Low pH induces co-ordinate regulation of gene expression in oesophageal cells

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The development of gastro-oesophageal reflux disease (GORD) is known to be a causative risk factor in the evolution of adenocarcinoma of the oesophagus. The major component of this reflux is gastric acid. However, the impact of low pH on gene expression has not been extensively studied in oesophageal cells. This study utilizes a transcriptomic and bioinformatic approach to assess regulation of gene expression in response to low pH. In more detail, oesophageal adenocarcinoma cell lines were exposed to a range of pH environments. Affymetrix microarrays were used for gene-expression analysis and results were validated using cycle limitation and real-time RT-PCR analysis, as well as northern and western blotting. Comparative promoter transcription factor binding site (TFBS) analysis (MatInspector) of hierarchically clustered gene-expression data was employed to identify the elements which may co-ordinately regulate individual gene clusters. Initial experiments demonstrated maximal induction of *EGR1* gene expression at pH 6.5. Subsequent array experimentation revealed significant induction of gene expression from such functional categories as DNA damage response (*EGR1-4*, *ATF3*) and cell-cycle control (*GADD34*, *GADD45*, *p57*). Changes in expression of *EGR1*, *EGR3*, *ATF3*, *MKP-1*, *FOSB*, *CTGF* and *CYR61* were verified in separate experiments and in a variety of oesophageal cell lines. TFBS analysis of promoters identified transcription factors that may co-ordinately regulate gene-expression clusters, Cluster 1: Oct-1, AP4R; Cluster 2: NF- κ B, EGRF; Cluster 3: IKRS, AP-1F. Low pH has the ability to induce genes and pathways which can provide an environment suitable for the progression of malignancy. Further functional analysis of the genes and clusters identified in this low pH study is likely to lead to new insights into the pathogenesis and therapeutics of GORD and oesophageal cancer.

Introduction

Over the past few decades, Western populations have witnessed a 10% annual increase in the incidence of oesophageal adenocarcinoma, whereas the incidence of oesophageal squamous carcinoma remains unchanged (1–3). Gastro-oesophageal acid reflux has been identified in epidemiological studies as a major risk factor in the pathogenesis of oesophageal adenocarcinoma (4). Oesophageal adenocarcinoma commonly arises from the presence of a premalignant lesion known as Barrett's oesophagus (BO). This is a common condition in which normal squamous epithelium is replaced by a metaplastic intestine-like epithelium containing goblet cells (2,5). Although the exact model for the development of BO has not been fully elucidated, the current paradigm indicates that it most commonly occurs in the setting of gastro-oesophageal reflux disease (GORD), in conjunction with as yet unidentified genetic and environmental factors (2,6–9).

The GORD refluxate consists of many different agents, such as stomach acid, bile acids (depending on the form of GORD), pepsins and gastrins (10), as well as metabolized oxidative products from the diet. As gastric acid is the major constituent of the refluxate and is postulated to be the cause of the initial inflammatory disease or oesophagitis, we focused our investigation on its modulatory role (11–13).

Previous work from our laboratory has demonstrated the ability of acidic or low pH conditions to induce pro-inflammatory signalling pathways, such as nuclear factor- κ B (NF- κ B) transcription factor activation (14,15). This activation is common in oesophageal adenocarcinoma where it inversely correlates with response to neo-adjuvant chemotherapy (14). It was of interest to expand the focus of this work to identify other transcriptional targets of low pH to provide further avenues for treatment. The early growth response gene 1 (*EGR1*) was an ideal candidate owing to its involvement in other inflammatory conditions and activation by carcinogens.

The EGR gene family of transcription factors encode nuclear proteins with zinc finger domains resembling those of the well characterized Sp1 transcription factor, which has been shown to have increased DNA binding activity under low pH conditions (16–18). There is increasing evidence for the involvement of *EGR1* in the regulation of immune response both in immune cells and epithelial cells (19,20). *EGR1* is rapidly and transiently induced by many extracellular signals, such as growth factors, cytokines and phorbol esters, in a variety of cell types including fibroblasts, epithelial and endothelial cells (21,22). In addition, *EGR1* is induced by diverse types of DNA damaging agents and mild oxidative stress. We hypothesized that *EGR1* may be induced by low pH in the oesophageal cell lines SKGT4 and OE-33 and may lead to the identification of other related genes involved in low pH signalling. A novel bioinformatic approach for the comparative analysis of transcription factor binding sites (TFBSs) was used to analyse data

Abbreviations: *ATF3*, activating transcription factor 3; BO, Barrett's oesophagus; BEBM, bronchial epithelial basal media; DAF, decay accelerating factor; EGRF (early growth response factor); *EGR1*, early growth response gene 1; GORD, gastro-oesophageal reflux disease; IL-8, interleukin-8; NF- κ B, nuclear factor- κ B; *NR4A2*, nuclear receptor 4A2; PTGS2, prostaglandin synthase-2; TFBS, transcription factor binding site.

derived from transcriptomic experiments. This identified distinct subsets of binding elements in the promoter regions that may co-ordinately control the transcription of particular gene clusters. In particular, the NF- κ B and early growth response factor (EGRF) binding elements were identified as potentially important mediators in low pH-based gene induction.

Materials and methods

Cells

OE-33 cells were obtained from the European Collection of Cell Cultures (Salisbury, UK) and maintained in culture with RPMI 1640 medium supplemented with 10% foetal calf serum, 100 U/ml penicillin, 100 μ g/ml streptomycin and 2 mM L-glutamine (Gibco BRL, Grand Island, NY) at 37°C in an atmosphere containing 5% CO₂. The SKGT4 cell line was obtained from Schrupp *et al.* (National Cancer Institute, MD) (23) and cultured as above. HET-1A cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in bronchial epithelial basal media (BEBM) media with growth supplements (Cambrex Bioscience, Berkshire, UK). For western blot analysis, cells were seeded in 6-well plates at a density of 5×10^5 cells/ml medium and grown to 80% confluence prior to experimentation. Cell viability was confirmed using acridine orange/ethidium bromide (AO/EB) and propidium iodide (PI) fluorescence staining (Sigma, Poole, UK). For gene-expression studies, 80 cm² culture flasks were seeded with $\sim 5 \times 10^6$ cells in order to obtain sufficient RNA for northern blotting, RT-PCR and DNA microarray analyses. Acidic media was made by titrating RPMI (without serum at a temperature of 37°C) with hydrochloric acid (0.4 M) to the required pH. Before acid treatment, SKGT4 cells were allowed to rest in serum-free media for 12 h before experimentation. No additive pH buffers were used; however, pH was monitored throughout the experiment and adjusted to within the range required. Resting control cells were mock titrated with equal volumes of water where required for each time point, to control for osmotic effects. RNA samples were taken after pH monitoring from the respective time points up to 4 h and at the same time as the appropriate time point controls. Experiments involving the HET-1A cell line were performed as above using BEBM media without the use of growth supplements.

Western blot analysis

Cells were lysed in 20 mM Tris-HCl (pH 7.5), 1% SDS, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.5 mM PMSF and 10 μ g/ml leupeptin (Sigma), followed by sonication and boiling. Protein extracts were resolved by SDS-PAGE and transferred to PVDF membrane (Millipore, Bedford, MA). Blots were blocked with 5% (w/v) dried skim milk in phosphate buffered saline (PBS) for 1 h at room temperature and then incubated for 1 h at room temperature with the primary polyclonal antibody to appropriate protein at a variable dilution in PBS/powdered milk. The immunoblots were then washed and incubated with an anti-rabbit, horseradish peroxidase-conjugated secondary antibody (DAKO, Bucks, UK). Immunodetection was accomplished by enhanced chemiluminescence.

RNA isolation and northern blot analysis

Total RNA was isolated using nucleospin extraction columns (Clontech, Palo Alto, CA) including a DNase step, while maintaining all samples on ice throughout the process. RNA samples (5 μ g) were run on 1.5% formaldehyde gels and integrity checked by UV transillumination. The RNA was transferred from formaldehyde gels to nylon membranes (Amersham Biosciences, Bucks, UK) by overnight capillary transfer. The membranes were cross-linked at 80°C for 2 h in an oven. Blots were checked for linear transfer by a methylene blue staining protocol (Sigma). Radiolabelled probes for northern blot analysis were prepared by PCR amplification of an appropriate region of the gene followed by incorporation of the labelled [³²P]dCTP nucleotide by random priming (Amersham, Aylesbury, UK). Templates for the probes were produced using the following primers: EGR-1 (forward 5'-CAGCTTGGTCAGTGGCCTAG-3' and reverse 5'-AATGTCAGTGTTCGGCGTG-3' from NCBI AJ24325) and GAPDH (forward 5'-CAGAACATCATCCCTGCCTCTAC-3' and reverse 5'-TTGAAGTCAGAGGAGACCACCTG-3' from NCBI J04038), synthesized by Invitrogen (Paisley, UK). Membranes were probed in Rapid-Hyb buffer (Amersham Biosciences) at 65°C overnight followed by washing with high stringency salt buffer.

Semi-quantitative RT-PCR analysis

Total RNA was isolated as above and was transcribed to cDNA using the RETROscript RT-PCR kit (Ambion, Huntingdon, UK). RNA (1 μ g) was reverse transcribed in a total volume of 20 μ l. Of this cDNA reaction, 5 μ l was subjected to PCR amplification with SuperTaq (Ambion) using appropriate

Table 1. RT-PCR primer sequences

Gene	Forward primer	Reverse primer
<i>MKP-1</i>	5'-GGTGATTTAGCGTCA-3'	5'-TGTGAAGGCAGACACCTA-3'
<i>CTGF</i>	5'-GCTCCCTGCATCTTCGG-3'	5'-GGCCCAACGGTTTG-3'
<i>EGR3</i>	5'-ACCAACGAGAAGCCCAA-3'	5'-GGGGTCGTGGAAAGACA-3'
<i>FOSB</i>	5'-ACCGTAGCTGAAGCTGA-3'	5'-ACTCGGCAGAGGGTGA-3'
<i>ATF3</i>	5'-CAAGAACGAGAAGCAGCA-3'	5'-CTGGGGCAGAAATGGGA-3'

primers. Primers used for RT-PCR analysis were as detailed in Table 1. Assessment of RT-PCR-derived products was performed via agarose gel electrophoresis at exponential points within respective reaction cycles.

Real-time RT-PCR analysis

Extraction of RNA and reverse transcription was performed as above. An aliquot of 1 μ l of the reverse transcription reaction was used in the real-time PCR reactions (20 μ l final volume) and performed in an ABI Prism 7700 thermocycler (Applied Biosystems, Foster City, CA). Primers for the genes (*ATF3*, *EGR3*, *CYR61*, *DUSP5*, *MAN2C*, *MNT*, *C8FW*) were obtained from the ABI 'Assay on Demand' website (<http://home.appliedbiosystems.com/>). Fold inductions were calculated using the cycle threshold $\Delta\Delta$ Ct method as previously described (24,25).

DNA microarray analysis

An aliquot of 10 μ g of total RNA from treated SKGT4 cells was reverse transcribed into single-stranded cDNA using the SuperScript Choice kit (Invitrogen, Paisley, UK). For this purpose, an oligo-dT primer containing a T7 RNA polymerase promoter (Genset) was utilized. Following double-stranded cDNA synthesis, biotin-labelled cRNA was generated by *in vitro* transcription using the BioArray RNA labelling kit (Enzo Diagnostics, Farmingdale, N.Y., USA). These complex cRNA targets, which are representative of the transcriptome of a particular sample, were hybridized against U133A arrays (Affymetrix, San Jose, CA). Detection was accomplished via a streptavidin-labelled fluorochrome (phycoerythrin) and laser scanning. The raw microarray data have been submitted to Gene Expression Omnibus for public access and may be obtained using the accession code GSE2144.

Inter-array comparison of gene-expression data

Normalization of data and inter-array comparisons of gene-expression profiles was carried out using Microarray Analysis Suite (MAS) version 5.0 (Affymetrix), together with Microsoft Access. In more detail, DNA microarray experiments were analysed using an approach based on the Mann-Whitney pairwise comparison test (26). To identify differentially expressed genes between any two samples, pairwise comparisons were performed using MAS. Lists of altered transcripts from different pairwise comparisons were sorted via Microsoft Access.

Cluster analysis

Detailed cluster analysis was performed using GeneSpring 4.0. Data were normalized initially by transforming the values <0.001 – 0.001 . Each measurement was divided by the 50th percentile of all measurements in that sample. The percentile was calculated with all raw measurements >0 and using all genes not marked as absent. Each gene-expression value was divided by the median of its measurement in all samples. If the median of the raw values was <0.01 , then each measurement for that gene was divided by 0.01. Significantly altered genes were identified by viewing the data in the form of an ordered list of significance from which the top 100 were chosen for analysis. Guided analysis in the form of similarity searches were performed, followed by *k*-means clustering. These lists were then compared with more unguided methods, such as hierarchical clustering, before attempting to identify individual promoter clusters.

Bioinformatic analysis of promoter regions

Promoter regions for genes residing within clusters of interest were identified and retrieved via the use of Genome Browser housed at University of California Santa Cruz (<http://genome.ucsc.edu/>). Retrieved promoter regions (1500 bp upstream of the predicted start site) from collated clusters of genes were comparatively analysed using the TFBS analysis tool MatInspector (<http://www.genomatix.de/>) (27). The resultant list of TFBSs identified for each cluster were filtered against each other and against a cluster of randomly selected non-changing genes to give a list of the most significant TFBSs for each cluster.

Results

Induction of *EGR1* mRNA by low pH in oesophageal cell lines

EGR1 is an early growth response gene whose expression can be induced by many different stimuli including cytokines, growth factors, DNA damaging agents and oxidative stress. To examine *EGR1* expression under conditions of low pH, SKGT-4 and OE-33 cells were treated with growth media whose pH had been altered with 0.4 M hydrochloric acid. Figure 1A shows western blotting data for the induction of the 80 kDa *EGR1* protein under conditions of varying pH for 1 h in both SKGT4 and OE-33 oesophageal cell lines. The pH ranges between 7 and 6, previously demonstrated to induce NF-KB (14), can clearly be seen to induce *EGR1* protein expression. Induction of *EGR1* mRNA was observed between pH 6.8 and 6.2 in both cell lines when the pH range was focused (Figure 1B). At pH 6.5 (chosen to reflect gene induction midpoint), maximal induction of *EGR1* was seen after 1 h (Figure 1C), which gradually returned to resting levels after 3 h. This is mostly likely reflective of the increase in pH within the media over this time period. A system of pH monitoring was then used to maintain the pH within the range observed for *EGR1* gene induction (Figure 1C); in this case, the level of *EGR1* mRNA expression was still increased up to 4 h post-initial treatment.

Identification of transcriptomic responses to conditions of low pH

To further examine the effect of low pH conditions on a transcriptomic level, we employed DNA microarray-based

gene-expression profile analysis. For this purpose, SKGT-4 cells were treated with low pH 6.5, together with pH monitoring, and analysed using Affymetrix U133A arrays, with ~20 000 transcripts being examined in this respect. A range of genes were identified as being consistently differentially expressed in conditions of low pH as compared with the resting state, with the following number of transcripts being affected over time: 116 transcripts for 30 min, 480 transcripts for 120 min, 115 transcripts for 180 min and 741 transcripts for 240 min. Figure 2 details those genes whose expression was upregulated across all time periods in comparison with the resting state. Of note, *EGR1* was identified from the DNA microarray studies as being one of the significantly altered genes, providing initial validation for the methodology employed. Moreover, additional members of the early growth response gene family, *EGR3* and *EGR4*, were induced by low pH conditions. Amongst the immune response genes, an increased expression of decay accelerating factor (DAF) was observed, which has previously been reported to be upregulated in BO (28). We also noted the expression of interleukin-8 (IL-8) and prostaglandin synthase (PTGS2 or COX2), both of which are upregulated in the Barrett's adenocarcinoma sequence (29–31). The transcription factors FOSB and JUN of the AP-1 transcription complex, known to have increased DNA binding potential under conditions of low pH, are upregulated in the differentiation/proliferation group (Figure 2) (32). Many of the identified differentially expressed genes have functional roles in stress response, proliferation, differentiation, inflammation and growth arrest. Other important transcription factors identified using this approach are the Barrett's associated genes activating transcription factor 3 (*ATF3*) and nuclear receptor 4A2 (*NR4A2*) (33).

Validation of gene-expression data

A cohort of genes were chosen for downstream validation, varying from growth factors (*CTGF*, *CYR61*, *C8FW*) to signalling molecules (*MKP-1*, *DUSP5*, *MNT*, *EGR3*, *ATF3*, *FOSB*). Changes observed in the DNA microarray studies of a subset of genes were confirmed by semi-quantitative RT-PCR analysis (Figure 3A). These included *MKP-1*, which was rapidly upregulated after 30 min, and *CTGF*, which remained upregulated across the time periods. Additionally, *ATF3* and *EGR3* were validated by this method, showing rapid induction by 30 min increasing incrementally over time. Real-time RT-PCR analysis permitted examination of a much broader range of gene-expression changes (Figure 3B and C). GAPDH was used to normalize all genes in the real-time RT-PCR analysis including *MAN2C*, which was chosen from the array dataset as a non-changing gene to determine baseline fluctuations in the experimental approach. *EGR3* and *ATF3* have similar induction profiles with maximal induction at 2 h, which may be indicative of similar transcriptional control mechanisms (Figure 3B). The *FOSB* gene was observed to be strikingly altered, with an ~200-fold increase in expression level observed at 120 min post-treatment as compared with that seen in the resting state (Figure 3B). Induction of *EGR3* and *ATF3* was also demonstrated on a protein level (Figure 3C). The importance of these genes in low pH signalling is highlighted by their responses in other oesophageal cell lines, such as OE-33 and the pre-neoplastic oesophageal cell line HET-1A (Figure 4). The oesophageal adenocarcinoma cell line OE-33 and the oesophageal squamous epithelial cell line HET-1A were treated with low pH 6.5

Fig. 1. Induction of *EGR1* protein and mRNA by low pH in SKGT4 and OE-33 oesophageal cells. Levels of *EGR1* mRNA and protein were determined by northern and western blot analysis, respectively. (A) Western blots from both oesophageal cell lines (SKGT4 and OE33) showing altered *EGR1* protein expression in response to varying pH conditions over 1 h. NS: Non-specific reference band. (B) *EGR1* mRNA expression over a focused pH range in both cell lines (SKGT4 and OE33) as determined by northern blotting. (C) Time-dependent induction of *EGR1* mRNA in SKGT4 cells stimulated with conditions of low pH with and without pH monitoring/adjustment (to maintain pH 6.5). Details the minutes following low pH treatment, with expression of *EGR1* mRNA appearing transiently without pH maintenance and continuously with pH maintenance. PMA and TNF α were used as positive controls for *EGR1* induction. R signifies resting pH of 7.4.

Unigene	Genbank	Affy ID	Gene Description	Time mins				Genespring										
				Mann Whitney (Log fold)				0 30 120 180 240										
				30	120	180	240	0	30	120	180	240						
Stress Response																		
Hs.283009	NM_020127	205807_s_at	Tuftelin 1 (TUFT1)	0.675	1.325	0.975	1.375											
Hs.204354	NM_004040	212099_at	Ras homolog gene family, member B (RHOB)	0.6	1.225	1.05	1.275											
Hs.51305	NM_012323	205193_at	v-maf fibrosarcoma oncogene homolog F (MAFF)	1.6	2.15	1.875	0.575											
		36711_at	..	1.6	1.575	1.875	2.175											
Hs.76556	NM_014330	202014_at	Apoptosis associated protein (GADD34)	0.925	0.475	0.975	1.075											
	NM_014330	37028_at	..	1.1	0.525	1.225	1.425											
Hs.171695	NM_004417	201041_s_at	Dual specificity phosphatase 1 (DUSP1)	3.15	1.15	2.25	2.475											
Hs.326035	NM_001964	201693_s_at	Early growth response 1 (EGR1)	1.7	-0.9	1.25	1.175											
Hs.74088	NM_004430	206115_at	Early growth response 3 (EGR3)	3.625	2.05	3.8	4.075											
Hs.110571	NM_015675	207574_s_at	Growth arrest and DNA-damage-inducible, beta (GADD45B)	1.4	1.5	1.65	1.8											
		209304_x_at	..	1.05	1.225	1.4	1.725											
		209305_s_at	..	0.925	0.775	1.2	1.325											
Hs.80409	NM_001924	203725_at	Growth arrest and DNA-damage-inducible, alpha (GADD45A)	0.65	1.3	1.15	1.425											
Hs.2128	NM_004419	209457_at	Dual specificity phosphatase 5 (DUSP5)	2.675	1.325	2.35	2.475											
Hs.3052	NM_001965	207767_s_at	Early growth response 4 (EGR4)	3	2.85	3.375	4.05											
Immune Response																		
Hs.179657	NM_002659	210845_s_at	Urokinase-type plasminogen activator receptor (PLAUR)	0.75	1.45	0.95	1.4											
		211924_s_at	..	0.675	1.375	0.8	1.225											
Hs.1369	NM_000574	201925_s_at	Decay accelerating factor for complement (DAF)	1.075	2.05	1.4	1.575											
		201926_s_at	..	0.825	1.6	1.125	1.275											
Hs.334924	NM_002309	205266_at	Leukemia inhibitory factor (LIF)	1.275	1.425	1.7	1.775											
Hs.75498	NM_004591	205476_at	Small inducible cytokine subfamily A, member 20 (SCYA20)	0.925	1.075	1.075	1.075											
Hs.132004	NM_013246	219500_at	Cardiotrophin-like cytokine (CLC)	1.2	1.425	1.625	1.95											
Hs.624	NM_000584	202859_x_at	Interleukin 8 C-terminal variant (IL8)	1.475	1.3	1.525	1.65											
Hs.82085	NM_000602	202628_s_at	Serine proteinase inhibitor, clade E member 1 (SERPINE1)	0.45	1.575	1.35	2											
		202627_s_at	..	1.2	1.625													
Hs.196384	NM_000963	204748_at	Prostaglandin-endoperoxide synthase 2 (PTGS2)	1.525	0.675	1.5	2.05											
Diff/Prolif																		
Hs.25497	NM_020310	204206_at	MAX binding protein (MNT)	0.675	1.275	1.125	1.275											
Hs.78465	NM_002228	201466_s_at	v-jun avian sarcoma virus 17 oncogene homolog (JUN)	1.675	1.275	1.975	2.15											
		201464_x_at	..	1.2	0.725	1.125	1.5											
Hs.8867	NM_001554	210764_s_at	cysteine-rich, angiogenic inducer, 61 (CYR61)	2.325	2.55	2.6	3.075											
		201289_at	..	2.175	2.5	2.6	2.775											
Hs.270833	NM_001657	205239_at	Amphiregulin (AREG)	1	1.8	1.3	1.6											
Hs.7837	NM_025195	202241_at	Phosphoprotein regulated by mitogenic pathways (C8FW)	1.675	1.275	1.575	1.775											
Hs.75511	NM_001901	209101_at	Connective tissue growth factor (CTGF)	2.075	2.4	2.45	2.45											
Hs.75678	NM_006732	202768_at	FBJ murine osteosarcoma viral oncogene homolog B (FOSB)	5.4	4.675	5.475	5.325											
Hs.283565	NM_005438	204420_at	FOS-like antigen-1 (FOSL1)	1.9	2.975	2.8	2.95											
Hs.75212	NM_002539	200790_at	Ornithine decarboxylase 1 (ODC1)	0.975	1.35	1.4	2.075											
Hs.92186	NM_016201	203002_at	Leman coiled-coil protein (LCCP)	1.05	1.25	1.25	1.525											
Signalling																		
Hs.460	NM_001674	202672_s_at	Activating transcription factor 3 (ATF3)	3.325	1.6	3.325	3.65											
Hs.82101	NM_007350	217997_at	pleckstrin homology-like domain, family A, mem 1 (PHLDA1)	0.8	1.175	1.1	1.275											
		217996_at	..	0.825	1	1.05	1.15											
Hs.77293	NM_014755	202657_s_at	SERTA domain containing 2 (SERTAD2)	0.9	1.45	1.575	2.05											
		202656_s_at	..	0.925	1.225	1.5												
Hs.171596	NM_004431	203499_at	Homo sapiens EphA2 (EPHA2)	0.675	0.525	0.725	0.625											
Hs.1119	NM_002135	202340_x_at	Nuclear receptor subfamily 4, group A, member 1 (NR4A1)	2.375	0.925	2.175	2.775											
Hs.82120	NM_006186	216248_s_at	Nuclear receptor subfamily 4, group A, member 2 (NR4A2)	2.075	0.925	2.175	2.575											
		204621_s_at	..	2.05	2.225	2.5												
		204622_x_at	..	1.95	0.8	2	2.45											
Other Functions																		
Hs.54451	NM_018891	207517_at	Laminin, gamma 2 (LAMC2)	1.4	1.6	1.5	1.85											
		202267_at	..	0.6	0.525													
Hs.5372	NM_001305	201428_at	Claudin 4 (CLDN4)	1.3	2.45	1.95	1.975											
Hs.12813	NM_015508	212665_at	DKFZP434J214 protein	0.575	0.775	1.45	1.625											
Hs.96427		213056_at	FERM domain containing 4B, KIAA1013 (FRMD4B)	1.2	1.325	1.425	1.45											
Hs.78452	NM_005415	201920_at	Solute carrier family 20, member 1 (SLC20A1)	0.5	0.65	0.8	0.725											
Hs.153405	NM_022817	205251_at	Period homolog 2 (PER2)	0.95	0.675	1.175	1.05											
		208518_s_at	..	0.8	0.675	1	1.05											
Hs.213289	NM_000527	202068_s_at	Low density lipoprotein receptor (LDLR)	0.75	1.15	0.875	1											
		202067_s_at	..	0.69	1.05	0.8	0.875											

Fig. 2. Consistently upregulated genes induced by pH 6.5 in the transcriptome of SKGT4 oesophageal cells. Experiment as defined by the Mann–Whitney pairwise comparison test. Comparisons were made between resting state and various time points after treatment, with fold difference values (on a log scale) indicated to the right. Genes are grouped by functional category of the corresponding protein as defined by LocusLink/Entrez Gene and literature search, along with other standard identifiers, namely the relevant UniGene and GenBank accession codes, as well as the Affymetrix transcript code. Dots (..) represent independent probe set for the same transcript. Corresponding GeneSpring block view of each differentially expressed gene across the various time periods post-treatment is shown to the right. Blue represents low-expression levels, which move through a range of colours to red for high level of expression.

as before and gene expression was assessed using real-time RT–PCR analysis (Figure 4). Overall, the data confirm that the gene inductions observed are not cell line specific and are maintained to different degrees in cell lines of different malignant stages.

Cluster analysis of DNA microarray data

Similar expression profiles for *EGR3* and *ATF3* were observed using the GeneSpring software and the subsequent transcript and protein validation (Figure 3), indicating that these genes are under similar transcriptional control. In order to assess this

Fig. 3. Validation of DNA microarray data from the low pH-induced transcriptome of SKGT4 oesophageal cells. Semi-quantitative (A) and real-time (B) RT-PCR analysis was used to examine the expression of indicated genes. In the context of real-time PCR analysis, fold changes for gene expression were calculated using the $\Delta\Delta C_t$ method, with GAPDH being used as the internal control gene and resting cells as the calibrator. (C) Western blot analysis of ATF3, EGR3 and Actin (loading control) expression in SKGT4 cells under various pH conditions. R signifies resting pH of 7.4.

possibility using promoter inspection software, it was necessary to identify distinct groups of profiles for comparison rather than one single profile. These profiles were obtained by clustering the top 100 most significantly altered genes as described by GeneSpring. Figure 5A details the unbiased hierarchical tree cluster of this ordered list, with three groups of genes identified and marked for *in silico* promoter analysis. We selected the three clusters for further promoter inspection based on the presence of verified and/or Barrett's related genes within the clusters.

Common regulatory elements within the promoter regions of individual clusters

A flow chart for the comparative promoter analysis is shown in Figure 5, beginning with the hierarchical tree and selected clusters in Figure 5A. Figure 5B details the expression profile of each of the clusters chosen for promoter analysis. Cluster 1 contains genes displaying a rapid increase in expression in response to low pH, such as *EGR3* and *ATF3*. Cluster 2 contains *CYR61* and *LDLR* genes that reach maximal

Fig. 4. Comparison of gene induction in the oesophageal adenocarcinoma cell lines SKGT4, OE-33 and squamous epithelial cell line HET-1A stimulated with low pH 6.5. Induction of mRNA was defined by real time RT-PCR in the oesophageal cell lines SKGT4 (A), OE-33 (B) and HET-1A (C). Fold changes for *ATF3*, *EGR3*, *FOSB*, *DUSP5*, *CYR61* and *MAN2C* were calculated using the $\Delta\Delta C_t$ method, with GAPDH being used as the internal control gene.

expression after 2 h, whereas Cluster 3 includes genes of the metallothionein family which have a latent maximal expression at 4 h. Promoter regions of the genes residing in the three clusters were examined for putative transcription factor binding sites which may co-ordinately regulate gene transcription. A fourth cluster of randomly selected non-changing genes was also chosen as an internal control for

Fig. 5. Co-regulatory analysis of the low pH-induced transcriptome of SKGT4 oesophageal cells using the promoters of gene sets defined by hierarchical clustering. (A) Hierarchical clustering map of the genes in the ordered list. Gene-expression profiles have separated into distinct clusters. Blue represents low-expression levels, which move through a range of colours to red for high level of expression. Three gene-expression clusters have been marked for further bioinformatic analysis. (B) Expression profiles of each of the three clusters demonstrating the similarities of the genes within the individual clusters in comparison to a random selection of non-changing profiles. (C) Gene nomenclature of those genes chosen for promoter analysis from each cluster inclusive of the control, non-changing gene set. (D) Statistically significant TFBSs found within the majority of gene promoters within each individual cluster after comparison with the TFBS sets of the other clusters and the control non-changing gene set.

randomly occurring TFBSs. We identified the increased or significant occurrence of the binding sites within each cluster in comparison with each other and with the control set described. Figure 5C lists the genes residing within each of the cluster described and the most significant set of transcription factor binding elements found in their promoter regions is detailed in Figure 5D. Interestingly, Cluster 1 (EGR genes) may be controlled by the stress response factor Oct-1 and Cluster 2 by factors such as NF-KB and EGRF. Both NF-KB and AP-1 have been shown to have increased DNA binding potential under conditions of low pH (14,32,34). Furthermore, genes upregulated early in response to low pH, such as those of the EGR family (Cluster 1), may be involved in the co-ordinate upregulation of the genes contained in Cluster 2 through the EGRF binding site identified. The co-ordinate relationship between the genes can be verified in correlation searches on the *ATF3* gene using Oncomine 2.0 (35). Using the array data from Welsh *et al.* (36) prostate cancer study (oesophageal correlation not available), a correlation search was performed for the Barrett's related gene *ATF3* (Figure 6) (33). A number of the co-regulated genes identified in this study (*EGR3*, *EGR1*, *FOSB*, *NR4A1*) correlated highly with *ATF3* expression, thus indicating that the clusters identified by this study may also occur *in vivo*.

Discussion

This study demonstrates that low pH induces a co-ordinate set of transcriptional responses in oesophageal cells, resulting in expression of genes involved in inflammation, stress response, proliferation and differentiation. This series of responses has been mapped to identify the impact of sequential transcriptional events. These findings identify genes induced by low pH which may be of potential importance in the pathogenesis of reflux oesophagitis and indirectly for the Barrett's cancer sequence.

The *in silico* transcription factor analysis applied in this study represents the first application of comparative binding site analysis to clustered data from a low pH gene induction study. Studies performed on cell lines with only one variable over time, i.e. low pH, are ideal candidates for TFBS analysis. This method identified TFBS sets found in the promoter regions which may co-ordinately regulate the genes within the given clusters. The incorporation of an expression cluster consisting of randomly selected non-changing genes is a method whereby randomly occurring non-functional TFBSs may be controlled and eliminated from the analysis. The discovery of increased presence of both NF-KB and EGRF binding elements in the promoter regions of Cluster 2 is of major

Fig. 6. Correlation searching in Oncomine cancer tissue database using *ATF3* demonstrates *in vivo* relationships of genes identified in this study. Heat map of a correlation search for the gene *ATF3* using the 'Welsh prostate array data' and the Oncomine 2.0 database. Detailing the similarity of gene-expression profiles to *ATF3* in numerous sets of microarray experiments performed on cancer tissue specimens.

interest for a number of reasons. The progress from GORD through to BO and adenocarcinoma passes through a gross inflammatory stage known as oesophagitis, and as with many other forms of inflammatory conditions can be characterized by the activation of the transcription factor NF-KB resulting in the expression of inflammation-related genes, such as *IL-8* (13,37). This master regulatory factor is also known to be upregulated in BO and overexpressed in 60% of oesophageal adenocarcinomas, where it inversely correlates with response to neo-adjuvant chemotherapy (14). Chromosome 4 on which NF-KB resides has also been shown to be amplified during oesophageal cancer progression (38–40). The above findings and previous reporting of NF-KB activation by low pH and bile acids lends weight to the importance of this transcription factor and its responsive genes in the progression of disease and as a potential therapeutic target. The promoters of Cluster 2 also contain the EGRF element which allows EGR1–4 to bind and activate transcription. This family of transcription factors are important inflammatory mediators and are often classed as oncogenes owing to their deregulation in many tumour types. EGR1, which is the most characterized member of the family can promote angiogenesis within tumour cores (41,42). The low pH responsive genes *EGR1* and *EGR3* identified in this study are members of the first expression cluster and this marks the beginning of what is termed a regulatory inference network (43,44). Transcriptional ordering or regulatory network inference is an area of bioinformatics under development as our understanding of gene networks and

promoter function grows. We believe the methods employed in this study can add to the tools available to analyse the vast amounts of data generated from transcriptomic experiments and assist in identifying sequential biological events. Further validation and dissection of this regulatory network using such techniques as chromatin immunoprecipitation will improve the current understanding of the relationship between promoter TFBSs and the genes they regulate.

In order to assess the effects of low pH on gene expression, we exposed oesophageal cells to a sustained change in pH over the period of the experiment rather than recapitulating the real-life scenario of intermittent brief exposures over a lifetime. We have previously shown in oesophageal and gastric cells that pH 6.5 can activate NF-KB, a key mediator of inflammation and disease progression (14,15), and the transcription factor EGR1 identified in this study. There have been other reports of AP-1 DNA binding activity at the pH ranges used in this study (32). Therefore, it was of interest to determine the genes downstream of these transcription factors induced by low pH conditions. Sustained exposure to pH levels <6 resulted in loss of induction of NF-KB and it would not have been possible to identify downstream effects of this transcription factor at lower pH levels (15). In addition, preliminary experiments (data not shown) demonstrated that continuous culture in pH 3.5 led to a significant loss in cell viability as determined by PI staining and MTT assay and, as such, was not suitable for detailed molecular analysis of low pH signalling.

The identification of *EGR1* as a low pH-responsive gene in oesophageal cells allowed for its use as a positive control gene in the subsequent set of transcriptomic experiments performed. The initial broad pH range used demonstrated *EGR1* protein induction from pH 7 to 6; however, when these experiments were performed on a transcriptional level with a more focused pH range, it was discovered that *EGR1* transcript induction occurred between pH 6.8 and 6.2. Therefore, our choice of pH 6.5 detailed previously was within the induction pH range for both *EGR1* protein and mRNA. The analysis of the data generated from array experimentation was critically assessed using two methods, the Mann–Whitney protocol developed by Jin *et al.* (26), followed by complementary analysis using GeneSpring, thus revealing genes whose expression had been altered by low pH conditions.

Subsequent to the array experimentation, the low pH-responsive genes identified were allotted into groups based on similarity in their function as described by literature searches. The data analysis identified a small subset of genes whose expression was downregulated but only by small fold changes that were deemed not statistically significant, hence their omission from Figure 2. The full dataset is available for assessment in the GEO repository (GSE2144). Interestingly, in this study the expression of two important phosphatases, *DUSP1* and 5, are upregulated. These genes are detailed in the signalling group (Figure 2) and are capable of dephosphorylating members of the SAPK, p38, JNK and MAPK family thus inactivating the pathways. Their induction by low pH at an early time point is indicative of genotoxic stress, environmental stress and MAP kinase pathway activation as this pathway attenuates its own induction in a pulsatile fashion (45,46). Although *DUSP1* is found in the cytoplasm where it may inhibit activation of the JNK pathway and thereby inhibit apoptosis, *DUSP5* resides in the nucleus and attenuates ERK activation (47,48). The induction and validation of the phosphatases in this study in conjunction with other members of the dual specificity phosphatases found in other low pH signalling models (49) lend weight to the importance of this family of proteins in low pH signalling. The stimulation of *IL-8*, urokinase-type plasminogen receptor and prostaglandin synthase 2 (*PTGS2* or *COX2*) by low pH allows the activation of the immune system which is often deregulated in many epithelial tumours and BO (13,37). The activation of these genes is a hallmark of NF- κ B activity which in turn controls the activation of the inflammatory response to stressful injury (13,40).

DAF, a complement regulatory protein, is another gene in the immune response category (Figure 2) induced by low pH conditions in this study that has previously been used as a biomarker for specialized intestinal metaplasia type BO (28). Other documented transcriptional responses to low pH confirmed in this study to have potential differential expression in BO include *CYR61*, which is a recently discovered angiogenesis factor, and Amphiregulin, which is an EGF receptor ligand from the differentiation and proliferation category (Figure 2) (33). *ATF3* and *NR4A2* orphan receptor, from the signalling group are also associated with BO (33). The expression of *ATF3*, a member of the ATF/CREB subfamily of basic-region leucine zipper (bZIP) proteins, can be induced by a number of stimuli, such as TGF- β , TNF- α , DNA damage and other stressful conditions (50). To date, this gene has been seen to perform many roles in the form of a transcriptional repressor, for example, the repression of the Id inhibitors of

differentiation through such mediators as Smad3 (51). Induction of this gene by low pH conditions in all three oesophageal cell lines used is indicative of oxidative damage to DNA, which can occur over time in acidic environments (50,52). The induction of some genes in both normal and adenocarcinoma cell types indicates that they may not be specific to the metaplasia→adenocarcinoma sequence, but it does not preclude their involvement in oesophageal inflammatory pathogenesis or carcinogenesis.

In conclusion, we have demonstrated that low pH conditions can alter the expression of genes with diverse functions which may have potential association with BO. Novel bioinformatic techniques identified regulators of low pH gene induction, such as NF- κ B and EGRF and the clusters of genes that they potentially regulate. Therapeutic intervention targeting these transcription factors and/or their regulatory mechanisms may be used to complement existing therapies to halt the development and progression of BO.

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