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Research Article

Enamel Matrix Derivative and TGF-Beta 1 Target Genes in Human Tongue Carcinoma Cells

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ABSTRACT

Enamel matrix derivative (EMD) can enhance proliferation and migration of different oral cell lines, including malignant oral carcinoma cells, *in vitro* and *in vivo*. The composition of EMD is not known, but part of the effects have been postulated to be caused by transforming growth factor-beta-1 (TGF-beta 1). This study aimed to compare target genes of EMD and TGF-beta 1 on highly malignant oral carcinoma HSC-3 cells. Microarrays were used to examine differentially expressed genes in HSC-3 cells after 6h and 24h incubations with EMD (200 µg/ml) or TGF-beta 1 (10 ng/ml). Gene Ontology (GO) enrichment analysis of the regulated genes was also conducted. After 6h and 24h of EMD treatments 42 and 12 genes, respectively, were statistically significantly ($P<0.05$) up- or down-regulated. However, as many as 393 and 346 genes were statistically significantly ($P<0.05$) up- or down-regulated by TGF-beta 1. Among the most up-regulated genes by both of the study reagents were MMP-9 and -10. The expression of MMP-10 by EMD treated carcinoma cells was also verified in protein level. In conclusion, TGF-beta 1 regulates more and mostly different genes compared with EMD, but both regulate the expression of matrix metalloproteinase genes in oral carcinoma cells.

Introduction

An extract of porcine enamel matrix, e.g., enamel matrix derivative (EMD), can promote periodontal regeneration by enhancing the proliferation and migration of periodontal ligament fibroblasts, osteoblasts and cementoblasts [1-3]. EMD can regulate the expression of genes related to extracellular protein synthesis, cell adhesion, cell growth and apoptosis in fibroblasts and osteoblast-like cell line (MG-63) [4-6]. Furthermore, EMD has been shown to modulate the expression of growth factors (transforming growth factor-beta 1 (TGF-beta 1) and platelet-derived growth factor), cytokines (interleukin-6), and MMPs [7-10].

The predominant compound of EMD is amelogenin (>90%) [11, 12]. However, several studies have shown EMD to be more effective than amelogenin in enhancing regeneration related factors [13-17]. Therefore, it is generally assumed that EMD contains other biologically active factors in addition to enamel proteins [11-13]. Results concerning cytokines and growth factors present in EMD or stimulated by EMD have been inconsistent, but a body of evidence suggests that at least some effects of EMD on gene expression require TGF-beta activity [18-22]. TGF-beta, and in particular TGF-beta 1, is a key regulator of epithelial homeostasis. TGF-beta 1 also acts as one of the major cytokines in carcinogenesis by stimulating angiogenesis, invasion and metastasis in oral carcinomas [23-26]. Recently, TGF-beta receptor inhibitors such as galunisertib have been developed with promising results in treating patients with advanced hepatocellular carcinoma [27]. A very recent review concluded that squamous cell carcinomas are an optimal cancer type to study the effectiveness of TGFβ inhibition due to the high prevalence of dysregulated TGF-beta signaling [28].

Previous studies have shown that TGF-beta 1 can directly induce expression of matrix metalloproteinases (MMPs) MMP-2 and MMP-9 in pre- and malignant epithelial cell lines, also of oral origin, as well as

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in endothelial cells [29-32]. Furthermore, it has been shown that TGF-beta 1 stimulates HSC-4 oral squamous cell carcinoma invasion through MMP-10 signaling [33]. In carcinomas, MMPs are important because of their ability to degrade extracellular matrix and basal membrane components and overexpression of MMPs, particularly MMP-2 and -9, are associated with poor prognosis in oral cancer [34-36]. We have previously shown EMD to be capable of enhancing MMP-2 and -9 release by HSC-3 carcinoma cells and to stimulate their migration. Furthermore, EMD promoted metastasis formation *in vivo* [8]. This effect of EMD on MMPs might be due to TGF-beta 1 activity in EMD.

As expected, most *in vitro* studies on EMD have focused on periodontal fibroblasts or bone cells. In contrast, the effects of EMD on other cell lines, particularly on malignant cells, have not been adequately studied. As EMD is widely used in regenerative dentistry, also patients with premalignant or malignant oral lesions may become subject to EMD treatments. In this study, which is also a part of an academic dissertation of MM, we aimed to find out and compare the target genes of EMD and TGF-beta 1 in HSC-3 oral carcinoma cells using Affymetrix microarrays [37]. Furthermore, the presence of MMP-10 in HSC-3 culture media was analysed by western blotting. Our hypothesis was that EMD and TGF-beta 1 have rather similar effects on the gene expression of HSC-3 oral carcinoma cells.

Materials and Methods

I Study Reagents

Lyophilized Enamel matrix derivative (EMD; Emdogain®) was provided by the manufacturer (Straumann, Basel, Switzerland). Recombinant human TGF-beta 1 was acquired from a supplier (Sigma-Aldrich, St. Louis, MO).

II Cell Cultures

The experiments were carried out using highly malignant HSC-3 tongue squamous cell carcinoma cells (tongue, Japan Health Science Resources Bank, JRCB 0623), cultured at 37°C in a humidified atmosphere and in their normal media as described earlier [8].

III Microarray Sample Preparation and Hybridization

1x106 HSC-3 cells/flask were let to grow overnight. The culture media was changed, and TGF-beta 1 (10 ng/ml) or EMD (200 µg/ml) was added, and untreated cells acted as a control. TGF-beta 1 and EMD concentrations used in were selected based on previous studies [8, 38]. The study cells were incubated for 6 h and 24 h and control cells for 24 h. Total cellular RNA was extracted using Qiagen RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Oslo, Norway). RNA quality and quantity were determined using the Agilent 2100 Bioanalyser (Agilent Technologies, DE, USA). Microarray experiments were carried out using the Affymetrix Human Genome U133 Plus 2.0 chip for analysis of over 47,000 transcripts. Total RNA (8 µg) from each sample was used for target cDNA synthesis according to the Affymetrix protocol (Link 1). Following the recommendations by Lee and Whitmore, three biological replicates were produced at each time point [39].

IV Data Deposition

The microarray data is publicly available at ArrayExpress (E-MEXP-2645).

V Microarray Data Analysis

The microarray data analysis was performed using open-source Chipster software (Link 2). For normalization, the Robust Multi-Array method was used. Empirical Bayes t-test was used to estimate the statistical significance of individual genes with a cut-off threshold of $P<0.05$. The fold change (FC) of the statistically significantly regulated genes is given in (Table 1 or in Supplement material S1 & S2).

For the differentially expressed genes, Gene Ontology (GO) enrichment analysis was performed using DAVID annotation tool (Link 3) according to Huang *et al.* [40]. The analysis was conducted using the list of all statistically significantly up- and down-regulated genes separately. The whole-genome list of the Human Genome U133 Plus 2.0 chip was used as a background. An EASE score (E-score), a modified Fisher Exact P-Value, was used to identify enriched categories. E-score <0.05 was considered as statistically significant. Furthermore, the enriched terms were grouped into functional clusters according to terms having similar biological meanings due to sharing similar gene members.

VI Western Immunoblotting

28,000 HSC-3 cells/well with three replicates were cultured in serum-free media with EMD (0, 100 or 200 µg/ml) for 24 h. Western blot for MMP-10 from culture media was carried out as previously described [41]. After SDS-PAGE gel electrophoresis, the membrane was incubated with human monoclonal MMP-10 antibody (1:1000, R&D systems, Minneapolis, MN).

Results

I 6 h EMD Treatment

A total of 29 genes were statistically significantly ($P<0.05$) up-regulated and 13 genes down-regulated in HSC-3 carcinoma cells after 6 h of EMD treatment compared to untreated control cells [(List of genes and fold changes (FC) in (Table 1)]. MMP-10 and PI3 were among the up-regulated genes. Claudin 1, transforming growth factor beta 2, epithelial membrane protein 1 (EMP1), and endothelin 1 were among the most down-regulated genes.

II 24 h EMD Treatment

After 24 h of EMD incubation, the regulated genes ($P<0.05$) were highly attenuated, showing only four up-regulated and eight down-regulated genes (Table 1). MMP-9 was the most significantly up-regulated, whereas surprisingly, MMP-10 was now the most down-regulated gene. Small proline-rich protein, annexin A10, TGF-beta-induced factor homeobox 1, neuregulin 1, and T-box 1 were down-regulated.

Table 1: Differentially expressed genes in HSC-3 cells, 6 h (A) and 24 h (B) after EMD administration. The two columns on the right present the genes, which were also regulated by TGF-β1 after 6 h (C) and 24 h (D).

Gene Symbol	Chr.	GenBank	Gene name	P (EMD)	FC (EMD)	P (TGF-β)	FC (TGF-β)
A) up-regulated genes in 6 h time point						***C) up-reg.***	
SCG5	15	NM_003020	secretogranin V (7B2 protein)	0,000	1,89	0,000	3,46
CFB	6	NM_001710	complement factor B	0,000	1,87	0,002	1,74
TMEPAI	20	AL035541	transmembrane, prostate androgen induced RNA	0,000	1,42	0,003	1,44
S100A8	1	NM_002964	S100 calcium binding protein A8	0,000	1,41	0,047	1,02
PI3	20	NM_002638	peptidase inhibitor 3, skin-derived (SKALP)	0,001	1,36	0,025	1,18
MMP10	11	NM_002425	matrix metallopeptidase 10 (stromelysin 2)	0,001	1,27	-	-
SLPI	20	NM_003064	secretory leukocyte peptidase inhibitor	0,002	1,27	0,029	1,15
SPRR1B	1	NM_003125	small proline-rich protein 1B (cornifin)	0,002	1,26	0,026	1,25
S100A9	1	NM_002965	S100 calcium binding protein A9	0,005	1,11	-	-
CDC2	10	AA749427	cell division cycle 2, G1 to S and G2 to M	0,006	1,09	-	-
DHRS2	14	AK000345	dehydrogenase/reductase (SDR family) member 2	0,011	1,01	0,010	1,27
FLRT2	14	NM_013231	fibronectin leucine rich transmembrane protein 2	0,016	0,97	0,005	1,35
ALG5	13	AF102850	asparagine-linked glycosylation 5 homolog	0,021	0,92	-	-
FAM76B	11	NM_144664	family with sequence similarity 76, member B	0,022	0,92	-	-
GJB2	13	M86849	gap junction protein, beta 2, 26kDa	0,024	0,90	0,001	1,50
GDPD1	17	R46180	glycerophosphodiester phosphodiesterase domain conta	0,024	0,90	-	-
PCDH7	4	BE644809	protocadherin 7	0,025	0,90	-	-
TLL2	10	AA582404	tolloid-like 2	0,030	0,87	0,002	1,55
PTGS2	1	NM_000963	prostaglandin-endoperoxide synthase 2	0,038	0,83	0,000	3,11
EDNRA	4	NM_001957	endothelin receptor type A	0,041	0,82	0,005	1,38
WDR5B	3	BF434228	WD repeat domain 5B	0,042	0,81	-	-
MCL1	1	AI806486	myeloid cell leukemia sequence 1 (BCL2-related)	0,042	0,81	-	-
HNRPDL	4	D89678	heterogeneous nuclear ribonucleoprotein D-like	0,042	0,81	-	-
FLRT2	14	AF169676	fibronectin leucine rich transmembrane protein 2	0,043	0,81	0,005	1,35
FXYD3	19	BC005238	FXYD domain containing ion transport regulator 3	0,044	0,81	-	-
C1S	12	BC007010	complement component 1, s subcomponent	0,044	0,80	0,018	1,18
SPRR1A	1	AI923984	small proline-rich protein 1A	0,044	0,80	-	-
KRCC1	2	NM_016618	lysine-rich coiled-coil 1	0,047	0,79	0,036	1,02
ZNF30	19	AI700188	zinc finger protein 30	0,049	0,79	-	-
A) down-regulated genes in 6 h time point						***C) down-reg.***	
KRTAP2-1	17	BC012486	keratin associated protein 2-1	0,000	-2,01	0,022	-1,28
OLR1	12	AF035776	oxidized low density lipoprotein (lectin-like) receptor 1	0,007	-1,08	-	-
CLDN1	3	NM_021101	claudin 1	0,010	-1,04	-	-
TncRNA	11	AU155361	trophoblast-derived noncoding RNA	0,014	-0,98	-	-
TGFB2	1	NM_003238	transforming growth factor, beta 2	0,020	-0,93	0,048	-0,93
EGLN3	14	NM_022073	egl nine homolog 3 (C. elegans)	0,028	-0,88	-	-
SFRS4	1	R05895	splicing factor, arginine/serine-rich 4	0,029	-0,87	-	-
FBXO32	8	AW006123	F-box protein 32	0,040	-0,82	-	-
VGLL1	X	BE542323	vestigial like 1 (Drosophila)	0,043	-0,81	0,001	-1,51
EMP1	12	BC017854	epithelial membrane protein 1	0,044	-0,80	0,023	-1,07
EDN1	6	J05008	endothelin 1	0,045	-0,80	-	-
ATRX	X	AA826176	alpha thalassemia/mental retardation syndrome X-linked	0,046	-0,80	-	-
LOC440836	22	BE048068	similar to MGC52679 protein	0,05	-0,78	-	-
B) up-regulated genes in 24 h time point						***D) up-reg.***	
MMP9	20	NM_004994	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatina	0,008	1,04	0,000	4,11
TMEM33	4	BF114679	transmembrane protein 33	0,038	0,82	0,001	1,39
NUCKS1	1	AW515443	nuclear casein kinase and cyclin-dependent kinase subs	0,040	0,81	-	-
OR5T2	11	M69039	olfactory receptor, family 5, subfamily T, member 2	0,043	0,80	-	-
B) down-regulated genes in 24 h time point						***D) down-reg.***	
MMP10	11	NM_002425	matrix metallopeptidase 10 (stromelysin 2)	0,000	-1,50	-	-
SPRR1A	1	AI923984	small proline-rich protein 1A	0,004	-1,14	-	-
ANXA10	4	AF196478	annexin A10	0,011	-1,00	-	-
ETV1	7	BE881590	ets variant gene 1	0,021	-0,91	-	-
TGIF1	18	AL832409	TGFB-induced factor homeobox 1	0,030	-0,85	0,016	-1,08
BDNFOS	11	BF674612	brain-derived neurotrophic factor opposite strand	0,041	-0,81	0,001	-1,47
NRG1	8	NM_004495	neuregulin 1	0,043	-0,80	-	-
TBX1	22	AF012130	T-box 1	0,046	-0,79	-	-

III 6 h TGF-Beta 1 Treatment

A total of 239 genes were statistically significantly ($P<0.05$) up-regulated and 154 genes down-regulated in HSC-3 cells after 6 h of TGF-beta 1 treatment compared to untreated control cells (complete gene list available as Supplement material, S1), The most up-regulated gene was MMP-9. MMP-10 and MMP-1 were also statistically significantly up-regulated. Furthermore, TGF-beta 1 up-regulated fibronectin 1, complement factor B, integrin α2, interleukin IL-8, PI3, integrin beta6, laminin and plasminogen activator (PLAU).

Most of the TGF-beta 1 down-regulated genes were related to cell proliferation and mitosis and some were related to HNSCC, for example

Neuromedin U, TGF-beta- induced homeobox 1, TGF-beta 2, paired-like homeodomain transcription factor 1 and periplakin.

IV 24 h TGF-Beta 1 Treatment

A total of 242 genes were statistically significantly (P<0.05) up-regulated and 104 genes down-regulated in HSC-3 carcinoma cells after 24 h of TGF-beta 1 treatment compared to untreated control cells (Supplement material, S2). 178 (74%) of the genes up-regulated by 6 h of TGF-beta 1 treatment were also regulated after 24 h. Of the down-regulated genes, 67 (44%) were down-regulated also after 24 h of treatment. No genes showed inverted regulation during the observation time.

MMP-9 was the most up-regulated gene by 24 h of TGF-beta 1 treatment. MMP-1 was also significantly up-regulated, whereas MMP-10 was not among the regulated genes. Furthermore, TGF-beta 1 up-regulated fibronectin 1, IL-6 signal transducer, superoxide dismutase SOD2, serine proteinase inhibitor SERINE 1, cyclin G2 and PLAU. Down-regulated genes included EMP1, paired-like homeodomain 1, TGF-beta-induced factor homeobox 1 and periplakin.

V GO Enrichment Analysis

GO enrichment analysis revealed several enriched terms after 6 h EMD treatment (Table 2). Furthermore, the cluster analysis revealed keratinocyte differentiation, inflammatory response and proteolysis to be the most up-regulated. Among the down-regulated genes, the most enriched clusters were cell growth and morphogenesis, circulatory system and cellular developmental processes. Because of the relatively few regulated genes by EMD after 24 h, the GO enrichment analysis was not performed.

Table 2: The 15 most enriched GO terms according to P-value by 6 h (A1/A2) of EMD and 6 h (B1/B2) and 24 h (C1/C2) of TGF-beta 1 treatments of HSC-3 cells. Table shows the term, p-value and number of genes (N. G.) related to various terms. *correspond to terms, which were the same for EMD and TGF-beta 1 after 6 h of treatment. " correspond to terms, which were the same for 6 h and 24 h of TGF-beta 1 treatments.

Enriched GO term	P-Value	N.G.	Enriched GO term	P-Value	N.G.
A1) EMD up-regulated terms (6 h)			***A2) EMD down-regulated terms (6 h)***		
direct protein sequencing *	0,001	12	circulatory system process	0,007	3
keratinocyte differentiation	0,002	3	blood circulation	0,007	3
inflammatory response *	0,002	5	cell growth	0,009	3
extracellular region *	0,002	8	regulation of cell size	0,009	3
Cornifin (SPRR)	0,006	2	growth	0,019	3
calcium	0,006	6	Keratin, high sulphur B2 protein	0,02	2
responce to wounding *	0,007	5	mesenchymal cell differentiation	0,023	2
protein binding/bridging	0,01	3	mesenchymal cell development	0,023	2
signal peptide	0,011	10	PMP-22/EMP/MP20/Claudin	0,023	2
signal	0,012	11	regulation of biological quality	0,023	4
calcium binding	0,012	3	tissue development	0,024	3
small proline-rich	0,014	2	keratin filament	0,027	2
calcium ion binding	0,016	6	responce to stress	0,04	4
peptidase activity	0,017	5	anatomical structure morphogenesis	0,046	4
defence responce *	0,018	5	system process	0,046	4
B1) TGF-β up-regulated terms (6 h)			***B2) TGF-β down-regulated terms (6 h)***		
response to external stimulus	<0.001	26	mitotic cell cycle	<0.001	18
direct protein sequencing *	<0.001	51	cell cycle phase	<0.001	16
organ development	<0.001	37	cell cycle process	<0.001	22
responce to wounding *	<0.001	20	mitosis	<0.001	13
immune system process	<0.001	28	M phase of mitotic cell cycle	<0.001	13
extracellular region *	<0.001	34	cell cycle	<0.001	23
defence responce *	<0.001	22	M phase	<0.001	13
inflammatory response *	<0.001	16	regulation of progression through cell c	<0.001	17
negative regulation of biological process	<0.001	32	cell division	<0.001	17
anatomical structure development	<0.001	47	cell cycle progression	<0.001	11
system development	<0.001	41	regultion of cell cycle	<0.001	14
Small chemokine, C-X-C/Interleukin 8	<0.001	5	mitosis	<0.001	8
responce to stress	<0.001	29	phosphoprotein	<0.001	55
bladder cancer	<0.001	7	protein binding	<0.001	73
negative regulation of cellular process	<0.001	30	cytoskeleton	<0.001	21
C1) TGF-β up-regulated terms (24 h)			***C2) TGF-β down-regulated terms (24 h)***		
Phosphoprotein	<0.001	87	biological regulation	<0.001	38
immune system process "	<0.001	30	regulation of biological process	<0.001	35
organ development "	<0.001	38	calcium ion homeostasis	<0.001	5
anatomical structure development "	<0.001	52	cellular calcium ion homestasis	<0.001	5
anatomical structure morphogenesis	<0.001	34	protein binding "	<0.001	49
direct protein sequencing "	<0.001	49	cellular metal ion homestasis	<0.001	5
system development "	<0.001	44	metal ion homestasis	<0.001	5
developmental process	<0.001	67	intracellular organelle	<0.001	53
myeloid cell differentiation	<0.001	9	organelle	<0.001	53
blood vessel development	<0.001	12	regulation of cellular process	<0.001	32
vasculature development	<0.001	12	cell proliferation	<0.001	11
responce to stress "	<0.001	30	cellular di-, tri-valent inorganic cation l	<0.001	5
response to external stimulus "	<0.001	22	di-, tri-valent inorganic cation homeost	<0.001	5
negative regulation of biological process "	<0.001	32	nucleus	<0.001	34
anatomical structure development "	<0.001	11	cellular cation homestasis	<0.001	5

After 6 h TGF-beta 1 treatment, GO analysis revealed several enriched terms (Table 2), which were grouped into functional clusters. The most enriched clusters among up-regulated genes were system development, chemokine activity/inflammatory response, hemopoiesis and angiogenesis. Those among down-regulated clusters were cell cycle processes, mitosis/ M-phase and cell growth. After 24 h TGF-beta 1 treatment, the most enriched clusters among up-regulated genes were developmental processes, inflammatory/defense response and angiogenesis. Down-regulated clusters were regulation of cellular processes, intracellular organelle and calcium homeostasis.

VI Comparison of the Regulated Genes by TGF-Beta 1 and EMD Treatment

TGF-beta 1 regulated markedly more genes than EMD after 6 h and 24 h of treatments. About half (55%) of the genes (n=16) up-regulated by 6 h of EMD treatment were the same as those up-regulated by the 6 h of TGF-beta 1 treatment. Five (31%) of the genes down-regulated by EMD were the same as those by TGF-beta 1 (Table 1). Of the 15 most enriched GO terms after 6 h of incubation, EMD and TGF-beta 1 both up-regulates five identical GO groups (Table 2). Of the four up- and eight down-regulated genes by 24 h of EMD treatment, two up- and two down-regulated genes were the same as with the TGF-beta 1 treatment (Table 1).

VII Western Blotting

We have previously shown that EMD can enhance the production of MMP-9 protein by HSC-3 cells analysed with ELISA immunosorbent assay and gelatin zymography [8]. In the present study, we wanted to confirm the presence of MMP-10 protein in HSC-3 culture media after 24 h of EMD treatment. Western blot confirms the result obtained with microarray. Untreated HSC-3 cells did not produce MMP-10, but the production was clearly induced by both EMD concentrations (100 and 200 µg/ml) (Figure 1).

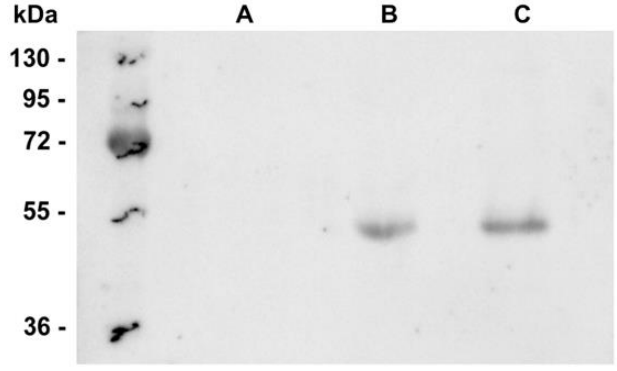

Figure 1: Western blot analysis of proMMP-10 (52 kDa) from the culture media of HSC-3 carcinoma cells after 24 h of EMD treatment. Lane A corresponds to control cells without EMD treatment. Lane B corresponds to EMD at the concentration of 100 µg/ml, and lane C to EMD at the concentration of 200 µg/ml.

Discussion

In the present study, we used microarrays to compare the effects of EMD and TGF-beta 1 on HSC-3 tongue carcinoma cell gene expression. HSC-3 carcinoma cells were highly more sensitive to TGF-beta 1 than to EMD. After 6 h of TGF-beta 1 treatment, a total of 393 genes were statistically significantly regulated, whereas 346 genes were regulated after 24 h of TGF-beta 1 incubation. However, EMD regulated only 48 genes after 6 h of treatment, and the effect was further attenuated after 24 h of incubation. In previous studies, EMD has regulated the expression of 202 genes in periodontal ligament fibroblasts and 107 genes in MG-63 osteosarcoma cells [5, 6]. Although there are some differences between the methods, the results confirm that the regulating effects of EMD on gene expression depend highly on the cell type investigated.

Several studies have suggested EMD to contain TGF-beta 1 as the principal bioactive factor, but the results are conflicting [2, 17, 18, 20, 42, 43]. Thus, the synergistic effect of various proteins, including amelogenin and unidentified mitogenic factors, is commonly proposed [18, 20]. In the present study, the number of regulated genes differed substantially according to study reagents. This indicates that EMD has only limited TGF-beta 1 activity on oral carcinoma cells. Nonetheless, EMD down-regulated the gene expression of TGF-beta 2 and TGF-beta-induced factor homeobox 1, as did also the recombinant TGF-beta 1. Curiously, in this assay, EMD and TGF-beta 1 did not regulate the expression of TGF-beta 1. However, in mammals, the TGF-beta isoforms (TGF-beta 1, TGF-beta 2 and TGF-beta 3] show 70-80% sequence identity, bind to the same cell surface receptors and elicit similar responses *in vitro*. Furthermore, it has been shown that elevated expression of either TGF-beta 1 or TGF-beta 2 results in more aggressive phenotype in human squamous cell carcinoma cells as well as in stromal fibroblasts of oral carcinoma [44, 45]. Consequently, it can be expected that TGF-beta isoforms have an overlapping regulation of their expression. In keeping with this hypothesis, it has been shown that both TGF-beta 1 as well as TGF-beta 2 regulate the expression of TGF-beta 1 gene in trabecular cells of porcine eyes [46]. To our knowledge, this is the first study to suggest that TGB-beta 1 can have effects on the expression of TGF-beta 2 in oral cancer cells. Furthermore, the result may suggest that the down-regulation of TGF-beta family members caused by EMD could be due to the slight amount presence of TGF-beta 1 in EMD.

Both TGF-beta 1 and EMD regulated the expression of MMPs. MMPs are centrally involved in malignant diseases by degrading basement membrane and extracellular matrix components [34]. In particular, MMP-2, -9 and -10 are up-regulated in oral carcinomas and correlates with the invasive potential and poor prognosis of HNSCCs [33, 35, 36, 47-49]. TGF-beta 1 has been shown to induce the expression of several MMPs, particularly MMP-9, in HNSCC cell lines, thereby favouring HNSCC invasion [30, 31, 50]. In this study, the MMP-9 was the most significantly up-regulated gene after 24 h of EMD treatment as well as after 6 and 24 h of TGF-beta 1 treatments. The results are also consistent with our previous studies, showing that the production of MMP-9 from cultured HSC-3 cells is significantly induced by EMD [8]. In addition, in this study, both EMD and TGF-beta 1 had significant effects on calcium homeostasis related GO terms. EMD up-regulated GO groups related to calcium and calcium-binding after 6 h of treatment, whereas TGF-beta 1 was observed to down-regulate calcium ion homeostasis after 24 h of incubation. Calcium has been observed to increase MMP-9 expression in normal and premalignant oral keratinocytes and to act via ERK1/2 and MAPK signaling pathways [51]. Furthermore, it has been

suggested that failures in these calcium-induced signaling pathways accompany the malignant transformation of the oral epithelium [51]. Our results are in keeping with these previous observations, and both EMD and TGF-beta 1 regulated MMP-9 and calcium homeostasis simultaneously, and the regulations of these factors are most likely related.

In addition to MMP-9, TGF-beta 1 can induce the expression of MMP-1 and MMP-10 by carcinoma cells [29, 49, 52, 53]. In line with the previous studies, MMP-1 was statistically significantly up-regulated in HSC-3 oral carcinoma cells after 6 and 24 h of TGF-beta 1 treatments. However, MMP-10, which is widely expressed in oral carcinomas but rarely in adjacent oral tissues, acted highly interestingly [54, 55]. MMP-10 was first among the most up-regulated genes after 6 h of TGF-beta 1 treatment but then absent after 24 h. Moreover, MMP-10 was the most up-regulated gene after 6 h of EMD treatment but was then surprisingly the most down-regulated gene after 24 h. MMP-10 was the only gene in this study possessing this kind of a drastic shift in regulation during 6 to 24 h of observation. Thus, the up- and down-regulation of MMP-10 by HSC-3 cells have to be exceptionally responsive to the study reagents and incubation time. A previous study has reported TGF-beta 1 to up-regulate MMP-10 production in HSC-4 but not in HSC-3 cells after a minimum of 48h incubation [33]. That study also found TGF-beta 1 to promote invasion through MMP-10 signaling [33]. In this study, untreated HSC-3 cells did not produce MMP-10 but EMD induced the production of MMP-10, which was detected in the culture media after 24 hours of incubation. Taken together, the activations of MMPs by TGF-beta 1, EMD or other MMPs seem to be interrelated and acts in complex cascades [30, 31, 56]. Also, other studies have suggested that MMPs [-2 and -9] acts co-operatively and are induced by MMP-10 [33]. Thus, further studies are needed to analyse the down-stream target genes and activation cascades of MMPs in oral carcinoma cells.

To further analyse the biological effects of the genes regulated by EMD and TGF-beta 1, a GO enrichment analysis was performed. Furthermore, the enriched terms were grouped into functional clusters according to terms having a similar biological meaning. However, these analyses have some limitations. A gene can be related to several different GO groups, and an extensive list of GO terms can be produced with only a small number of genes. Therefore, we did not conduct the GO enrichment analysis from the few genes regulated by EMD after 24 h. The GO analysis revealed that the most relevant up-regulated GO clusters by 6 h of EMD treatment were inflammatory response and proteolysis. In addition, the most important GO clusters among EMD down-regulated genes were cell growth and morphogenesis. These were almost similar for TGF-beta 1.

After 6 h of TGF-beta 1 treatment, the up-regulated genes in HSC-3 cells were related to inflammatory response and angiogenesis. However, TGF-beta 1 also had a pronounced effect on the growth and proliferation of HSC-3 cells, as the three most down-regulated clusters were all related to these terms. After 24 h of TGF-beta 1 incubation, angiogenesis and inflammatory/defense were still among the three most significantly up-regulated clusters. However, cell growth and mitosis were not anymore among the down-regulated clusters. Thus, this result suggests that TGF-beta 1 can suppress carcinoma cell growth and mitosis at first, but then its carcinogenesis promoting effects, for example induction of angiogenesis, become more expressed and may exceed former anti-cancer activity. This observation is in keeping with, and further explains, the previous results showing that TGF-beta 1 can first inhibit and at a later stage, promote carcinogenesis [25, 26].

This study was the first microarray to compare the effects of EMD and TGF-beta 1 on invasive oral carcinoma cells. TGF-beta 1 was observed to be more effective and shows a different gene expression pattern compared with EMD. However, both EMD and TGF-beta 1 statistically significantly induced the expression of MMP-9, but their effects on MMP-1 and -10 varied.

Supplementary Materials

The Supplementary Tables S1 & S2 are available online (Link 4).

Author Contributions

Conceptualization and methodology: MM, TS, TS; assays and investigation: MM, STV; data curation: MM, STV; writing-original draft preparation: MM; writing-review and editing: STV, TS, TS. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

None.

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