IFs, expression of caldesmon (CaD) isoforms, l-CaD (low *Mr*-form which is widely distributed in non-muscle tissues and cells) and h-CaD (high *Mr*-form which dominantly expressed in differentiated smooth muscle cells), has been implicated to modulate EMyT phenotype.[28] Expression of unphosphorylated and Ser^{789}-phosphorylated l-CaD detectable at 75 kDa was increased in spindle FIB and NuB1 cells compared with cobblestone EPI and FuB1/NuB2 cells, respectively (Figure 1b). Notably, the presence of Ser^{789}-phosphorylated h-CaD detectable at 120 kDa was found in both spindle FIB and NuB1 cell lines (as well as HeLa cells, not shown), but absent in cobblestone cells. This phosphorylation may indicate that spindle cells had adopted a phenotype of differentiated smooth muscle cells.[28,29] Thus, α-SMA, l-CaD, as well as phospho l- and h-CaD expression, were associated with spindle phenotype of immortalized human gingival keratinocytes.

Preneoplastic characteristics of spindle and cobblestone cell lines *in vitro*

The ability of preneoplastic cells to form branching morphogenesis in response to Matrigel has been used to determine the morphogenetic re-programming potential of epithelial cells.[30] Spindle NuB1 and FIB cells were resistant against Matrigel-induced differentiation by forming tubules with branching morphogenesis (Figure 2a). Cobblestone EPI and NuB2, and FuB1 cells were differentiated by Matrigel to form non-proliferative round cells. Tubules from spindle cells were lacking in lumen. NuB1 cells appeared to produce more extensive meshwork with branching-tubule lengths longer than FIB cells (Figure 2a). To identify a myofibroblast-like property among spindle and cobblestone cell lines, we determined contractility function by employing a collagen contraction assay.[26,27] Collagen gels containing NuB1 or FIB cells were able to contract immediately after detachment of gels from the culture dish, resulting in reduced diameters of collagen gels (Figure 2b). Cobblestone NuB2 cells did not contract.

Next, we determined the preneoplastic potential of our cell lines by measuring anchorage-independent growth and invasiveness. We have previously reported that FIB cells exhibited anchorage-independent growth.[23] NuB1 cells exhibited anchorage-independent growth with greater extent than FIB cells (Figure 2c). For the invasion assay using a Boyden chamber, we have previously shown that FIB cells are more invasive than EPI cells.[31] Similar to anchorage-independent growth, NuB1 cells were more superior to FIB cells (Figure 2d). Cobblestone cells exhibited no anchorage-independent growth and invasiveness. Taken together, increased tubulogenesis, contractility, anchorage-independent growth and invasiveness of spindle cells indicated enhanced preneoplastic potentials compared with cobblestone cells.

To associate muscle-specific genes (Figure 1b) and preneoplastic characters (Figures 2a–d) to MEF2B, we next correlated the spindle phenotype with mRNA expression of MEF2B as well as non-muscle myosin heavy chain (MYH9) which is an actomyosin contractile apparatus mediated by SRF[15] and MEF2.[16] MyH9 has been shown to correlate with cytoskeletal dynamics of metastasis[15] and was therefore used in our study as a positive transcription control for plasticity and EMyT. Compared with cobblestone NuB2 and FuB1 cells, mRNA expression of MEF2B and MYH9 was increased in NuB1 cells, indicating a correlation of MEF2B with the spindle and EMyT phenotypes (Figure 2e).

EGF treatment of cobblestone cells induces myofibroblast protein expression

It has been recognized that autocrine/paracrine signaling due to tumor microenvironment cytokines, such as EGF,

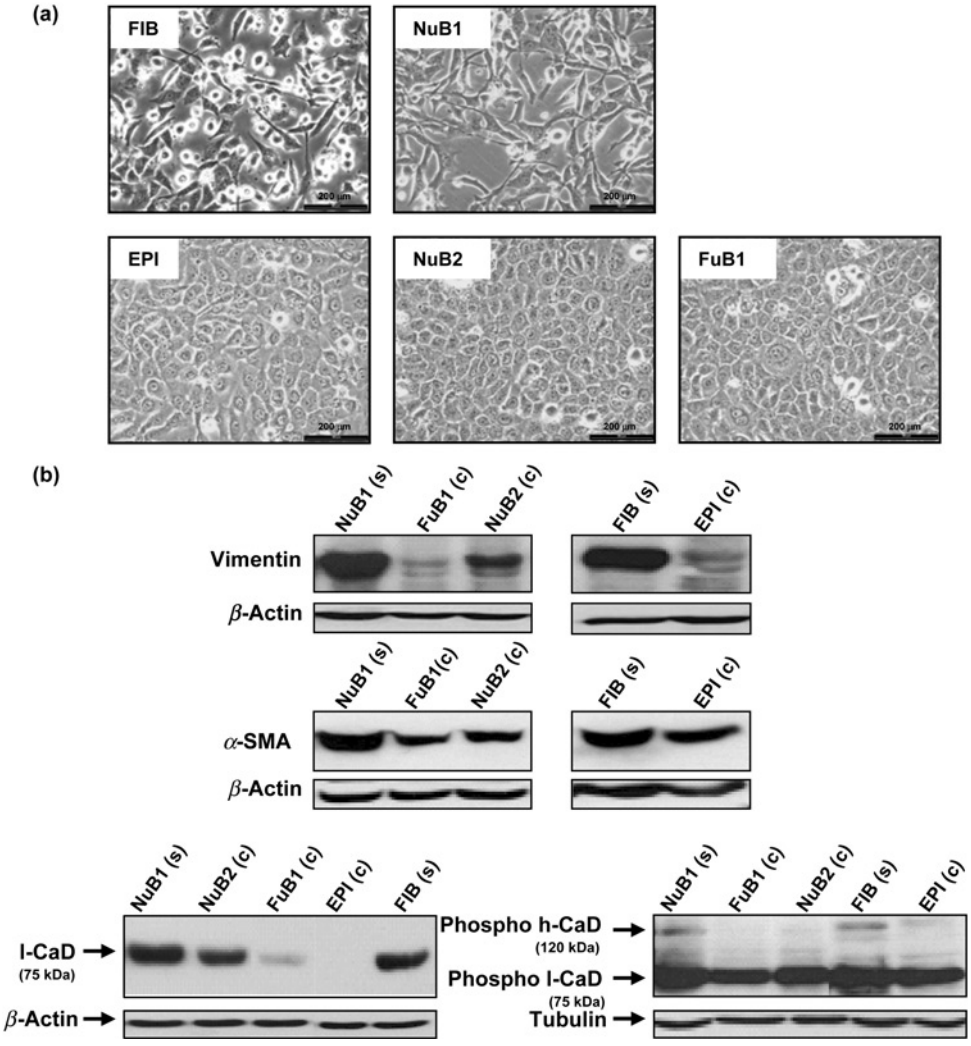

Figure 1 Morphology, phenotypes and expression of intermediate filament proteins of human immortalized keratinocytes used in this study. (a) Two distinctive phenotypes with fibroblast-like spindle-shape (FIB and NuB1 cells) and epithelium-like cobblestone (EPI, NuB2 and FuB1 cells) morphology were characterized in this study. FIB and EPI cells were the paired cell lines generated from chronic ethanol treatment of GM16. NuB1, NuB2 and FuB1 were among cell lines generated from overexpression of the E6/E7 oncogenes of human papillomavirus type 16 (HPV16) in GM16 cells with oncogenic promoter Nox1. The bar scale represents 200 μm. (b) Protein expression of intermediate filaments was compared among spindle-shape (labeled with 's' in brackets) and cobblestone (marked with 'c' in brackets). Compared with cobblestone cells, spindle-shaped FIB and NuB1 cells showed increased expression of vimentin, α-smooth muscle actin (α-SMA), non-muscle caldesmon (l-CaD for low *Mr* caldesmon at 75 kDa) and phosphorylated-l-CaD (Ser^{789}phospho-l-CaD). Spindle cells also expressed phospho-muscle-type h-caldesmon (h-CaD for high *Mr* caldesmon at 120 kDa). Direct comparison of protein expression could be made between EPI/FIB pair and FuB1/NuB1 pair since these pairs were cultivated to the same passage number. Cobblestone NuB2 cells were at lower passages than FuB1 and NuB1

largely regulates the morphological and invasive phases of the EMyT phenotype.[32] After 24-h treatment of cobblestone NuB2 and FuB1 cells with EGF (100 ng/mL), morphological changes with multinucleated cells were observed showing elongated projections correlating with features of plasticity and motility (Figure 3a). EGF treatment of NuB2 and FuB1 cells significantly increased expression of vimentin and fibronectin in cell lysates (Figure 3b). While not observed in whole-cell lysates, expression of α-SMA, phospho l-CaD and fibronectin was increased in cytoskeletal fractions of EGF-treated NuB2 and FuB1 cells compared with controls (Figure 3b). EGF combined with bone morphogenic proteins was similarly capable of inducing morphological changes and expression of these myofibroblast proteins (please see Supplementary Figure 1 at http://ebm.rsmjournals.com/lookup/suppl/doi:10.1258/ebm.2011.011261/-/DC1). While antibody against MEF2B is not available, anti-pan MEF2 could detect the translocation of MEF2 from the cytosol to the nucleus upon EGF treatment (Figure 3c). Thus, EGF treatment of cobblestone cells induced plasticity toward the EMyT phenotype concomitant with increased expression of myofibroblast proteins, and activation of MEF2 translocation to the nucleus.

Differential effects of MEF2B knockdown on myofibroblast proteins

Because MEF2B is not specific to differentiated muscle cells and is also present in embryonic carcinoma cells as well as fibroblast cell lines,[20,21] we next determined whether MEF2B expression was correlated with EGF-induced plasticity of cobblestone NuB2 cells. Indeed, increases in

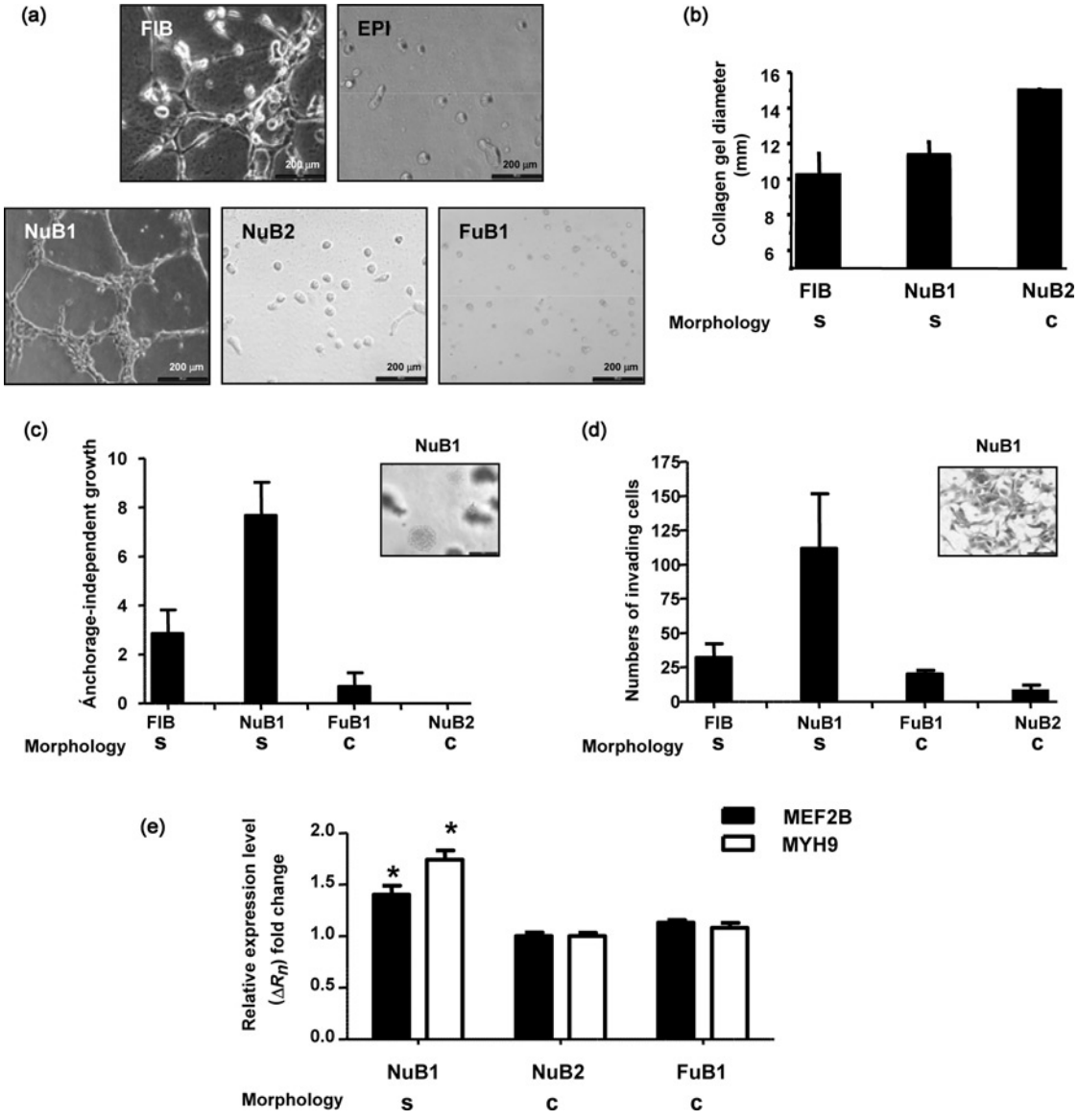

Figure 2 Characterization of preneoplastic potentials of spindle and cobblestone cell lines. (a) Ability of cells to form tubules (tubulogenesis assay) was compared by plating cells on Matrigel and cultured in Dulbecco's modified Eagle's medium containing 1% fetal calf serum, and phase-contrast pictures were taken three days later. Spindle FIB and NuB1 cells were de-differentiated when plated on Matrigel, showing extensive elongated and stretched morphology. Cobblestone EPI, NuB2 and FuB1cells did not form elongated mesh network. Bar scale represents 200 μm. (b) Contractility properties of spindle (morphology labeled 's') and cobblestone (morphology labeled 'c') cells were assessed by mixing them in collagen gel. Gel was detached from the culture dish, and collagen gel shrinking was an indicator of contractility. The original diameter of collagen gels before detachment was 15 mm. The reduced diameter of gels containing FIB or NuB1 cells demonstrated cellular contractility. Cobblestone NuB2 cells did not contract, maintaining ~15 mm gel diameter (mean $\pm$ SD, $^*P < 0.05$, $n = 3$). Anchorage-independent growth and invasiveness of spindle and cobblestone cells are shown in (c) and (d), respectively. Spindle cells formed more colonies on soft agar and were more invasive than cobblestone cells. Phase-contrast pictures shown in the insets were soft agar colonies and invading NuB1 cells. (e) Spindle NuB1 cells expressed increased levels of myocyte enhancing factor 2B (MEF2B) mRNA as well as the known metastasis SRF-dependent transcription factor MyH9 compared with those of cobblestone NuB2 and FuB1 cells. TaqMan® realtime polymerase chain reaction (PCR) were representatives from two experiments ($^*P < 0.05$, mean $\pm$ SEM, $n = 4$ for each PCR)

MEF2B and MyH9 mRNA were found upon EGF treatment of NuB2 cells for two and four hours (Figure 4a); this was confirmed by increased MEF2 protein in control transfected cells (Figure 4b). To investigate whether MEF2B could regulate expression of myofibroblast proteins, we performed MEF2B knockdown. Transfection of NuB2 cells with MEF2B siRNA markedly reduced MEF2B mRNA (please see Supplementary Figure 2 at http://ebm.rsmjournals.com/lookup/suppl/doi:10.1258/ebm.2011.011261/-/DC1) with only weak reduction of MEF2 protein (Figure 4b). In control siRNA-transfected cells, EGF treatment increased expression of MEF2 as well as all mesenchymal (vimentin and fibronectin) and myogenic (l-CaD and α-SMA) proteins (Figure 4b). Under basal condition, MEF2B knockdown unexpectedly increased expression of mesenchymal vimentin and to a lesser extent fibronectin. However, MEF2B-knockdown abolished EGF-induced upregulation of MEF2, vimentin and l-CaD proteins as well as l-CaD activity (indicated by phospho l-CaD expression). MEF2B knockdown also further enhanced fibronectin expression in EGF-treated

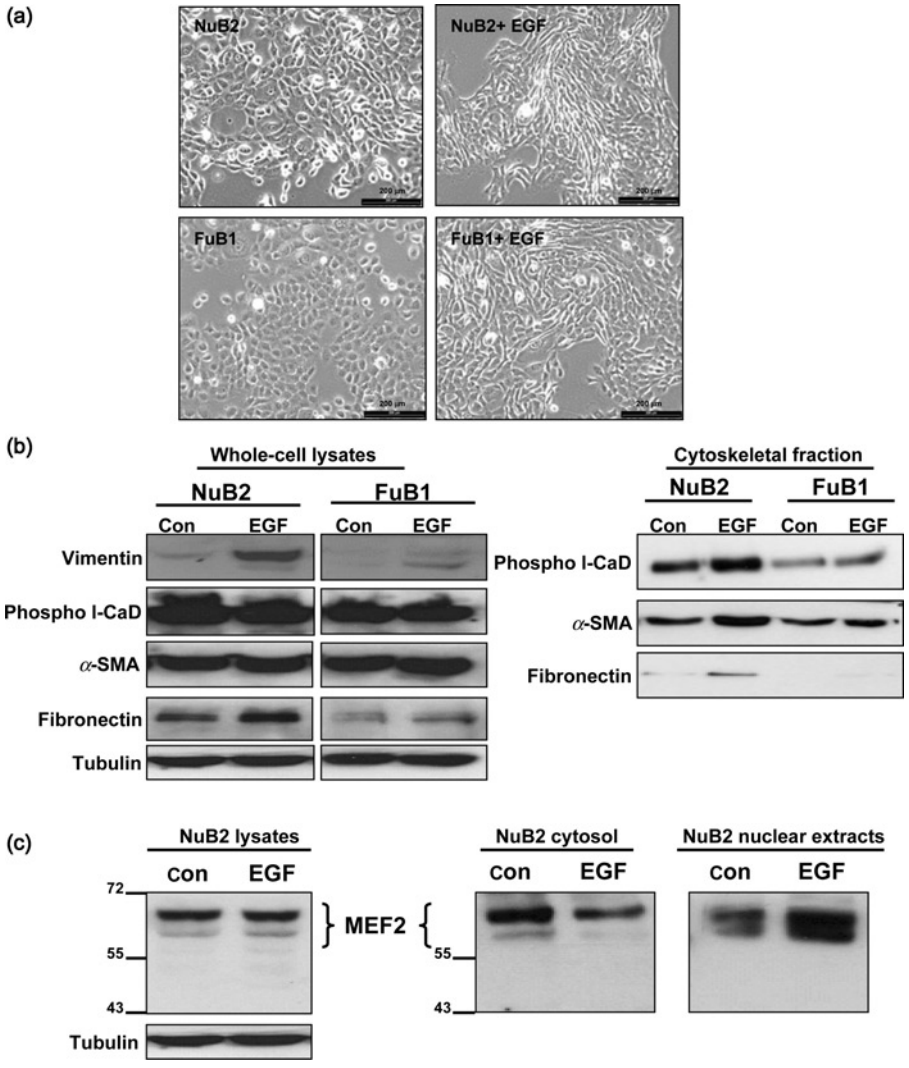

Figure 3 Epidermal growth factor (EGF) treatment of cobblestone cells induces plasticity and motile morphogenesis. (a) NuB2 or FuB1 cells were treated with EGF (100 ng/mL) for 24 h in serum-free medium. EGF induced motile, elongated and spiral morphology in some cells, indicating a transition towards spindle phenotype. Bar scale represents 200 μm. (b) EGF (100 ng/mL) treatment of NuB2 and FuB1 cells for 24 h induced increased protein expression of vimentin and fibronectin in cell lysates. β-Tubulin was used as loading control. Increased expression of phospho l-CaD (low molecular weight non-muscle caldesmon), α-smooth muscle actin (α-SMA) and fibronectin proteins was more evident in the cytoskeleton fraction in NuB2 and FuB1 cells treated with EGF (100 ng/mL) for 24 h. Cytoskeletal fraction of 7.5 μg protein of each treatment group was loaded for Western blot analysis. (c) After 24-h treatment of NuB2 cells, EGF induced MEF2 activation as indicated by increased MEF2 in the nucleus concomitant with decreased MEF2 expression in the cytosol. No alteration of MEF2 expression in cell lysates was observed

cells. With or without EGF treatment, expression of α-SMA was not affected by MEF2B knockdown. Hence, MEF2B exhibited differential regulation in blocking expression of vimentin and fibronectin, and, on the other hand, promoting activation of l-CaD.

Discussion

Despite the reports of the presence of MEF2B in embryonic carcinoma cells[20] and fibroblasts,[21] investigations into the role of MEF2B in cell transformation, oncogenic potentials and induction of EMyT have not been reported. Cell lines generated from immortalized gingival keratinocytes were used as a model experimental system to study the contribution of MEF2B to EMyT and cellular plasticity. Spindle cell lines expressing increased myofibroblast proteins exhibited increased tubulogenesis, contractility and anchorage-independent growth, and were more invasive than cobblestone cells. EGF treatment of cobblestone cells upregulated the expression of MEF2B as well as myofibroblast proteins. We also provided evidence that MEF2B blocked mesenchymal vimentin and fibronectin expression, but promoted myogenic l-CaD activation. Differential regulation of myofibroblast proteins by MEF2B may determine cellular fates such as migration and plasticity of human gingival keratinocytes.

Our HPV16 E6/E7-immortalized human gingival keratinocyte cell lines represent invaluable tools to investigate the potential role of myogenic transcription factor MEF2B in EMyT of keratinizing oral cells. A previous study has shown that HPV16 E6/E7 transfection under the control of α-SMA promoter produces immortalized cells which exhibit anchorage-independent growth,[33] thus providing a support for the feasible generation of our preneoplastic spindle cells

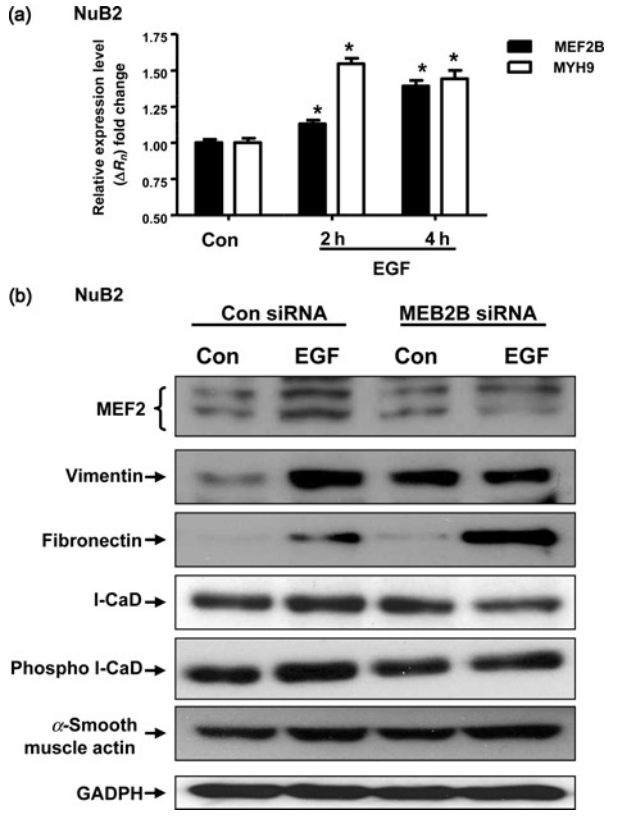

Figure 4 Epidermal growth factor (EGF) induces myocyte enhancing factor 2B (MEF2B) expression and MEF2B differentially regulates myofibroblast proteins. (a) EGF treatment of NuB2 cells for two or four hours increased mRNA expression of MEF2B and MyH9. TaqMan® realtime polymerase chain reaction (PCR) data were representatives from two experiments ($^*P < 0.05$, mean $\pm$ SEM, $n = 4$ for each PCR). (b) EGF treatment increased protein expression of MEF2, vimentin, l-CaD (low molecular weight non-muscle caldesmon), phospho l-CaD, α-smooth muscle actin (α-SMA) and fibronectin in control siRNA-transfected NuB2 cells. Upon MEF2B knockdown of NuB2 cells, the EGF effects were abolished in up-regulating MEF2, vimentin, l-CaD and phospho l-CaD proteins. MEF2B knockdown did not affect α-SMA but further increased fibronection expression. NuB2 cells were transfected with 50 nmol/L control or MEF2B siRNAs for 18 h in Dulbecco's modified Eagle's medium (DMEM) + 10% fetal calf serum, and subsequently treated with EGF (100 ng/mL) in serum-free DMEM for further 24 h

which exhibited increased myogenic gene expression. NuB1 and FIB cells also were more invasive. They also expressed elevated levels of α-SMA and h-CaD which are markers of, respectively, early and late differentiation in smooth muscle development.[34] We found that h-CaD was also present in the human cervical cancer Hela cell line. As h-CaD is expressed in some soft tissue tumors of the skin,[35] h-CaD may thus be ubiquitously expressed in soft tissue tumors of mucosa origin. Cell contractility is commonly regarded as a feature of mesenchymal cells and is acquired by cells that have undergone EMyT.[36] Spindle cell lines were thus classified as more transformed than cobblestone cell lines. The expression of vimentin, α-SMA, l-CaD and h-CaD in our spindle cell lines is consistent with a high frequency of myogenic genes in myofibroblast lesions of skin and soft tissues,[10,11] including myofibromas and myosarcomas of the oral cavity.[12–14]

The direct role of MEF2B on EMyT-associated proteins was studied upon MEF2B knockdown of cobblestone NuB2 cells, and differential effects were observed. Under basal conditions, MEF2B knockdown increased both mesenchymal vimentin and fibronectin protein expression. MEF2B knockdown reversed EGF-induced upregulation of MEF2 protein, suggesting that MEF2B may transactivate other MEF2s. With EGF treatment, MEF2B knockdown inhibited vimentin but further enhanced fibronectin expression. MEF2B's ability to block expression of both mesenchymal proteins may reflect an adaptive response mechanism in spindle cells and during EGF treatment of cobblestone cells. The mechanism of this negative regulation and differential effects between vimentin and fibronectin requires further investigations. We speculate that MEF2B knockdown may have removed an unknown factor that inhibited transcription of vimentin and fibronectin. However, we observed that EGF-induced upregulation of vimentin was removed upon MEF2B knockdown, indicating a positive regulation. This is consistent with positive regulation of vimentin by MEF2A/C/D in regenerating skeletal muscle.[37] Because MEF2B was reported to bind to the canonical MEF2-binding site,[23] MEF2B may also be able to transactivate other MEF2 isoforms that are transcribed by the same MEF2-binding site.[38] Upon EGF treatment, MEF2B may transactivate MEF2A/C/D activity, thereby upregulating expression of vimentin.

MEF2 is one of the key downstream effectors of mitogenic signaling pathways via serum-inducible expression of c-jun which positively regulates cell-cycle progression.[39] This supports the role of MEF2 in mitogenesis of cancer cells as evidenced by the presence of MEF2 in carcinosarcomas[18] and fibromas.[19] EGF treatment increased l-CaD and its activity was reversed by MEF2B knockdown. MEF2B, however, did not regulate basal expression of l-CaD. Increased l-CaD and activity upon EGF treatment is consistent with the physiological role of caldesmon on cell proliferation and migration.[29] As MEF2B knockdown did not have any effects on α-SMA, this suggested that other transcription factors, such as myocardin-related transcription factors, [4] may regulate this protein.

In conclusion, we showed that MEF2B expression was correlated with spindle phenotype and activation of myofibroblast proteins by EGF. The role of MEF2B in cellular plasticity was evident in two ways: one with blocking expression of mesenchymal proteins and the other way by proliferative activation of l-CaD. On availability of anti-MEF2B antibody, it may be pertinent to determine MEF2B, vimentin and caldesmon expression in cancer tissues, such as rhabsomyosarcoma[14] and soft tissue myxoma[40] of the gingiva.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. QS, AS and WC conducted the experiments; JB and WS supplied antibodies and MEF2B plasmids; and WS and QS wrote the manuscript. QS and AS contributed equally to this work.

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REFERENCES

1 Petersen OW, Lind Nielsen H, Gudjonsson T, Villadsen R, Rønnov-Jessen L, Bissell MJ. The plasticity of human breast carcinoma cells is more than epithelial to mesenchymal conversion. *Breast Cancer Res* 2001;**3**:213–7

2 Rønnov-Jessen L, Petersen OW, Koteliansky VE, Bissell MJ. The origin of the myofibroblasts in breast cancer. Recapitulation of tumor environment in culture unravels diversity and implicates converted fibroblasts and recruited smooth muscle cells. *J Clin Invest* 1995;**95**:859–73

3 Zhang Z, Yuan XM, Li LH, Xie FP. Transdifferentiation in neoplastic development and its pathological implication. *Histol Histopathol* 2001;**16**:1249–62

4 Masszi A, Speight P, Charbonney E, Lodyga M, Nakano H, Szaszi K, Kapus A. Fate-determining mechanisms in epithelial–myofibroblast transition: major inhibitory role for Smad3. *J Cell Biol* 2010;**188**:383–99

5 Weintraub H, Tapscott SJ, Davis RL, Thayer MJ, Lasser AB, Miller AD. Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. *Proc Natl Acad Sci USA* 1989;**86**:5434–38

6 Boukamp P, Chen J, Gonzales F, Jones PA, Fusenig NE. Progressive stages of 'transdifferentiation' from epidermal to mesenchymal phenotype induced by MyoD1 transfection, 5-aza-2′-deoxycytidine treatment, and selection for reduced cell attachment in the human keratinocyte line HaCaT. *J Cell* 1992;**116**:1257–71

7 Huang HY, Antonescu CR. Epithelioid variant of pleomorphic liposarcoma: a comparative immunohistochemical and ultrastructural analysis of six cases with emphasis on overlapping features with epithelial malignancies. *Ultrastruct Pathol* 2002;**26**:299–308

8 Nascimento AF, Bertoni F, Fletcher CD. Epithelioid variant of myxofibrosarcoma: expanding the clinicomorphologic spectrum of myxofibrosarcoma in a series of 17 cases. *Am J Surg Pathol* 2007;**31**:99–105

9 Lazard D, Sastre X, Frid MG, Glukhova MA, Thiery JP, Koteliansky VE. Expression of smooth muscle-specific proteins in myoepithelium and stromal myofibroblasts of normal and malignant human breast tissue. *Proc Natl Acad Sci USA* 1993;**90**:999–1003

10 Perez-Montiel MD, Plaza JA, Dominguez-Malagon H, Suster S. Differential expression of smooth muscle myosin, SMA, h-caldesmon, and calponin in the diagnosis of myofibroblastic and smooth muscle lesions of skin and soft tissue. *Am J Dermatopathol* 2006;**28**:105–11

11 Kumar S, Perlman E, Harris CA, Raffeld M, Tsokos M. Myogenin is a specific marker for rhabdomyosarcoma: an immunohistochemical study in paraffin-embedded tissues. *Mod Pathol* 2000;**13**:988–93

12 Montgomery E, Speight PM, Fisher C. Myofibromas presenting in the oral cavity: a series of 9 cases. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2000;**89**:343–8

13 Rodini CO, Pontes FS, Pontes HA, Santos PS, Magalhães MG, Pinto DS Jr. Oral leiomyosarcomas: report of two cases with immunohistochemical profile. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2007;**104**:e50–8

14 Chi AC, Barnes JD, Budnick S, Agresta SV, Neville B. Rhabdomyosarcoma of the maxillary gingiva. *J Periodontol* 2007;**78**:1839–45

15 Medjkane S, Perez-Sanchez C, Gaggioli C, Sahai E, Treisman R. Myocardin-related transcription factors and SRF are required for cytoskeletal dynamics and experimental metastasis. *Nat Cell Biol*2009;**11**:257–68

16 Yu YT, Breitbart RE, Smoot LB, Lee Y, Mahdavi V, Nadal-Ginard B. Human myocyte-specific enhancer factor 2 comprises a group of tissue-restricted MADS box transcription factors. *Genes Dev* 1992;**6**:1783–98

17 Edmondson DG, Cheng TC, Cserjesi P, Chakraborty T, Olson EN. Analysis of the myogenin promoter reveals an indirect pathway for positive autoregulation mediated by the muscle-specific enhancer factor MEF-2. *Mol Cell Biol* 1992;**12**:3665–77

18 Yamazaki K. Electron microscopy and immunohistochemistry studies of pulmonary carcinosarcomas expressing the transcription factor MEF-2 and showing significant cell-to-cell, cell-to-matrix, and epithelial–mesenchymal interactions. *Virchows Arch* 2004;**444**:542–53

19 Yamazaki K. An ultrastructural and immunohistochemical study of elastofibroma: CD 34, MEF-2, prominin 2 (CD133), and factor XIIIa-positive proliferating fibroblastic stromal cells connected by Cx43-type gap junctions. *Ultrastruct Pathol* 2007;**31**:209–19

20 Hidaka K, Morisaki T, Byun SH, Hashido K, Toyama K, Mukai T. The MEF2B homologue differentially expressed in mouse embryonal carcinoma cells. *Biochem Biophys Res Commun* 1995;**213**:555–60

21 Molkentin JD, Firulli AB, Black BL, Martin JF, Hustad CM, Copeland N, Jenkins N, Lyons G, Olson EN. MEF2B is a potent transactivator expressed in early myogenic lineages. *Mol Cell Biol* 1996;**16**:3814–24

22 Morisaki T, Sermsuvitayawong K, Byun SH, Matsuda Y, Hidaka K, Morisaki H, Mukai T. Mouse Mef2b gene: unique member of MEF2 gene family. *J Biochem* 1997;**122**:939–46

23 Chamulitrat W, Schmidt R, Chunglok W, Tomakidi P. Epithelium-like and fibroblast-like phenotypes derived from HPV16 E6/E7-immortalized human gingival keratinocytes following chronic ethanol treatment. *Eur J Cell Biol* 2003;**82**:313–22

24 Chamulitrat W, Huber A, Riedel HD, Stremmel W. Nox1 induces differentiation resistance in immortalized human keratinocytes generating cells that express simple epithelial keratins. *J Invest Dermatol* 2007;**127**:2171–83

25 Dobashi Y, Suzuki S, Sugawara H, Ooi A. Involvement of epidermal growth factor receptor and downstream molecules in bone and soft tissue tumors. *Hum Pathol* 2007;**38**:914–25

26 Mayer DCG, Leinwand LA. Sarcomeric gene expression and contractility in myofibroblasts. *J Cell Biol* 1997;**139**:1477–84

27 Ngo P, Ramalingam P, Phillips JA, Furuta GT. Collagen gel contraction assay. *Methods Mol Biol* 2006;**341**:103–9

28 Sobue K, Hayashi K, Nishida W. Expressional regulation of smooth muscle cell-specific genes in association with phenotypic modulation. *Mol Cell Biochem* 1999;**190**:105–18

29 Kordowska J, Huang R, Wang CL. Phosphorylation of caldesmon during smooth muscle contraction and cell migration or proliferation. *J Biomed Sci* 2006;**13**:159–72

30 Takeuchi K, Sakurada K, Endou H, Obinata M, Quinlan MP. Differential effects of DNA tumor virus genes on the expression profiles, differentiation, and morphogenetic reprogramming potential of epithelial cells. *Virology* 2002;**300**:8–19

31 Chamulitrat W, Sattayakhom A, Herold-Mende C, Stremmel W. Human papillomavirus 16 E6/E7-immortalized human gingival keratinocytes with epithelial mesenchymal transition acquire increased expression of cIAP-1, Bclx and p27(Kip1). *Exp Dermatol* 2009;**18**:1067–9

32 Wilkins-Port CE, Higgins PJ. Regulation of extracellular matrix remodeling following transforming growth factor-beta1/epidermal growth factor-stimulated epithelial–mesenchymal transition in human premalignant keratinocytes. *Cells Tissues Organs* 2007;**185**:116–22

33 Loeuillet C, Douay L, Hervé P, Chalmers DE. Preservation of the myofibroblastic phenotype of human papilloma virus 16 E6/E7 immortalized human bone marrow cells using the lineage limited alpha-SMA promoter. *Cell Growth Differ* 2001;**12**:233–42

34 Frid MG, Shekhonin BV, Koteliansky VE, Glukhova MA. Phenotypic changes of human smooth muscle cells during development: late expression of heavy caldesmon and calponin. *Dev Biol* 1992;**153**:185–93

35 D'Addario SF, Morgan M, Talley L, Smoller BR. h-Caldesmon as a specific marker of smooth muscle cell differentiation in some soft tissue tumors of the skin. *J Cutan Pathol* 2002;**29**:426–9

36 Yamauchi Y, Kohyama T, Takizawa H, Kamitani S, Desaki M, Takami K, Kawasaki S, Kato J, Nagase T. Tumor necrosis factor-alpha enhances both epithelial–mesenchymal transition and cell contraction induced in A549 human alveolar epithelial cells by transforming growth factor-beta1. *Exp Lung Res* 2010;**36**:12–24

37 Akkila WM, Chambers RL, Ornatsky OI, McDermott JC. Molecular cloning of up-regulated cytoskeletal genes from regenerating skeletal muscle: potential role of myocyte enhancer factor 2 proteins in the activation of muscle-regeneration-associated genes. *Biochem J* 1997;**325**:87–93

38 Ramachandran B, Yu G, Li S, Zhu B, Gulick T. Myocyte enhancer factor 2A is transcriptionally autoregulated. *J Biol Chem* 2008;**283**:10318–29

39 McKinsey TA, Zhang CL, Olson EN. MEF2: a calcium-dependent regulator of cell division, differentiation and death. *Trends Biochem Sci* 2002;**27**:40–7

40 Perrotti V, Rubini C, Fioroni M, Piattelli A. Soft tissue myxoma: report of an unusual case located on the gingiva. *J Clin Periodontol* 2006;**33**:76–8

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