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Modulation of Cell-cell Contacts during Intestinal Restitution *In Vitro* and Effects of Epidermal Growth Factor (EGF)

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Key Words
Cell migration • Wound healing • Restitution • EGF • CXCR4 • EMT • Metastasis • Tight junctions • Claudin • JAM • LI-cadherin

Abstract
Mucous epithelia represent a major barrier to the outside world and are capable of undergoing rapid repair after injury by cell migration, a process called "restitution". Here, a sensitive RT-PCR method was applied allowing systematic gene expression analysis of separated stationary and migratory non-transformed IEC-18 and IEC-6 cells after scratch wounding. The focus was on genes related to cell-cell contacts. Furthermore, the effect of epidermal growth factor (EGF) on gene expression was studied. Most of the genes investigated here were down-regulated in migratory cells. Many of the alterations are expected to affect the permeability of tight junctions. Also the nectin-afadin complex of adherens junctions was modulated as well as the expression of both the chemokine receptor CXCR4 and the EGF receptor. Of note, restitution was not accompanied by the epithelial-mesenchymal transition (EMT). EGF treatment severely affected the expression of genes important for cell-cell contact and cell communication such as selected tight junction components, CXCR4, and TFF3. Many of these genes are known to be involved in EMT and metastasis. Of special note, most of the expression changes induced by EGF are in contrast to the changes observed in migratory cells.

Introduction

Mucous epithelia cover the delicate surfaces of our body such as the respiratory, the alimentary, and the urogenitary tracts. Here, they represent highly interactive barriers allowing essential communication with the environment as well as ensuring the physical integrity of these surfaces. Major elements of the barrier are specialized epithelial cell-cell contacts such as tight junctions (TJs), adherens junctions, and desmosomes [1]. Furthermore, a series of defense mechanisms have developed in order to protect, regenerate and repair these epithelial barriers following constant exposure to a variety of noxious agents. The various defenses are of special importance in preventing chronic inflammation, which is a critical component of cancer progression [2]. For

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example, some 90% of fatal malignancies in adult humans are of epithelial origin. An important component in the early stages of mucosal repair is a process called "restitution", which starts within minutes following a superficial damage [3, 4]. Here, re-epithelialization is driven by migration of neighbouring cells into the wounded area.

Cell migration is essential not only for wound healing by restitution, but also for embryonic development, immunity (including inflammation), angiogenesis, and tumor metastasis [5]. While cells of the immune system may be considered constitutive migratory cells, most other cells are only migratory in a specific phase.

Under certain circumstances, epithelial cells may undergo an epithelial-mesenchymal transition (EMT) to become migratory [6]. Typical examples are migration processes during embryonic development and tumor progression (metastasis). The EMT is characterized by trans-differentiation of epithelial cells to fibroblastoid, motile cells, which is accompanied by a change to a mesenchymal gene expression program [7, 8]. Hallmarks are down-regulation of epithelial-specific genes (e.g., tight- and adherens-junction proteins such as occludin and E-cadherin, respectively) and induction of various mesenchymal genes such as vimentin, N-cadherin, and α-smooth muscle actin (α-SMA) [9-11]. It has been established for some time that transforming growth factor β (TGFβ) and epidermal growth factor (EGF) are key regulators of the EMT [9, 12] and both stimulate EMT even synergistically [13].

However, in spite of the many similarities there are a few crucial differences between cell migration processes during epithelial wound repair and cancer [14]. One major difference is that the EMT is not complete in wound repair [14]. This picture has been confirmed recently by studying mucosal restitution *in vitro* using the non-transformed rat intestinal cell line IEC-18 [15]. Both this cell line and the related cell line IEC-6 belong to the best studied *in vitro* models for restitution [16]. These cell lines share features of undifferentiated crypt cells and retain the potential to differentiate into the different intestinal cell lineages, i.e., enterocytes, goblet, Paneth and enteroendocrine cells. A method was developed allowing a systematic gene expression analysis of separated stationary and migratory IEC-18 cells after scratch wounding [15]. Of special note, full EMT was not detectable in migratory cells. However, motile cells reversibly lost markers of terminal differentiation [such as stromal cell-derived factor (SDF-1) and lysozyme] and changed to a phenotype that assists the process of restitution by up-regulating the expression of genes such as plasminogen activator inhibitor-1, transforming growth factor α (TGFα), heparin-binding EGF-like growth factor (HB-EGF), α-SMA, ornithine decarboxylase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [15].

There are no systematic data concerning modulation of cell-cell contacts during restitution *in vitro* so far. Thus, studying cell-cell contacts in stationary and migratory cells would be a very promising goal because epithelial cells undergoing restitution *in vivo* gradually interconvert between two types of cell polarity, i.e., they change from polarized epithelial cells (with apical-basal polarity) to polarized migrating cells (with planar polarity) [17].

Recently, we reported that EGF treatment of scratch-wounded IEC-18 cells resulted in enhanced cell migration with a significantly increased number of fibroblastoid cells in the migratory zone and an increased internalization of E-cadherin [18]. Similar results were reported for gastric cells [19]. This is indicative of the EMT [20]. Thus, the work presented here focuses on two major questions: How are cell-cell contacts changed during restitution and what are the effects of EGF? These goals were achieved by systematic expression analysis of selected genes (many of them were related to cell-cell contacts) in both separated stationary as well as migratory cells.

Materials and Methods

Culture and scratch wounding of the non-transformed IEC-18 cell line (from the ileum of newborn rats) as well as RT-PCR analysis of separated stationary and migratory cells (i.e., about 10 rows of cells directly behind the migratory front) was as described in detail previously [15]. In short, cells were seeded at a density of 300.000 cells per culture dish (10 cm diameter) at day 0, medium was changed on day 2 and was replaced with "starvation medium" on day 5. Scratch wounding was on day 7 when cells showed differentiation and clear formation of cell-cell contacts. Stationary as well as migratory cells were collected on day 9.

Alternatively, also the non-transformed epithelioid IEC-6 cell line from the crypts of the intestine of adult rats [21] was included in this study, which was purchased from the „Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH" (DSMZ; Braunschweig, Germany). Culture conditions were similar as for IEC-18 cells, with the exception that these cells were supplemented with 5% AC2 (Cell Concept, Umkirch, Germany) instead of 5% fetal calf serum. „Starvation medium" contained a reduced AC2 content (0.2% v/v). IEC-6 cells were seeded at a density of 600.000 cells per culture dish (10 cm diameter).

Table 1. Oligodeoxynucleotides used for RT-PCR analysis and calculated size of the products. F11r = Jam1, Gja1 = connexin 43, Jup = gamma catenin = plakoglobin, Marvel = tricellulin, Mrps18a = mitochondrial ribosomal protein S18A, Myo6 = MyosinVI, Pvrl1 (poliovirus receptor-related 1) = HveC = Nectin-1, Tjp = zonula occludens (ZO).

Genes (Acc. No.)	Primer pairs (nucleotide positions)	RT-PCR	Size (bp)	Intron -spanning
Ocln (NM_031329.2)	MB479 (1332)ACCCAGACCACTATGAAACC(1351)	57°C, 28 cycles	417	yes
	MB 480 (1748)TCTCACTTCTCCAGCAACC(1730)			
Cldn1 (NM_031699.1)	MB469 (510)GATGAAGTGCAAAAGATGTGG(530)	57°C, 35 cycles	228	yes
	MB470 (737)ACAGGAGCAGGAAAGTAGG(719)			
Cldn2 (XM_236535.1)	MB471 (251)TGGGGCTATTAGGCACATC(269)	57°C, 35 cycles	513	no
	MB472 (763)GTGAGCAGGAAAAGCAGAG(745)			
Cldn3 (NP_031700.2)	MB824 (1277)TTGCCACCTGATTACCTGG(1295)	57°C, 35 cycles	175	no
	MB825 (1451)TGAAAAATACGAAACGGCCC(1432)			
Cldn4 (NM_001012022.1)	MB711 (758)CAACGAAAAGCCCTACTCC(776)	57°C, 35 cycles	441	no
	MB712 (1198)GAATGTTTGCTAACATGACCAG(1177)			
Cldn5 (NM_031701.2)	MB791 (987)CCCTGCTCAGAACAGACTAC(1006)	57°C, 35 cycles	246	no
	MB792 (1232)TGCCCTTTTTAACGCATTCC(1213)			
Cldn6 (XM_001055688.1)	MB793 (281)TGTCATCACCCTCCTCATTG(300)	57°C, 35 cycles	119	no
	MB794 (399)TGACACCAGAGATGAGCAC(381)			
Cldn7 (NM_031702.1)	MB714 (419)CAACATCATCACAGCCCAG(437)	57°C, 35 cycles	293	yes
	MB715 (711)CTACCAAAGCAGCAAGACC(693)			
Cldn8 (NM_001037774.1)	MB795 (118)ACATTGTCGTGTTTGAGAACC(138)	57°C, 35 cycles	269	no
	MB796 (386)GATGAAGAAGATGATTCCAGCC(365)			
Cldn9 (NM_001011889.1)	MB1536 (982)GGTGCTTCGGGACTTGATAA(1001)	60°C, 35 cycles	393	no
	MN1537 (1374)GGGACTGCTTGGGCTAGACT(1355)			
Cldn10 (XM_001074876.1)	MB797 (157)ACCGCCACTTATTTTGCC(174)	57°C, 35 cycles	519	yes
	MB798 (675)TCCTTCTCCGCCTTGATAC(657)			
Cldn11 (NM_053457.2)	MB602 (606)CACCATCTGGTTTCCTGTATG(626)	57°C, 35 cycles	213	no
	MB604 (818)TAGACATGGGCACTCTTGG(800)			
Cldn12 (XM_001067835.1)	MB799 (894)CCTCAAGCATTTAGAGAGAACC(915)	57°C, 28 cycles	346	yes
	MB800 (1239)CAACTCTTCAATCCCTTCACC(1219)			
Cldn14 (BC091387.1)	MB801 (894)GACCACGAATGATGTGGTG(912)	57°C, 35 cycles	115	yes
	MB802 (1008)GATGAGAGACAGGGATGAGG(989)			
Cldn15 (XM_222085.4)	MB803 (410)ATACTTGCTGGAGCCTGTG(428)	57°C, 35 cycles	295	yes
	MB804 (704)TGCCATATTTACCAAAGCTGAC(683)			
Cldn16 (NM_131905.2)	MB805 (647)ATGAGCCACACATTAAAGTCC(667)	57°C, 35 cycles	209	yes
	MB806 (855)AAACAACCCAAAGACCCAG(836)			
Cldn17 (XM_001070924.1)	MB807 (183)TTTCAGCTTTCATCGGCAG(201)	57°C, 35 cycles	308	no
	MB808 (490)CAGAACGAAGATACCAGTCAC(470)			
Cldn18 (NM_001014096.1)	MB809 (347)CATTGGCAGCATGGATGAC(365)	57°C, 35 cycles	271	yes
	MB810 617)AGCAATGCACATCATCACAC(598)			
Cldn19 (NM_001008514.1)	MB811 (450)AGGTGCTCTCTTCCTCTTG(469)	57°C, 35 cycles	132	yes
	MB812 (581)AACAGAGCTGGACCAAATTC(562)			
Cldn20 (XM_001060166.1)	MB813 (202)TACTCCATTCTGTCGCTCC(220)	57°C, 35 cycles	316	no
	MB814 (517)ACATCGCTGAAATGAACCC(499)			
Cldn22 (XM_001059255.1)	MB815 (484)AGCTGAACGAGATGGAAAAC(503)	57°C, 35 cycles	149	no
	MB816 (632)ATTGGACAGGGACATCAGG(614)			
Cldn23 (NM_001033062.1)	MB817 (880)ATGGATGTGCTTGAGGGAGAAG(901)	57°C, 35 cycles	100	no
	MB818 (979)AGTCACAGGGCAGCGAATTTTG(958)			
F11r (NM_053796.1)	MB497 (1800)ACAGCAGATGCCAAGAAAAC(1818)	57°C, 30 cycles	384	yes
	MB498 (2040)TCACACCAGGAATGACGAG(2021)			
Jam2 (NM_001034004.1)	MB707 (308)AAAACCCCAAAGAAGACCAC(327)	57°C, 35 cycles	554	no
	MB708 (861)TTGAGAACATCTACTTGCATCC(840)			
Jam3 (NM_001004269.1)	MB709 (186)GACTCACAGACAAATGACCC(205)	57°C, 30 cycles	461	yes
	MB710 (646)TGCACAGCACTGAAAACC(629)			
Marvel (XM-345145.3)	MB765 (371)CTCTCCAGCAAGACCAAAC(389)	57°C, 28 cycles	668	no
	MB766 (1038)CTATCTGACCTCCTTCTACCC(1018)			
Cdh1 (NM_031334.1)	MB435 (2406)TGAAGAGGGAGGTGGAGAAG(2425)	57°C, 28 cycles	446	yes
	MB436 (2851)GGAGCTGAAAGACCACTGAC(2832)			
Cdh2 (NM_031333)	MB645 (2571)GATGAAACGCCGGGATAAAG(2590)	57°C, 28 cycles	440	yes
	MB646 (3010)GTCCCCAGTCATTCAGGTAG(2991)			
Cdh17 (NM_053977.1)	MB677 (1084)CCTCACCCCATCAAAATCAC(1103)	57°C, 35 cycles	315	yes
	MB678 (1398)GCTACTACCCAAGACTTCATTC(1377)			
Pvrl1 (XM_236210.4)	MB501 (387)AGCTCCACAGAGACTTGAC(405)	57°C, 30 cycles	231	no
	MB502 (617)AGCACACAAGACAAGAACAG(598)			
Gja1 (NM_012567.2)	MB848 (131)CAAGGAGTTCCACCAACTTT(150)	57°C, 30 cycles	314	yes
	MB849 (444)ATGATCTGAAGGACCCAGAA(425)			
Tjp1 (XM_218747.2)	MB481 (4907)TATCCAAACCAGACCCACC(4925)	57°C, 27 cycles	571	yes
	MB482 (5377)CCTCATCTTCATCTTCTTCCAC(5356)			
Tjp2 (NM_053773.1)	MB735 (1413)CCCTCATCAACATCCCATCTC(1433)	57°C, 28 cycles	448	yes
	MB736 (1860)CCAGCCACAAATATGCCAAC(1841)			
Tjp3 (XM_001069839.1)	MB733 (1992)TTGCTATGAAGAGACTGACAAC(2013)	57°C, 30 cycles	204	yes
	MB734 (2195)AGAAGATGACGACAGGGTAG(2176)			
Cng (XM 227472.4)	MB769 (1270)TTCAAATCCACCCCAGACC(1288)	57°C, 28 cycles	590	yes
	MB770 (1859)TCCTCTACTCGTTGTTTACCC(1839)			
Af6 (NM-013217.1)	MB771 (3993)CTGAAGCCTATCCTATCCCTAC(4014)	57°C, 28 cycles	592	yes
	MB772 (4584)TCCTCTCTCTTTTTCCTCTCTG(4563)			
Ctnna1 (NM_001007145.1)	MB767 (723)CCCATCCTCTACACTGCATC(742)	57°C, 23 cycles	599	yes
	MB768 (1321)ACCTCCTTCTCGTTTCCATTC(1301)			
Ctnnb1 (NM_053357.2)	MB691 (655) ACCCTACACAACCTTTCCC(673)	57°C, 28 cycles	406	yes
	MB692 (1060)TAGAGCAGACAGACAGCAC(1042)			
Jup (NM_031047.2)	MB950 (1730)GACAAGGACGACATCACAGA (1759)	57°C, 30 cycles	339	yes
	MB951 (2068) GATCTCCTCCATCCTCACAC (2049)			
Cxcr4 (NM_022205.3)	MB1350 (1)GTGCAGCAGGTAGCAGTGAC(19)	57°C, 35 cycles	301	yes
	MB1351 (301)GCAGCCGGTACTTGTCTGTC(283)			
Egfr (NM 031507.1)	MB705 (481)AATGCTCTCTACGAAAACACC(501)	57°C, 35 cycles	293	yes
	MB706 (773)CAGTTCTCCTCTCCTCTTCC(754)			

(continued next page)

Genes (Acc. No.)	Primer pairs (nucleotide positions)	RT-PCR	Size (bp)	Intron -spanning
Egf (NM 012842.1)	MB641 (3337)CGATGGGTACTGCCTCAATG(3356)	57°C, 35 cycles	291	yes
	MB642 (3627)TTGCTGCTTGACTCTTCGG(3609)			
Tff1 (NM_057129.1)	MB43 (1)GATCACTCGTGGTCTTCCC(19)	57°C, 35 cycles	446	yes
	MB44 (446)ATCAGGCCGAATTTGAGGG(428)			
Tff2 (NM_053844.1)	MB45 (1)GCAGAACACCCAGGTCCAG(19)	57°C, 28 cycles	555	yes
	MB46 (555)TTGGAAAGACACTGTGTC(537)			
Tff3 (NM_013042.1)	MB47 (1)GAAGTTTGCGTGCTGCCAT(19)	57°C, 35 cycles	384	yes
	MB48 (384)GGACTCCCAGCTCCTATAT(366)			
Tnc (DQ916292)	MB729 (66)AAGCGGGCATAAATCCCAG(86)	57°C, 28 cycles	298	unknown
	MB730 (365)CCAGAACCACCCAGTAGAAG(346)			
Fn1 (NM_019143.2)	MB675 (4710)TTCTATCACCCTCACCAACC(4729)	57°C, 22 cycles	448	yes
	MB676 (5157)GACACTGATGCTGTTGTCC(5139)			
Mylk3 (NM_001110810.1)	MB1492 (1631)AACACCGGGTAGTGAGCATC(1650)	60°C, 30 cycles	267	yes
	MB1493 (1897)GGGTGAAGCTGTTCTTGCTC(1878)			
Myo6 (XM_236444.4)	MB703 (1851)CACCAGAAGCACAAAGACC(1833)	57°C, 28 cycles	455	yes
	MB704 (2305)TCCAGCACAGACACCATAC(2287)			
Acta2 (NM_031004.1)	MB671 (257) CGATAGAACACGGCATCATCAC(278)	57°C, 23 cycles	457	yes
	MB672 (713) TCCAGAGCGACATAGCACAG(694)			
Gapdh (NM_017008.3)	MB751 (684)ACATCATCCCTGCATCCAC(702)	57°C, 22 cycles	330	yes
	MB752 (1013)ATACCAGGAAATGAGCTTCAC(992)			
Actb (NM_031144.2)	MB830 (818) AGGTCATCACTATCGGCAAT(837)	57°C, 22 cycles	266	yes
	MB831 (1083) CTCAGGAGGAGCAATGATCT(1064)			
Actb-promoter region (NW_047369.2)	MB1510 (28641)TTCCTCAATCTCGCTTTCTC(28660)	57°C, 35 cycles	209	------
	MB1511 (28849)GGTTTTATAGGACGCCACAG(28830)			
Mrps18a (NM_198756.1)	MB891 (184)AACTCCAGATCCCCCTAATC(203)	57°C, 28 cycles	393	yes
	MB892 (576)CTTCTGGAGTAGCGGACATT(557)			

In the present study, scratch wounded cells were also treated with EGF on day 7 as follows. The wounded cell cultures were rinsed gently and repeatedly (up to 5 times) with starvation medium to remove residual cell debris and then incubated at 37°C with 10 ml of fresh starvation medium supplemented with 5 nM EGF [Mr: 6222; purchased from Sigma, Taufkirchen, Germany; stock solution at 50 µg/ml in EGF vehicle (EGFV), i.e., 10 mM acetic acid and 0.1% bovine serum albumin]. In the case of the control cells, the supplement was omitted and replaced by adequate volumes of solvents (i.e., PBS or EGFV). Then, the cells were incubated in 6.5% (v/v) CO_2 at 37°C for 48 h. The cells were washed with starvation medium (lacking EGF) at the end of the migration period, i.e. day 9, and sorted for RT-PCR analysis as described previously.

Total RNA was isolated using Trizol Reagent (Invitrogen, Karlsruhe, Germany) and digested with DNAse. RNA concentration was determined with a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Peqlab Erlangen, Germany). The relative expression levels of selected genes was monitored using the specific primer pairs as listed in Table 1. As a control for the integrity of the cDNA preparations, transcripts for β-actin or mitochondrial ribosomal protein S18A were amplified in parallel reactions. The cDNA was also checked for contaminating chromosomal DNA by amplification of a promoter sequence from the β-actin gene (oligonucleotides MB1510 and 1511; see Table 1).

For selected transcripts a semi-quantitative analysis of the bands was performed in 4-9 independent experimental series. The images of the ethidium bromide-stained agarose gels were recorded with a camera (Herolab E.A.S.Y. 429K, Herolab GmbH, Karlsruhe, Germany) and the intensity of the bands was analyzed with Genesnap software (Syngene Europe, Cambridge, UK). The subsequent statistical analyses of the densitometric data was realized with the Excel 2003 software package (Microsoft.Office, USA) using the Student's t-test. The error bars in Figure 3 represent +SEM. Significant differences between the mean values of the experimental groups are indicated by asterisks ($P \leq 0.05$: one asterisk, $P \leq 0.01$: two asterisks, $P \leq 0.001$: three asterisks).

Results

The following genes known for their direct role in cell-cell contact formation were selected for RT-PCR analysis: occludin, claudins-1-12/14-20/22/23, junction adhesion molecules (JAMs) 1/2/3, tricellulin, ZO-1/2/3, cingulin; N-cadherin, LI-cadherin, nectin 1, afadin-6, α-catenin, β-catenin, γ-catenin; connexin 43. Furthermore, additional genes were included in this study such as the chemokine receptor CXCR4, the EGF receptor, EGF, TFF1-3, tenascin C, fibronectin 1, myosin light chain kinase (Mylk3/MLCK), and myosin 6. For comparison, transcripts for α-SMA, GAPDH or β-actin were amplified. The expression profiles of these genes were monitored in IEC-18 cells 48 h after scratch wounding (EGF-treated cells as well as control cells treated with PBS or EGFV, respectively). Both the stationary cells as well as the migratory cells were isolated and analyzed separately. The results of a typical experimental series performed in plastic culture dishes are shown in Fig. 1. RT-PCR profiles are shown only for those genes that gave clear and reproducible results (some of the genes investigated resulted in only very weak or non-detectable RT-PCR signals, e.g., many claudins). Furthermore, some of these genes were analyzed also in IEC-6 cells in order to demonstrate specific EGF effects (Fig. 2).

Transcripts for β-actin were amplified as a control for the amount and integrity of the cDNAs. All samples

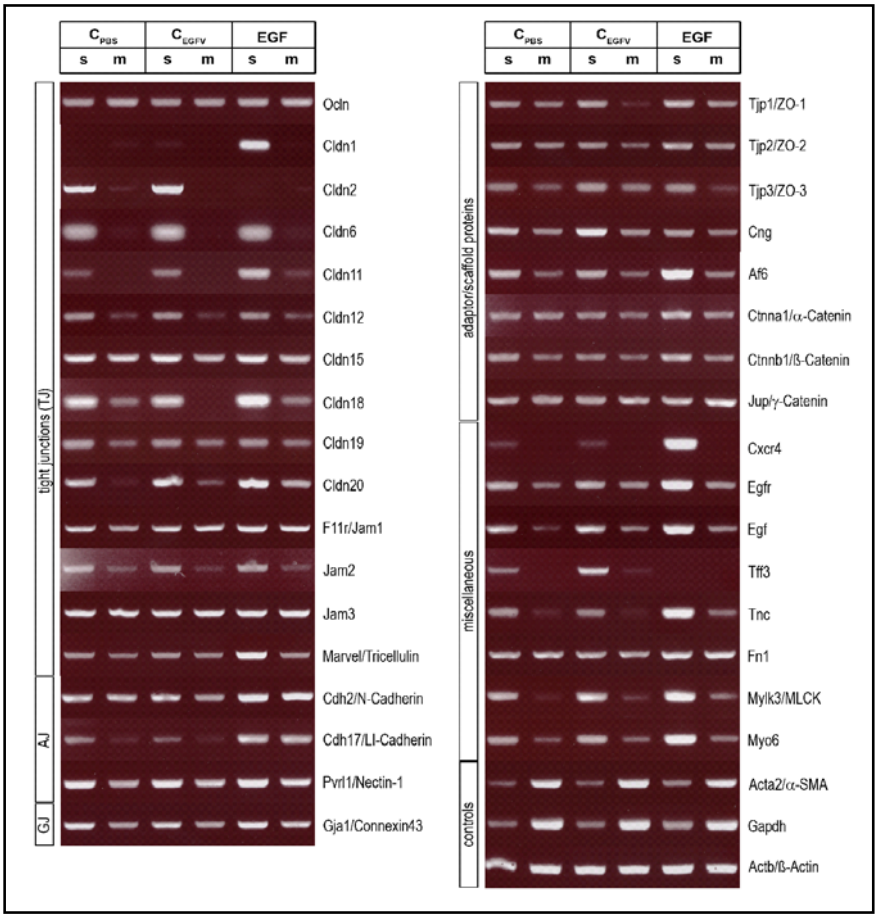

Fig. 1. RT-PCR analysis (IEC-18 cells). Gene expression concerning tight junction components [occludin, claudins, junction adhesion molecules (JAM), tricellulin], adherens junction components [N-cadherin, LI-cadherin, nectin-1], the gap junction component connexin 43, adaptor/scaffold proteins [zonula occludens (ZO), cingulin, afadin 6, catenins], miscellaneous proteins [Cxcr4, EGF receptor, EGF, Tff3, tenascin C, fibronectin 1, myosin light chain kinase (MLCK), myosin 6], and various controls [α-smooth muscle actin (α-SMA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-actin] was monitored in EGF-treated IEC-18 cells and control cells (C_{PBS} and C_{EGFV}) 48 h after scratch wounding. Both the stationary (s) and migratory (m) cells were isolated and analyzed separately. The integrity of the cDNAs was tested by monitoring the transcripts for β-actin.

showed comparable β-actin transcript levels or a slight increase in migratory cells. In contrast, both α-SMA and GAPDH transcripts were markedly elevated in all migratory cells when compared with the corresponding stationary cells.

Characteristic expression patterns were obtained when stationary and migratory IEC-18 cells were compared (Fig. 1). Generally, many genes were consistently down-regulated in migratory cells such as claudins-2, 6, 11, 12, 18, 19, 20, JAM2, ZO-1, 3, cingulin, LI-cadherin, nectin-1, afadin-6, β-catenin, connexin 43, CXCR4, EGF receptor, EGF, TFF3, tenascin C, Mylk3/MLCK, and myosin 6. Furthermore, the following genes showed a tendency to be weakly down-regulated in motile cells: claudin-1 and 15, tricellulin, and ZO-2. In contrast, a second group of genes did not show consistent differences in the expression levels of stationary and migratory cells. Genes belonging to this group are occludin, JAM1 and 3, N-cadherin, α-catenin, γ-catenin, fibronectin 1, and β-actin. A third group of genes was selected as controls because these genes were already known to be up-regulated in migratory cells [15]. This control group consisted of α-SMA and GAPDH only. For selected genes, such as claudins and various EGF

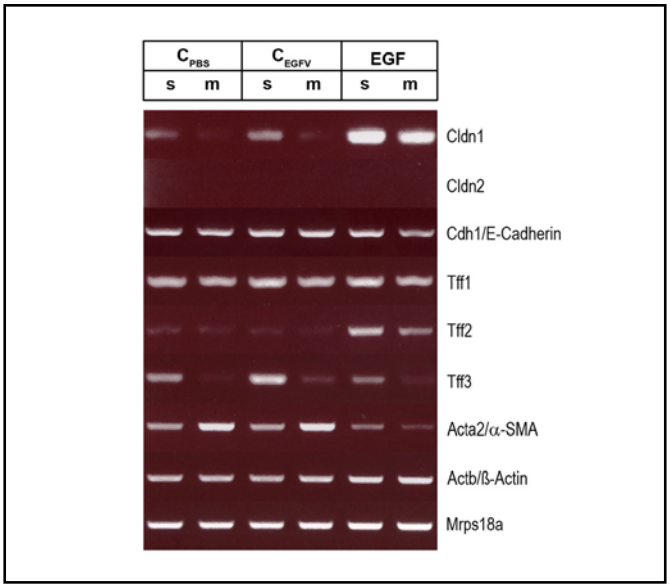

Fig. 2. RT-PCR analysis (IEC-6 cells). Gene expression concerning claudin-1, claudin-2, E-cadherin, Tff1, Tff2, Tff3, α-smooth muscle actin (α-SMA), β-actin, and mitochondrial ribosomal protein S18A was monitored in EGF-treated IEC-6 cells and control cells (C_{PBS} and C_{EGFV}) 48 h after scratch wounding. Both the stationary (s) and migratory (m) cells were isolated and analyzed separately. The integrity of the cDNAs was tested by monitoring the transcripts for β-actin.

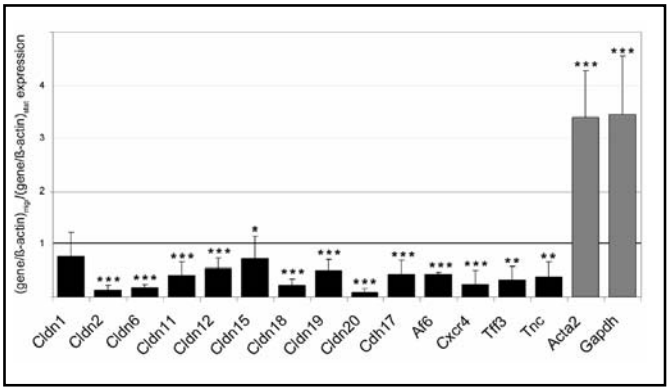

Fig. 3. Semi-quantitative RT-PCR analysis (IEC-18 cells). Shown are the transcript ratios of selected genes in migratory and stationary cells both normalized against β-actin (relative gene expression levels), i.e. $(\text{gene}/\ \beta\text{-actin})_{migr}/(\text{gene}/\ \beta\text{-actin})_{stat}$, based on the data from 4-11 independent experiments. Values below 1 indicate declined expression of the selected gene in migratory cells (black bars); whereas values above 1 are characteristic of up-regulation in migratory cells (grey bars). The relative expression level of the following genes was investigated: claudins, cadherin17/LI-cadherin, afadin-6, Cxcr4, Tff3, tenascin C, Acta2/α-SMA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Significant differences between stationary and migratory cells are indicated by asterisks: *** extremely high significance ($P \leq 0.001$), ** high significance ($P \leq 0.01$), * significance ($P \leq 0.05$).

regulated genes (LI-cadherin, afadin-6, CXCR4, TFF3, and tenascin C), a semi-quantitative RT-PCR analysis was performed using 4-9 independent experimental series (Fig. 3). For comparison, the two known up-regulated genes (α-SMA and GAPDH, [15]) were included. Clearly, down-regulation was significant for all the selected genes, with the exception of claudin-1. Furthermore, as expected, up-regulation of α-SMA and GAPDH transcripts was also significant.

EGF treatment of IEC-18 cells also changed the expression profile characteristically (Fig. 1). Some genes were consistently up-regulated by EGF, such as claudin-1 and 18, tricellulin, N-cadherin, LI-cadherin, afadin-6, CXCR4, EGF receptor, EGF, tenascin C, Mylk3, and myosin 6. Significance of up-regulation was demonstrated by semi-quantitative RT-PCR analysis for selected genes (claudin-1 and 18, LI-cadherin, afadin-6, CXCR4, and tenascin C) in 4-9 independent experimental series (data not shown). Markedly down-regulated by EGF were claudin-2 and TFF3 expression (significance was demonstrated by semi-quantitative RT-PCR analysis of 7 and 4 independent experimental series, respectively; data not shown). A similar picture appeared after studying IEC-6 cells (Fig. 2). Here, additionally TFF2 expression was up-regulated by EGF (in contrast, TFF2 transcripts were not detectable in IEC-18 cells). Furthermore, the α-SMA transcript level was down-regulated by EGF (in contrast to IEC-18 cells).

Discussion

Modulation of Gene Expression during Intestinal Restitution

A number of genes playing a direct role in cell-cell contact formation were down-regulated in migratory IEC-18 cells (Figs. 1 and 3). These genes include components of tight (claudins-2, 6, 11, 12, 18-20, JAM2) and adherens junctions (LI-cadherin, nectin-1), their respective adaptor and scaffold proteins (ZO-1, 3, cingulin, β-catenin, afadin-6) as well as gap junctions (connexin 43). Furthermore, claudin-1 and 15 transcripts show a tendency to be down-regulated in motile cells. However, other components of tight (occludin, JAM1 and 3, tricellulin) and adherens junctions (N-cadherin) as well as some adaptor proteins (ZO-2, α-catenin, γ-catenin) did not show marked alterations during restitution.

Expression of the classical adherens junction protein E-cadherin was not significantly changed neither during restitution of IEC-6 (Fig. 2) nor IEC-18 cells [15]. Interestingly, the E-cadherin transcript level was much higher in IEC-6 cells than in IEC-18 cells (data not shown) which might be indicative of a more differentiated state in IEC-6 cells. However, the reduced expression of the atypical cadherin LI-cadherin as well as the nectin-afadin complex, and connexin 43 clearly indicate that adherens and gap junctions were selectively altered in motile cells. Of note, afadin-6 serves as a scaffold protein between cell membrane-associated proteins and the actin cytoskeleton and a reduction in afadin-6 levels has been reported to accelerate epithelial wound closure due to impaired E-cadherin-dependent intercellular adhesion [22]. Thus, reduction of afadin-6 expression levels in migratory IEC-18 cells is expected to enhance restitution.

Of note, also myosin 6 expression is down-regulated in migratory IEC-18 cells. Myosin 6 is required for E-cadherin-mediated cell migration [23]. In contrast to most other motor proteins it is trafficking towards the minus end of the actin filament.

Generally, the data indicate that tight junctions were markedly modified during restitution of IEC-6 cells particularly by down-regulation of all claudins detectable (Fig. 3) as well as JAM2. Claudins are the major determinants of the barrier function of tight junctions with

a complex expression pattern along the intestine and they create highly selective paracellular pores (channels) for specific ions [24]. Most pronounced were down-regulation of claudin-2 and 6. Interestingly, claudin-2 is known for inducing lower transepithelial resistance and claudin-6 overexpression induces a barrier dysfunction [25]. Down-regulation of specific claudins in migratory cells could be related with that of ZO-1 because ZO-1 plays a specific role for claudin polymerization during formation of TJs around the adherens junctions [26, 27]. Down-regulation of ZO-3 might also be connected with that of ZO-1 because both proteins appear to be associated and even co-immunoprecipitate [26]. Specific down-regulation of JAM2 is also interesting, because only this JAM is solely enriched in apical ZO-1-positive regions and it is known for its heterophilic interactions with JAM3 as well as various integrins [28]. Furthermore, ZO-1 is indispensable for cingulin to be recruited to TJs [26]. Thus, down-regulation of cingulin in migratory cells could well be related with that of ZO-1. However, down-regulation of cingulin is also of special interest because it is a critical player in linking TJ signaling and gene expression, particularly that of claudin-2 and ZO-3 [1].

The observation that occludin is not down-regulated in migratory IEC-18 cells argues strongly against EMT during restitution. This view is in agreement with a previous study [15].

Other genes, whose expression is markedly down-regulated in migratory IEC-18 cells, are those encoding TFF3 and MLCK. This is in agreement with the reduced terminal differentiation of migratory cells [15] because TFF3 is a typical secretory product of intestinal goblet cells [29] and MLCK is normally restricted to well differentiated enterocytes [30]. MLCK expression is low in proliferating intestinal crypt cells [30]. Particularly, down-regulation of MLCK is highly interesting because MLCK is known to effect cell migration and TJ permeability. For example, activated MLCK catalyzes the activation of MLC enhancing cell migration [31]. Furthermore, MLCK is well established in the regulation of intestinal TJ permeability, particularly in absorptive enterocytes [30]. Here, a reduction of MLCK levels decreased TJ permeability [30]. Thus, MLCK is expected to be a major player during restitution.

Furthermore, migratory IEC-18 cells show decreased transcript levels of the chemokine receptor CXCR4 as well as the EGF receptor which would also be in line with reduced terminal differentiation regulated by TJ signaling [1]. Expression of CXCR4 has been observed in enterocytes as well as in IEC-6 cells [32, 33]. Interestingly, down-regulation of CXCR4 in migratory cells parallels that of its major ligand SDF-1 [15].

Furthermore, also EGF is down-regulated in migratory IEC-18 cells. This is somewhat surprising because other EGF receptor ligands such as TGFα and HB-EGF are up-regulated in migratory IEC-18 cells [15]. Thus, EGF seems to differ in its function by rather promoting differentiation of IEC-18 cells; whereas TGFα and HB-EGF enhance restitution [15].

EGF Induces Expression of Genes Involved in EMT and Metastasis

From the time-lapse studies of scratch-wounded IEC-18 cells [18] the question arises whether the typical motogenic and morphogenic effects of EGF are accompanied by specific changes in gene expression, particularly in migrating cells.

As a hallmark, EGF treatment caused a characteristic inverse regulation of the tight junction genes claudin-1 and 2 in IEC-18 cells. Claudin-1 was strongly up-regulated in EGF-treated cells; whereas claudin-2 was markedly down-regulated. The latter is in agreement with the observation that MAPK signaling is known to down-regulate claudin-2 expression [24]. The differential regulation of claudin expression by EGF is not accompanied by altered expression of the tight junction protein occludin which is in complete agreement with previously published results [34]. The observed inverse change in claudin expression in IEC-18 cells after EGF treatment would be well placed to increase synergistically the transepithelial electrical resistance [25, 34] and is contrary to the change observed in patients with ulcerative colitis [35]. Paradoxically, claudin-1 up-regulation has been found in colorectal cancer and claudin-1-overexpressing cells produced more metastasis than cells expressing low levels of claudin-1 [25]. This effect is explained by misregulated tight junction assembly. Other TJ genes up-regulated by EGF are claudin-18 and tricellulin. Claudin-18 represents a typical gastric claudin [36]; whereas the transmembrane protein tricellulin participates in the epithelial barrier and organization of not only tricellular but also bicellular TJs [24]. In this context it is also interesting that MLCK expression is up-regulated in EGF-treated cells because MLCK is well established in the regulation of intestinal TJ permeability [30]. Taken together, EGF-treatment is expected to change dramatically TJ permeability and transepithelial resistance of IEC-18 cells by regulating the expression of a variety of crucial genes.

LI-cadherin (also known as cadherin-17) is also up-

regulated by EGF. This atypical cadherin is associated with metastasis of gastric carcinoma to the lymph nodes and its expression correlates with tumor invasion and poor prognosis in gastric cancer [37]. Thus, the increased expression of LI-cadherin could also be responsible for the tendency of EGF-treated IEC-18 cells to leave the formation and to migrate as individual cells with a fibroblast-like morphology [18].

EGF treatment also resulted in a markedly increased afadin-6 expression. Afadin-6 is a negative regulator of Rap-induced cell adhesion and overexpression of afadin-6 has been reported to inhibit cell adhesion to fibronectin [38]. Of note, fibronectin 1 has been localized in IEC-6 cells exclusively in regions of cell-cell contacts [39]. Also the myosin 6 expression is up-regulated by EGF. This actin-based motor protein is required for E-cadherin mediated cell migration.

Furthermore, N-cadherin expression slightly increased in EGF-treated cells when compared with control cells. This could be a sign for a gradually increased EMT when compared with untreated cells.

The extracellular matrix protein tenascin C is another consistently up-regulated gene in IEC-18 cells after EGF treatment. It is a typical mesenchymal protein induced by the EMT and plays a crucial role during wound healing and metastasis [7, 40, 41]. Thus, tenascin C is certainly a major candidate responsible for the morphogenic effect of EGF on IEC-18 cells [18]. This view would also be in line with regulation of tenascin C expression via ERK/MAPK signaling [40].

One typically up-regulated gene by EGF is that encoding the chemokine receptor CXCR4. This result is in line with a recent report concerning fibrocytes [42]. The observed up-regulation of CXCR4 expression is very important because this chemokine receptor is most widely expressed in malignancy and it clearly contributes to tumor metastatic capacity [43]. For example, down-regulation of CXCR4 resulted in reduced migration and invasion *in vitro* and the cells formed fewer metastases *in vivo* [44]. Furthermore, the expression of the EGF receptor as well as its ligand EGF were up-regulated by EGF. This autocrine stimulation of EGF expression is in line with a previous report on IEC-6 cells [45].

To our surprise, TFF3 expression was markedly down-regulated in both IEC-6 and IEC-18 cells after EGF treatment. To our knowledge, this is the first report describing such an effect. If this effect is of any physiological or pathological relevance is not clear to date. However, it might explain decreased TFF3 levels observed in ulcerative colitis tissues [46]. Furthermore,

Gene fct.	Migration-responsive genes s>m	Migration-responsive genes s<m	EGF-regulated genes	
Cell-cell contacts	Cldn-2, 6, 11, 12, 15, 18, 19, 20		Cldn1	↑
			Cldn2	↓
			Cldn18	↑
	Jam2		Marvel/Tricellulin	↑
	Cdh17/LI-Cadherin		Cdh2/N-Cadherin	↑
	Pvrl1/Nectin-1		Cdh17/LI-Cadherin	↑
	Gja1/Connexin43			
	Tjp1/ZO-1			
	Tjp3/ZO-3			
	Cng			
	Af6		Af6	↑
	Ctnnb1/ß-Catenin			
Receptors/ligands	Cxcr4		Cxcr4	↑
	Egfr		Egfr	↑
	Cxcl12/SDF-1[15]			
	Egf	Tgfa/TGFα[15]	Egf	↑
		Hbegf [15]		
ECM	Tnc		Tnc	↑
		Serpine1/PAI-1[15]		
Cytoplasm	Mylk3/MLCK		Mylk3/MLCK	↑
	Myo6		Myo6	↑
		Acta 2/α-SMA		
		Gapdh		
		Odc1 [15]		
Differ. marker	Tff3		Tff3	↓
	Lyz [15]			
	Anpep [15]			
Trans	Msi1 [15]			
	Gata5 [15]			

Table 2. Gene expression in IEC-18 cells. Shown are genes which are down-regulated (s>m) or up-regulated in migratory cells (s<m) as well as EGF-regulated genes (based on results shown here or previously [15]). The genes were grouped according to their functions (cell-cell-contacts,receptors/ligands, extracellular matrix components, cytoplasmic components, intestinal differentiation markers, and regulators of a transcription).

the decreased TFF3 expression might also influence the migration pattern of IEC-18 cells [18]. In contrast, TFF2 expression was up-regulated by EGF in IEC-6 cells only; whereas TFF1 expression was not altered following EGF treatment. The up-regulation of TFF2 by EGF is in agreement with a previous report using AGS-E cells [47]. The corresponding TFF proteins are well known for their key roles during regeneration and repair of mucous epithelia, particularly by enhancing restitution [4].

Of special note, most of the expression changes induced by EGF are in contrast to the changes observed in migratory cells. For example, expression of claudin-1, claudin-18, LI-cadherin, afadin-6, CXCR4, EGF receptor, EGF, tenascin C, MLCK, and myosin 6 was up-regulated by EGF; whereas migratory cells showed down-regulation of these genes. Thus, EGF clearly acts as a motogen enhancing the migration speed of IEC-18 cells [18] but it

induces an expression pattern which is partly inverse to the expression pattern of non-transformed migratory IEC-18 cells. This discrepancy may be the reason why these cells do not show a cooperative migration pattern and behave more like metastatic cells [18].

Conclusions

Taken together, there are characteristic changes in gene expression concerning cell-cell contacts and the epithelial barrier during restitution of non-transformed IEC-18 cells *in vitro* (summarized in Tab. 2). Generally, the corresponding genes were typically down-regulated in migratory cells. Many of the alterations are expected to severely affect the permeability of TJs. Furthermore, the nectin-afadin complex of adherens junctions is modulated; whereas the E-cadherin expression was not significantly changed. Also expression of both the chemokine receptor CXCR4 and the EGF receptor genes are down-regulated in migratory cells, as well as expression of terminal differentiation markers. Of special note, the results clearly strengthen our previous report that restitution of IEC-18 cells is not accompanied by the EMT [15].

Furthermore, EGF treatment severely affected the expression pattern of crucial genes important for cell-cell contact and cell communication (summarized in Tab. 2). Particularly concerned were selected TJ components (such as claudins-1, 2, and 18), the chemokine receptor CXCR4, and TFF3. Interestingly, most of the effects induced by EGF were in contrast to the expression changes induced by migration. Generally, many of the EGF-induced expression changes affected genes known to be involved in EMT and metastasis. This is in good agreement with the scattered migration and fibroblast-like morphology of EGF-treated cells [18, 19]. The *in vitro* results obtained here might also be of relevance for *in vivo* situations because EGF and other EGF receptor ligands are present at the luminal side of most mucous epithelia [48]. However, EGF is assumed to have beneficial effects *in vivo* after its physiological release from salivary and Brunner's glands and even intravenous infusion of EGF in an infant with necrotising enterocolitis has been reported [49]. Maybe, the naturally occurring rather complex combination of EGF and other growth factors might create a cocktail which optimally supports barrier integrity and repair of mucous epithelia by ensuring cooperative migration during restitution in spite of the presence of EGF. The method used here and previously [15] might facilitate systematic investigations in this direction.

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