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Expression profile of genes regulated by activity of the Na-H exchanger NHE1

Luanna K Putney[1,2] and Diane L Barber*[1]

Address: [1]Department of Stomatology, University of California San Francisco, San Francisco, CA 94143, USA and [2]Office of Research Technology Transfer Center, University of California, Davis, Davis, CA 95616, USA

Email: Luanna K Putney - lkputney@yahoo.com; Diane L Barber* - barber@itsa.ucsf.edu

* Corresponding author

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Abstract

Background: In mammalian cells changes in intracellular pH (pH_i), which are predominantly controlled by activity of plasma membrane ion exchangers, regulate a diverse range of normal and pathological cellular processes. How changes in pH_i affect distinct cellular processes has primarily been determined by evaluating protein activities and we know little about how pH_i regulates gene expression.

Results: A global profile of genes regulated in mammalian fibroblasts by decreased pH_i induced by impaired activity of the plasma membrane Na-H exchanger NHE1 was characterized by using cDNA microarrays. Analysis of selected genes by quantitative RT-PCR, TaqMan, and immunoblot analyses confirmed results obtained from cDNA arrays. Consistent with established roles of pH_i and NHE1 activity in cell proliferation and oncogenic transformation, grouping regulated genes into functional categories and biological pathways indicated a predominant number of genes with altered expression were associated with growth factor signaling, oncogenesis, and cell cycle progression.

Conclusion: A comprehensive analysis of genes selectively regulated by pH_i provides insight on candidate targets that might mediate established effects of pH_i on a number of normal and pathological cell functions.

Background

Intracellular pH (pHi) homeostasis is exquisitely controlled. Variations in pH_i both reflect and determine changes in a number of cellular processes, including adhesion, proliferation, metabolism, and programmed cell death. How pH_i responds to and regulates distinct cellular processes has primarily been determined by evaluating protein activities. Although effects of pH_i on gene expression have been determined in yeast [1] and bacteria [2], we know little about how pH_i regulates gene expression in metazoan cells.

In metazoan cells pH_i homeostasis is maintained by a number of H^+ translocating mechanisms, primarily localized at the plasma membrane. In mammalian fibroblasts, a predominant regulator of pH_i is the Na-H exchanger, NHE1. NHE1 is an H^+ extruder, catalyzing an electroneutral exchange of extracellular Na^+ for intracellular H^+ and regulating pH_i and cell volume homeostasis. NHE1 activity is increased in response to growth factors and oncogenes [3,4], and increases in NHE1 activity and pH_i promote cell cycle progression [5], increased proliferation [6,7], and cell survival [8]. NHE1 activity is necessary for a number of cytoskeleton-associated processes including cell shape determination [6], remodeling of cell-substrate

adhesion complexes [6,9,10], and directed cell migration [9,11,12]. NHE1-dependent increases in pH_i also play an essential role in cell transformation and the development of malignant progression [13,14] and NHE1-deficient cells have a markedly reduced capacity for tumor growth in vivo [15].

In this study we used cDNA microarray analysis to determine changes in steady-state gene expression in fibroblasts stably a mutant NHE1 lacking ion translocation activity compared with fibroblasts stably expressing wild-type NHE1. Consistent with a role for NHE1 in cell growth regulation, the unbiased microarray analysis indicated that in the absence of NHE1 activity there are significant changes in the expression pattern of genes related to growth factor signaling, growth and oncogenesis, and DNA synthesis and cell cycle control.

Results and Discussion

Global gene profiling

Recent evidence indicates that in addition to the function of NHE1 in ion translocation and pH_i homeostasis, the exchanger also acts as a scaffold to assemble signaling complexes and as a plasma membrane anchor for the actin-based cytoskeleton [3,6]. To selectively impair only ion translocation by NHE1, we engineered an ion translocation-defective NHE1 containing an isoleucine substitution for glutamine 266 (NHE1-E266I). In cells expressing NHE1-E266I, the scaffolding and actin anchoring functions of NHE1 are retained, but ion translocation is absent [5,6]. Wild-type NHE1 (LAPN cells) and NHE1-E266I (LAPE cells) were stably expressed in NHE1-null LAP1 cells, which are derived from NHE1-expressing Ltk-mouse muscle fibroblasts [16,17]. As previously reported [5] NHE1 expression in LAPN and LAPE cells, as determined by immunoblotting, is similar and steady-state pH_i in the continuous presence of serum and HCO_3^- is ~7.35 for LAPN cells and ~7.10 for LAPE cells. The presence of HCO_3^- allowed the function of anion exchangers contributing to pH_i homeostasis in the absence of ion translocation by NHE1.

For DNA microarray analysis, significant regulation of genes in LAPE cells compared with LAPN cells was defined as a fold change > 1.5 with a p value of < 0.05 from five independent cell preparations and microarray hybridizations. Of the 6,500 probe sets, 198 or 3.05% were significantly different in LAPE cells. Two widely used approaches to analyze DNA microarray data include hierarchial clustering of genes with similar expression patterns [18] and grouping of biologically related genes into processes or pathways [19,20]. We used the latter strategy to group genes regulated by NHE1 activity into related biological pathways or processes. Genes were grouped according to key-words representing functional categories and GenMAPP, developed by the Conklin laboratory at the University of California, San Francisco [20,21], was used to visualize gene expression data on maps representing biological pathways. The advantage of a pathway-based analysis is that it provides a global perspective of functionally-related genes. Pathway-based grouping indicated a substantial number of differentially expressed genes associated with growth factor/hormone signaling and growth and oncogenesis (Fig. 1). The caveat of pathway-based analyses is that based on key-word representation, some genes are implicated in multiple biological processes. Hence, we listed all genes exhibiting significant changes in LAPE cells compared with LAPN cells (Table 1). Data in Table 1 are grouped according to biological function with absolute changes indicated.

Growth factor and oncogenic signaling

The expression of a substantial number of genes encoding proteins related to growth factor/hormone signaling and growth and oncogenesis was altered in LAPE cells compared with LAPN cells. A schematic cascade of growth factor signaling indicates that a number of genes regulated in LAPE cells function in Ras-dependent signaling (Fig 2A). Activation of many of these signaling proteins, including serotonin (5HT) [22] and thrombin (PAR2) [23] receptors, Ras [13], Raf [24], PLCγ1 [25], and Erk [26,27] stimulates NHE1 activity. Increased NHE1 activity and a resulting intracellular alkalinization are thought to be necessary for oncogenic transformation [14] and tumor development [15]. Immunoblot analysis confirmed increased protein expression of Fyn, PLCγ1, and ERK2 in LAPE cells compared with LAPN cells (Fig. 2B), In LAPE cells, the global increased expression of a number of genes involved in growth factor and oncogenic signaling suggests a feedback response to acidic or osmotic stress. Alternatively, because the proliferative response is suppressed in LAPE cells [5] (Fig. 3), increased growth factor signaling could result from a feedback mechanism to maintain cell proliferation.

Expression of a number of transcription factor genes acting downstream of growth factor signaling was also differentially regulated in LAPE cells compared with LAPN cells (Fig. 2A). Fra1 and Fra2 are Fos proteins and components of the AP1 transcription factor. They form dimmers with Jun transcription factors to regulate a number of cell processes including differentiation, proliferation, and oncogenic transformation [28]. The regulated expression of two members of the C/EBP (CAAT/enhancer binding protein) family of transcription factors was confirmed. RT-PCR was used to confirm decreased expression of C/EBPδ (Fig. 2C), which dimerizes with C/EBPβ in response to Ras-ERK signaling to regulate adipocyte [29,30] and epidermal [31] differentiation. Increased expression of GADD153 (CHOP), which is a transcription factor in the

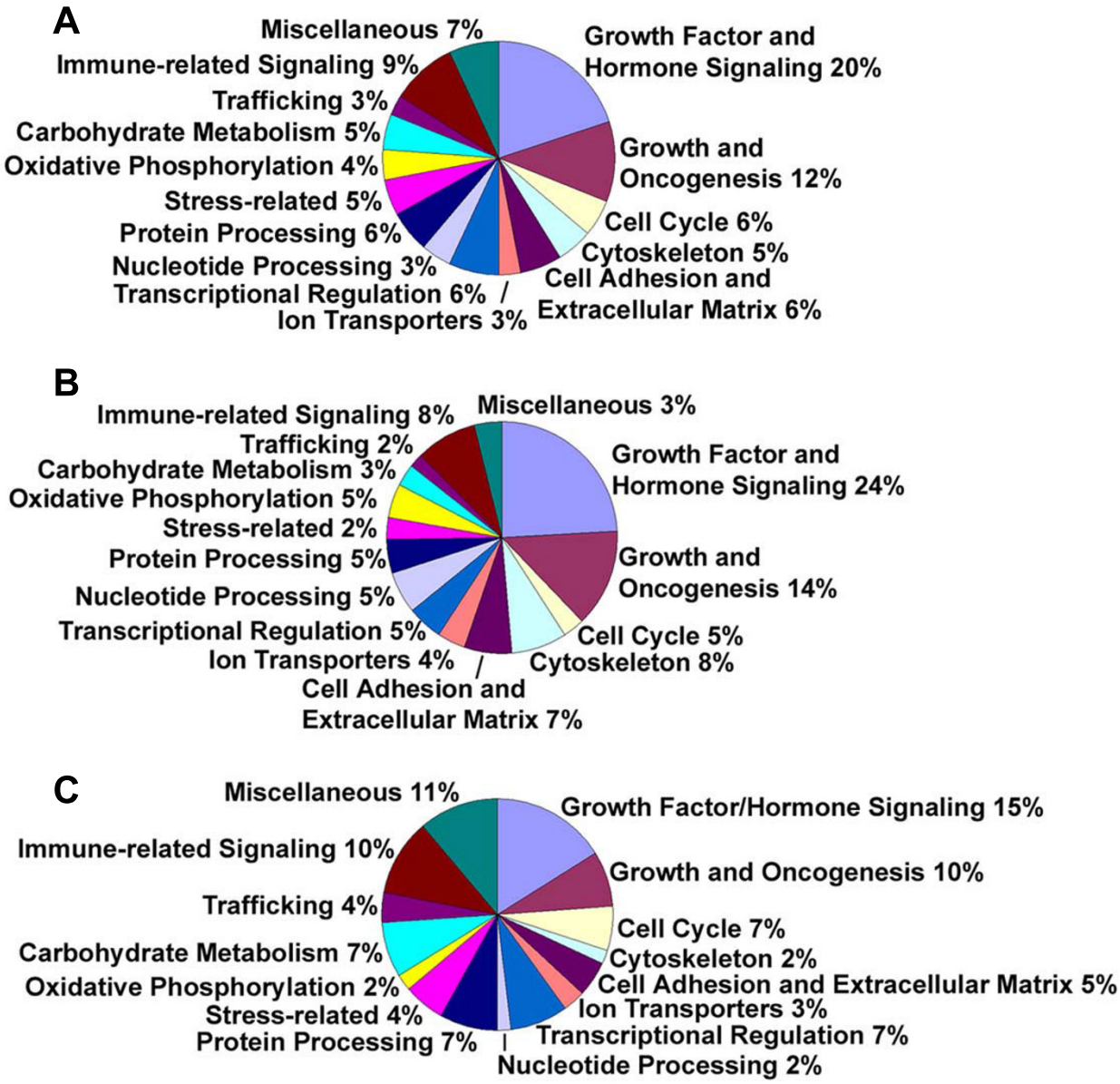

Figure 1
Relative functional clustering of genes differentially regulated in LAPE cells. Percentage of genes in the indicated functional categories that were regulated (A), had increased expression (B), and decreased expression in LAPE cells compared with LAPN cells ($p < 0.05$, $n = 5$).

C/EBP family, was confirmed by TaqMan analysis (Fig. 2D). GADD153 dimerizes with other C/EBP isomers to inhibit their binding to C/EBP binding sites in the promoters of a number of genes involved in differentiation and mitogenesis [32,33]. Expression of GADD153 increases in response to DNA damage [34], and oxidative stress [35,36], hence its role in cell cycle progression is also included in Figure 3.

Table 1: Differential Gene Expression in LAPE Cells Relative to LAPN Cells

Growth Factor/Hormone Signaling	**Accession**	**fold-change**
phospholipase C gamma 1	W65065	4.1
uPAR	X62701	3.5
MDK1 (neuronal tyrosine kinase receptor)	X79082	3.2
FGF-6	M92416	3.1
TC21 ras-like protein	W91283	3.1
tyrosine kinase SEK receptor precursor	W53668	3.0
GC Binding Protein	Z36270	3.0
FGFR-4	X59927	2.9
casein kinase II alpha	AA153726	2.8
FYN tyrosine protein kinase	W35964	2.6
ERK2	W51403	2.5
PIP5KII	P48426	2.5
P2X purinoceptor 3 (ATP receptor)	AA050453	2.3
IRG47 GTP binding-protein	M63630	2.3
p120GAP	P09851	2.2
PDGF/VEGF member	X99572	2.1
FGF-4	X14849	2.0
CAP adenylyl cyclase-associated protein	L12367	2.0
N-ras	X13664	1.9
protein-tyrosine phosphatase epsilon precursor	U35368	1.7
guanine nucleotide binding protein G(K) alpha	W64628	1.6
brain-derived neurotrophic factor	X55573	1.6
Grg1 groucho-related gene 1 protein	U61362	1.5
SOS 2 (ras GEF)	Z11664	1.5
proteinase activated receptor 2, PAR2	Z48043	-1.8
receptor of activated protein kinase C (RACK1)	AA024231	-1.8
PAK p21-activated kinase	AA117286	-2.0
guanine nucleotide binding protein gamma-7	W64628	-2.1
ERF1 EGF-response factor 1	W33538	-2.3
Fgd1 (faciogenital dysplasis) (Cdc42 GEF)	U22325	-2.7
5-HT5B serotonin receptor	X69867	-2.7
chemokine receptor type 4	P70658	-2.7
A-raf	AA104043	-3.4
FGF-7	Z22703	-3.5
GRK5	W36620	-3.7
Emr1 receptor (EGF-7 TM family)	U66890	-4.3
gliostatin (PD-ECGF)	AA008687	-5.9
MAPKK 3	W29331	-8.2
Growth and Oncogenesis	**Accession**	**fold-change**
semaphorin E	X85994	5.0
Evi-1 proto-oncogene	X54989	3.1
interferon-inducible protein 9–27	P13164	2.5
testis-specific c-abl protein	J02995	2.5
MAF proto-oncogene	W77346	2.3
MUC18 melanoma-associated antigen	AA088962	2.3
B94 TNF-α-induced early response gene	L24118	2.1
calpactin I light chain	M16465	2.1
Fra 1	U34245	2.0
cell division protein FTSH homolog	AA014057	1.9
ALL-1 zinc finger protein HRX	W62585	1.9
LAF-4 lymphoid nuclear protein	U34361	1.7
calpactin I heavy chain	D10024	1.6
insulin-induced growth response protein CL-6	AA030483	1.5
mage-like protein	W51344	-1.9
TRAF4	X92346	-2.1
rearranged mutant c-myb gene	M13990	-2.1
Ing1	AF177757	-2.2
Fra 2	P15408	-2.8
ERV1	AA034842	-2.9
MAGE-11	W51344	-3.4
MCF2 Dbl proto-oncogene	W98059	-4.0
HSP 90 alpha	AA117183	-9.1
membrane glycoprotein	Z22552	-10.7
Cell Cycle/DNA Replication	**Accession**	**fold-change**
GADD153	X67083	45.7
wee1 kinase	D30743	2.3
RAD54 DNA-repair gene	X97796	1.9
14-3-3 protein tau/theta	W61758	1.7
G1/S-specific cyclin D1	P25322	1.7
cell division-associated protein BIMB.	AA165880	-1.5
proliferating-cell nuclear antigen	AA088121	-1.7
CKS-2 cyclin-dep kinases regulatory subunit 2	X54942	-1.8
GADD45	AA138777	-2.1
gas1	X65128	-2.5
SKCDC25	Q02342	-4.1
FLAP endonuclease-1; FEN-1	AA072149	-7.5
Cytoskeleton	**Accession**	**fold-change**
clip 170 (restin)	W13214	4.2
septin 2 (NEDD5 PROTEIN)	W51490	3.6
KIF4 kinesin-like protein	AA109999	3.4
neuraxin	AA048974	2.1
kinesin light chain 1	W81858	2.1
axonemal dynein heavy chain	Z83815	1.9
septin 4 (BRAIN PROTEIN H5)	AA020101	1.8
gelsolin	J04953	1.7
myosin regulatory light chain 2, smooth muscle isoform	W18383	-4.5
NF2 neurofibromatosis type 2 isoform 1	X74671	-5.7
Cell Adhesion and Extracellular matrix	**Accession**	**fold-change**
mast cell protease 5 precursor	AA032912	6.3
osteopontin	X51834	2.3
neural cell adhesion molecule (NCAM-140)	X07233	2.1
anti-von Willebrand factor antibody NMC-4 kappa chain	U90238	2.1
inter-alpha-inhibitor H2	X70392	2.0
galectin-3	P16110	1.8
extensin precursor	W75015	1.7
E-selectin ligand-1 (ESL-1)	X84037	-1.6
microfibril associated glycoprotein precursor (MAGP)	W08049	-1.8
integrin beta-5 subunit precursor	W14823	-2.0
lectin lambda	U56734	-3.3
type IV collagenase	Z27231	-26.0
Ion Transporters	**Accession**	**fold-change**
CMP-sialic acid transporter	Z71268	2.5
V-ATPase A	U13837	2.2
AKR voltage-gated potassium-channel (KCNA4)	U03723	2.1
potassium channel protein NGK2	Y07521	2.0

Table 1: Differential Gene Expression in LAPE Cells Relative to LAPN Cells *(Continued)*

glucose transporter type 4 insulin-responsive (GT2)	M23383	-4.0
synaptic vesicle amine transporter	AA166512	-7.1
V-ATPase E	W50167	-10.4
Transcriptional Regulation	**Accession**	**fold-change**
histone H3.1	X16496	6.2
retinoic acid-binding protein	X51715	3.7
HNF-3/Forkhead homolog II	Q61575	3.2
transcription regulatory protein MCP-1 (POU 1)	D13801	3.0
NfiA2-protein (nuclear factor 1)	Y07691	1.8
transcription factor C1	U53925	-1.5
GATA-6	U51335	-1.6
zinc finger protein 91	Q05481	-1.9
winged-helix gene, htlf	Y12656	-2.2
C/EBP delta	X61800	-2.5
retinoid X receptor-beta	X66224	-2.9
HLX homeo box protein	X58250	-3.8
Nucleotide Processing	**Accession**	**fold-change**
myoblast cell surface antigen	W98426	5.6
activator 1 37 KD subunit	W85565	3.6
RNA polymerase II large subunit	M12130	3.0
DNA-directed RNA polymerase III largest subunit	W54015	3.0
uridylate kinase	AA114781	1.8
U1RNA-associated 70-kDa protein	X15769	-1.6
U6 snRNA-associated protein	W34985	-3.3
Protein Processing	**Accession**	**fold-change**
eukaryotic peptide chain releasing factor GTP-binding subunit	AA105072	2.5
putative ATP-dependent RNA helicase PL10	AA125293	2.4
40S ribosomal protein S10	W13807	2.1
tryptophanyl-tRNA synthetase	AA051240	1.8
ubiquitin carboxyl-terminal hydrolase (protease 4)	W50538	1.6
ribosomal protein L32	K02060	-1.8
elongation factor TS (forms complex with EF-tu)	W70475	-1.9
ubiquitin carboxyl-terminal hydrolase (protease 8)	AA087408	-2.4
elongation factor 2 (EF-2)	P05086	-2.6
phenylalanine – tRNA synthetase	AA020069	-3.8
threonyl-tRNA synthetase	AA051240	-4.5
elongation factor TU	AA088054	-5.4
Stress-related	**Accession**	**fold-change**
glutathione peroxidase	AA038094	2.9
thioredoxin-dependent peroxide reductase 2	W85659	1.6
superoxide dismutase 3 (SOD3)	X84940	-1.5
thioredoxin-dependent peroxide reductase 1	W88176	-2.1
glutathione S-transferase, GSTT1	X98055	-3.2
24p3 lipocalin	X81627	-23.7
Carbohydrate Metabolism	**Accession**	**fold-change**
pyruvate kinase M2	AA168931	3.8
hexokinase	P24049	2.4
phosphofructose kinase-2	P70265	2.3
acetyl-Coenzyme A acetyltransferase 2	BC000408	-1.8
Ldh-2	X51905	-1.9
ERV1	AA034842	-2.9
galactokinase 2	AA145750	-2.9

Table 1: Differential Gene Expression in LAPE Cells Relative to LAPN Cells *(Continued)*

citrate transport protein	AA108822	-2.9
phosphorylase B kinase gamma catalytic subunit	AA015461	-3.7
fructose-1,6-bisphosphatase	P19112	-4.0
lactate dehydrogenase	P00338	-4.0
Electron Transport and Oxidative Phosphorylation	**Accession**	**fold-change**
ATP synthase (subunit D)	P31399	6.4
NADH-ubiquinone oxidoreductase (complex I)	BC002772	2.3
cytochrome C oxidase VIa	U08439	2.2
ATP synthase P1precursor (subunit C)	W16250	2.0
ATP synthase (subunit A)	W49135	1.8
mitochondrial inner membrane protease subunit 1	AA009014	-1.5
cytochrome P450IIIA	D26137	-3.9
Trafficking	**Accession**	**fold-change**
beta adaptin	P21851	1.9
rab10	AA119194	1.5
rab8	P22128	3.3
rab11b	L26528	-1.5
BRAIN PROTEIN I47(similar to yeast SEC 17)	W55684	-1.7
PROTEIN TRANSPORT PROTEIN SEC22	AA023107	-1.8
SYNAPTOBREVIN 2	AA072236	-4.6
Immune-related Signaling	**Accession**	**fold-change**
anti-DNA immunoglobulin heavy chain IgG	U55461	3.5
immunoglobulin rearranged kappa chain	ET62056	3.4
complement receptor type 2 precursor (CR2)	W98124	2.6
thymocyte B cell antigen precursor	AA068606	2.3
immunoglobulin alpha heavy chain	J00475	2.2
interferon gamma receptor second chain	U69599	1.8
pre-B cell enhancing factor precursor	W59723	1.8
interferon beta type 2	V00756	1.7
anti-DNA immunoglobulin light chain IgG	U55604	1.6
FK506-binding protein precursor (FKBP-13)	AA163272	-1.5
immunoglobulin light chain Fv-fragment	Y10941	-2.3
Ig 1B4.B5 heavy chain mRNA for mouse cytochrome c	ET61726	-2.3
immunoglobulin-like receptor PIRA1	U96682	-2.8
CD10 neutral endopeptidase (pre-B cell differentiation)	M81591	-3.1
immunoglobulin variable region, heavy chain	X95878	-3.3
immune-responsive gene 1 (Irg1)	L38281	-3.9
anti-DNA immunoglobulin heavy chain IgG	U55550	-4.1
immunoglobulin light chain variable region	ET61272	-7.8
immunoglobulin heavy chain variable region	ET62261	-8.2

Table 1: Differential Gene Expression in LAPE Cells Relative to LAPN Cells *(Continued)*

Miscellaneous	Accession	fold-change
parotid secretory protein	X01697	4.0
amyloid-like protein 1 precursor	Q03157	4.0
liver receptor homologous protein	M81385	1.7
oncomodulin	Z48238	-1.6
tctex-1	M25825	-1.6
beta-hydroxysteroid dehydrogenase type 2	X90647	-2.8
C57BL/6J ob/ob haptoglobin	M96827	-2.9
angiotensin-converting enzyme	J04947	-3.2
Swiss Webster demilune cell-specific salivary gland protein	W15826	-7.1
neurexophilin 1	U56651	-7.8

Cell cycle regulation

NHE1 activity has a permissive effect in promoting cell proliferation [6,7] and cDNA microarray analysis indicated a number of genes with roles in DNA synthesis and cell cycle control had altered expression in LAPE cells compared with LAPN cells. We recently reported [5] that the proliferative rate of LAPE cells is ~3 to 4-fold less than that of LAPN cells and that LAPE cells lack a pH-dependent timing of cell cycle progression that is specifically associated with delayed G2/M entry and transition. Consistent with these findings there was an upregulation of genes associated with G2/M arrest and DNA repair responses (Fig. 3A). Decreased protein expression of GADD45 and FEN1 was confirmed by immunoblotting (Fig. 3B,3C). The decrease in FEN1 in LAPE cells was most marked when cells were synchronized by a double thymidine block, and released from the block for 3 to 9 hours (Fig. 3C). GADD45, which acts in DNA repair, is generally upregulated with cell cycle arrest [37,38], and FEN1 is thought to play an essential role in DNA replication and in base excision repair [39,40]. Although decreased expression of GADD45 and FEN1 in LAPE cells appears paradoxical, recent findings indicate that a decrease in GADD45 would contribute to p53 instability [38] and FEN1 is stimulated by proliferating nuclear antigen [41], which is decreased in LAPE cell (Fig. 3A). Additional growth arrest and DNA damage-inducible proteins, including Rad54 and GADD153, were upregulated. Rad54 functions in homologous recombination repair pathways to maintain telomere length [42] and it facilities chromatin remodeling [43], which correlates with increased histone H3 in LAPE cells (Fig. 2). As described above, increased GADD153 expression, which was confirmed TaqMan analysis (Fig. 5B), is induced by growth arrest, DNA damage, and environmental stress. Despite an established role for GADD153 in inducing apoptosis [44,45], and its increased expression in LAPE cells (~45-fold), there was no indication that LAPE cells have increased necrosis or apoptosis compared with LAPN cells.

Consistent with LAPE cells having delayed G2/M entry and progression [5], the array analysis indicated upregulation of genes negatively regulating G2/M, including 14-3-3 θ and Wee1 kinase. Increased expression of Wee1 kinase, which induces inhibitory phosphorylation of Cdc2 on tyrosine 15 [46], was confirmed by TaqMan analysis (Fig. 3D). Moreover, there was a downregulation of genes associated with promoting cell cycle progression, including CDC25 (Fig 3A) and CKS-2 (Fig. 3A), and involved in chromatin assembly, including histone H3 (Fig. 2A). Although cDNA array analysis of asynchronous LAPE cells did not indicate a change in cyclin B1 expression, we found that with a time-dependent release of cells from a double thymidine block, cyclin B1, as indicated by immunoblotting, was significantly downregulated in LAPE cells compared with LAPN cells (Fig. 3C). Hence, loss of NHE1 activity likely decreases the stability of cyclin B1 protein rather than decreasing cyclin B1 gene expression. Decreased cyclin B1 expression and increased Wee1 kinase expression is consistent with our previous finding that Cdc2 kinase activity is inhibited in LAPE cells compared with LAPN cells [5].

Carbohydrate metabolism, electron transport and oxidative phosphorylation

A global pattern of metabolic genes differentially regulated in LAPE cells would favor glycolysis and oxidative phosphorylation, possibly in response to reduced ATP (Fig. 4A). Paradoxically, expression of genes encoding enzymes that regulate substrate entry for glycolysis was decreased. Decreased expression of the glucose transporter Glut-4 was confirmed by TaqMan analysis (Fig 4B). Additionally, decreased phosphorylase kinase would favor decreased conformational change of phosphorylase *b* to phosphorylase *a*, and reduced glycogen breakdown to glucose 6-phosphate and decreased galactokinase would favor decreased utilization of galactose for glycolysis. Key regulators of glycolytic flux, however, including hexokinase, phosphofusctose kinase, and pyruvate kinase were upregulated in LAPE cells compared with LAPN cells. Increases in hexokinase and phosphofusctose kinase, which catalyze the first and second ATP ultilization steps of glycolysis, respectively, and in pyruvate kinase, which catalyzes the final reaction of glycolysis, would favor increased production of pyruvate. Phosphorylation of fructose 6-phosphate by phosphofusctose kinase is a rate-determining reaction and the activity of phosphofusctose kinase is stimulated by low AMP and inhibited by high ATP and by citrate. In LAPE cells, a decrease in citrate transport protein would likely decrease cytosolic citrate, a negative regulator of phosphofusctose kinase, and indirectly increase phosphofusctose kinase activity. Glycolytic

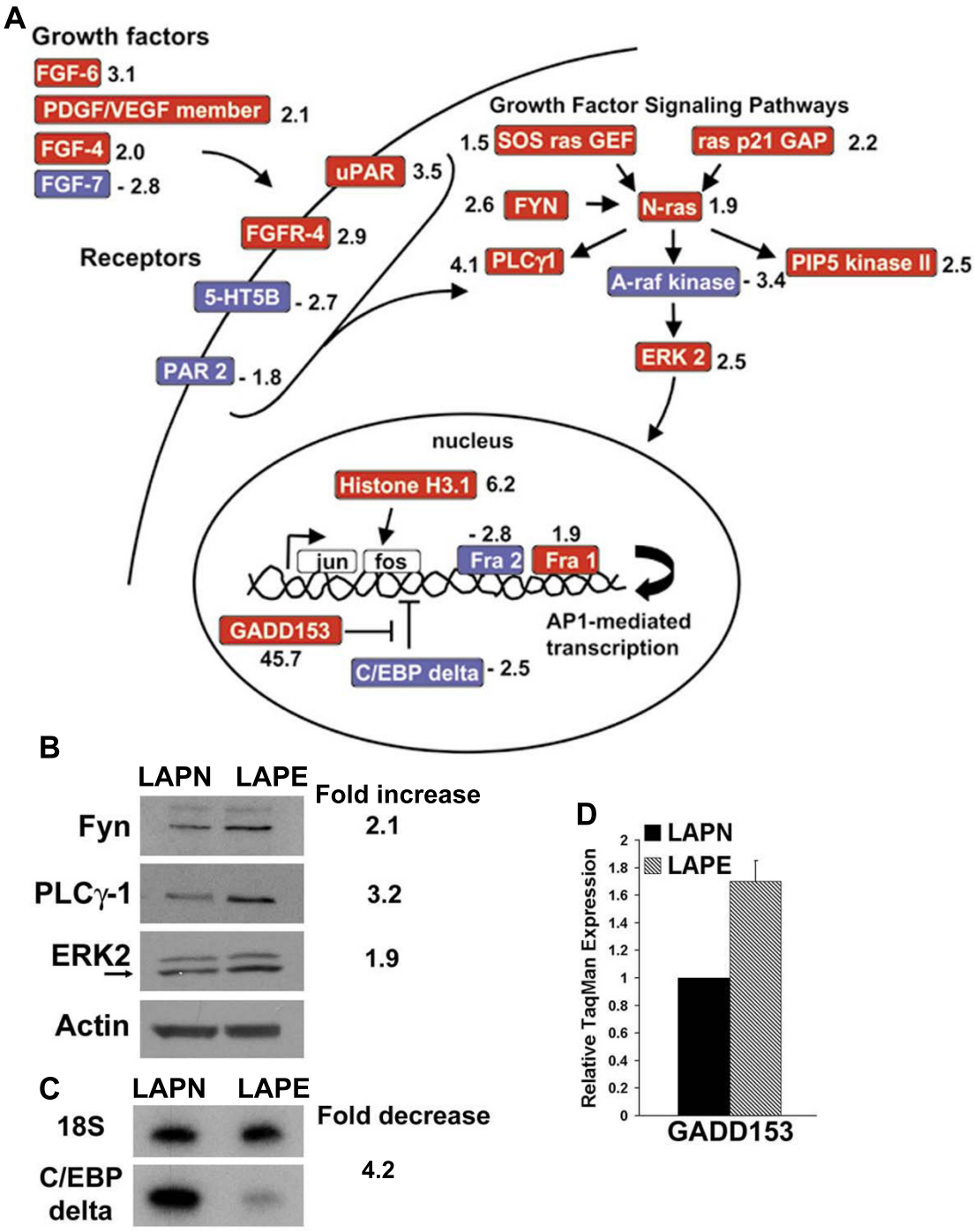

Figure 2

Genes differentially regulated in LAPE cells grouped as functioning in growth factor signaling and transcriptional regulation. A. Schematic diagram of growth factor signaling and transcriptional regulation. Red indicates genes with increased expression in LAPE cells compared with LAPN cells, and blue indicates genes with decreased expression. B. Immunoblot analysis of the indicated proteins confirmed increased protein expression in LAPE cells predicted by GeneChip data. C. Relative RT-PCR for the transcription factor C/EBP delta confirmed GeneChip data of increased expression in LAPE cells compared with LAPN cells. D. TaqMan analysis confirmed increased expression of GADD153 in LAPE cells compared with LAPN cells. Data in A represent the means of fold-increase or – decrease in LAPE cells ($p < 0.05$, $n = 5$). Data in B, C, and D are representative of 2 to 3 separate cell preparations.

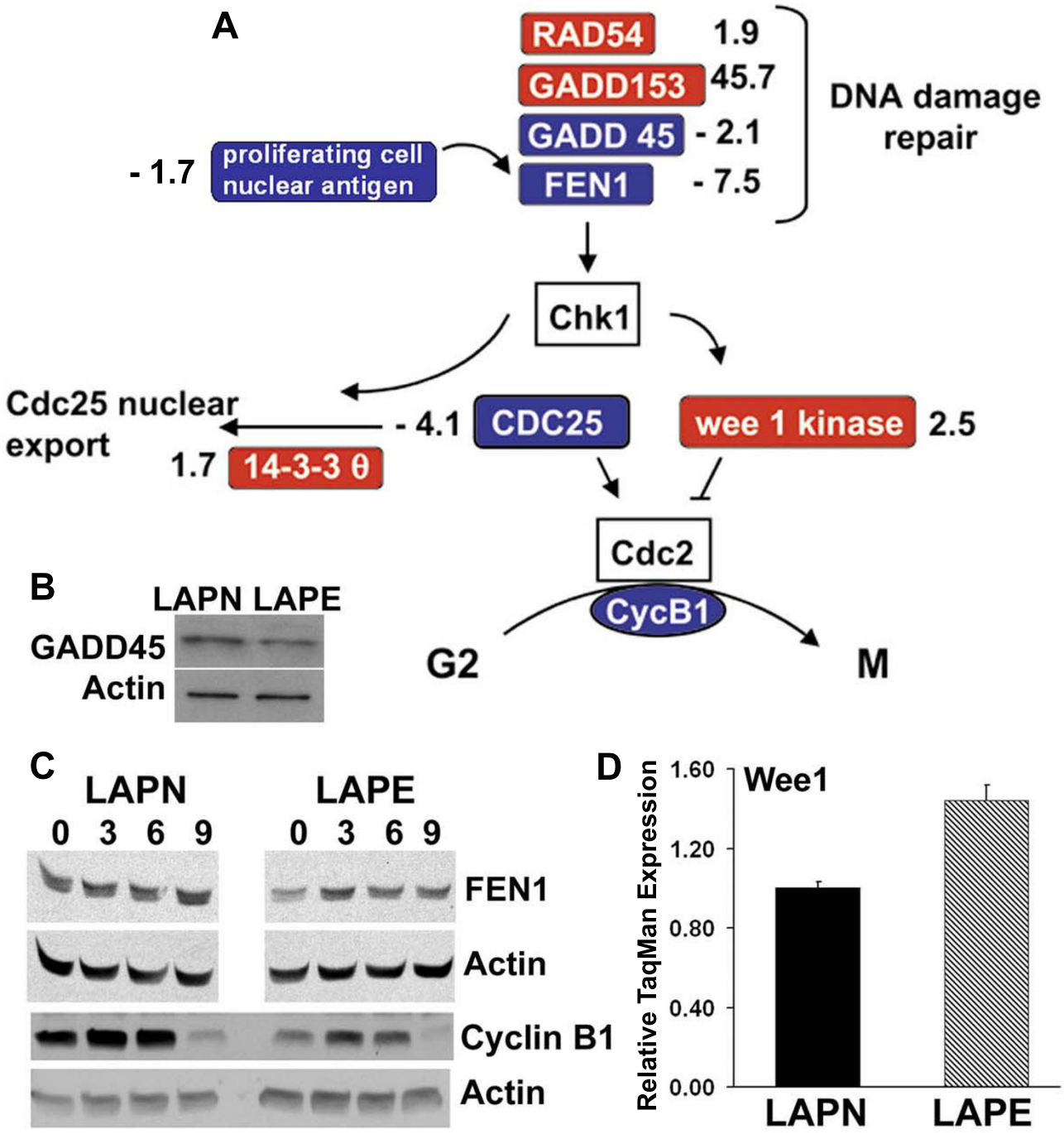

Figure 3

Genes differentially regulated in LAPE cells grouped as functioning in the G2/M transition of cell cycle progression and DNA damage checkpoint. A. Schematic diagram of G2/M regulation. Red indicates genes with increased expression in LAPE cells compared with LAPN cells, and blue indicates genes with decreased expression. B. Immunoblot of GADD45 confirmed decreased protein expression in LAPE cells compared with LAPN cells. C. Immunblotting for FEN1 and cyclin B1 at the indicated times after release from a double thymidine block. C. Relative TaqMan expression of Wee1 kinase confirmed GeneChip data of increased Wee1 expression in LAPE cells compared with LAPN cells. Data in A represent the means of fold-increase or – decrease in LAPE cells ($p < 0.05$, $n = 5$). Data in B and C are representative of 2 to 3 separate cell preparations. Data in D represent the mean ± s.e.m. of 3 separate cell preparations.

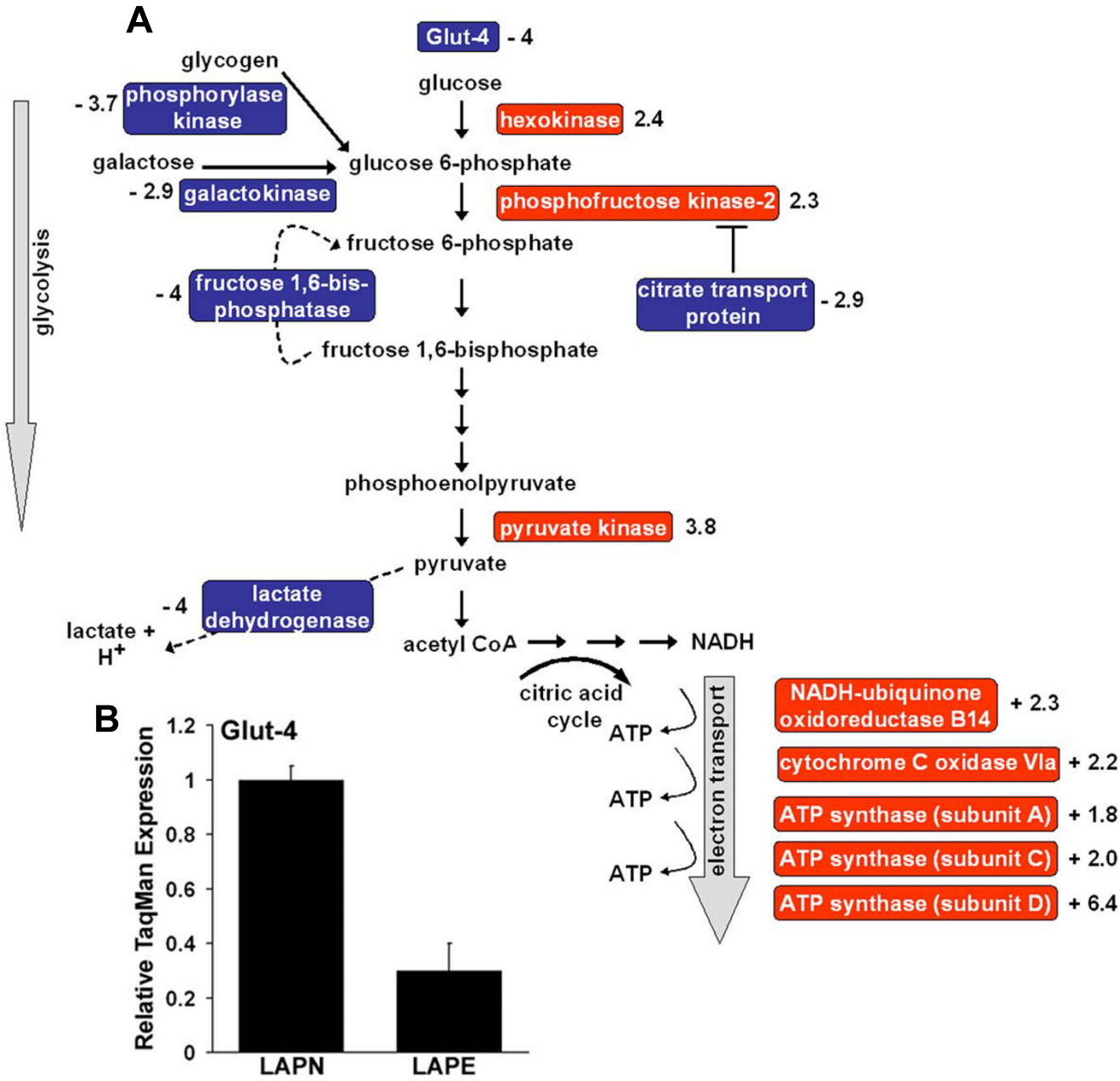

Figure 4

Genes differentially regulated in LAPE cells grouped as functioning in glycolysis, electron transport and oxidative phosphorylation. A. Schematic diagram of carbohydrate metabolism and oxidative phosphorylation. Red indicates genes with increased expression in LAPE cells compared with LAPN cells, and blue indicates genes with decreased expression. B. Relative TaqMan expression of Glut-4 confirmed GeneChip data of decreased Glut-4 expression in LAPE cells compared with LAPN cells. Data in A represent the means of fold-increase or – decrease in LAPE cells ($p < 0.05$, $n = 5$). Data in B are representative of 2 separate cell preparations.

flux and NADH would also be favored by decreased expression of fructose 1,6-bis-phosphate, which limits substrate recycling, and lactate dehydrogenase, which catalyzes the reduction of NADH by pyruvate to yield NAD^+ and lactate. An established metabolic difference in oncogenic transformed cells compared with normal cells

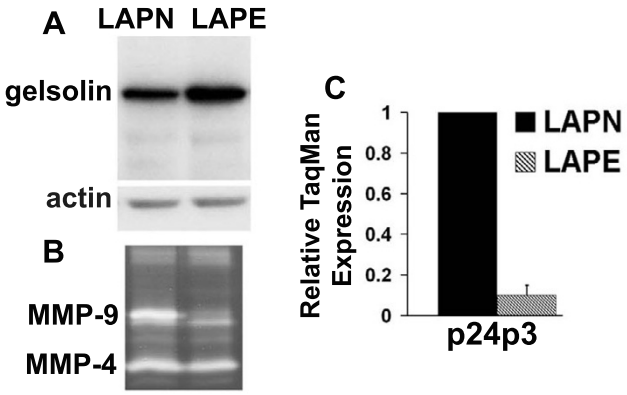

Figure 5
Expression of cytoskeleton and extracellular matrix genes differentially regulated in LAPE cells. A. Immunoblotting for gelsolin (top panel) and zymography for type IV collagenase (MMP-9) activity (bottom panel) confirmed increases and decreases, respectively, in LAPE cells compared with LAPN cells observed with GeneChip data. B. Relative TaqMan analysis indicated decreased p24p3 expression in LAPE cells, consistent with GeneChip data.

is increased lactic acid production [47] and decreased lactate dehydrogenase in LAPE cells correlates with increased NHE1 activity being necessary for oncogenic transformation [14] and tumor development [15].

Consistent with increased glycolysis and pyruvate production in LAPE cells, key enzymes favoring ATP production by electron transport and oxidative phosphorylation were increased (Fig. 4A). Increases in NADH-ubiquinone oxidoreductase and cytochrome *c* oxidase would favor oxidation of NADH. Increases in subunits A, C, and D of ATP synthase would increase endergonic synthesis of ATP. ATP synthase in the inner mitochondrial membrane is a proton translocator, extruding protons into the mitochondrial matrix. Whether decreased cytosolic pH in LAPE cells compared with LAPN cells alters the pH of the mitochondrial matrix and proton-electromotive force powering ATP synthesis remains to be determined. Collectively, the profile of gene expression in LAPE cells suggests equilibrium towards increased glycolytic flux and ATP production.

Cytoskeleton and extracellular matrix

cDNA array analysis indicated that loss of NHE1 activity in LAPE cells was associated with the regulation of a number of genes involved in cytoskeleton organization, cell adhesion, and extracellular matrix assembly. The regulation of several genes correlates with reported effects of NHE1 activity and pH_i on cell shape determination [6], cell polarity [9], actin-filament bundling [9,48], cell-substrate adhesion [9,10] and cell migration and metastasis [9,11,14]. NHE1 acts as an anchor for actin filaments by binding directly members of the ERM (ezrin, radixin, moesin) family of actin binding proteins, and in LAPE cells, expression of NF2 (merlin), a tumor suppressor protein and member of the ERM family, was downregulated. Consistent with a role for NHE1 activity in cell polarity and actin dynamics, loss of NHE1 activity was associated with decreased expression of myosin regulatory light chain, and increased expression of gelsolin (Fig. 5A), a pH-dependent actin severing and capping protein. Although regulation of the microtubule-based cytoskeleton by NHE1 and pH_i have previously not been reported, a number of microtubule-related genes, including Clip 170, KIF4, kinesin light chain, and a dynein heavy chain, were upregulated in LAPE cells.

Consistent with NHE1-dependent cell adhesion and migration, LAPE cells had a marked (~26-fold) decrease in type IV collagenase (MMP-9) expression, and zymography confirmed that activity of MMP-9, but not activity of MMP-4, was selectively decreased in LAPE cells compared with LAPN cells (Fig. 5B). Correlating with a decrease in MMP-9 expression, LAPE cells also had similar marked (~24-fold) decrease in the expression of lipocalin 24p3, which was confirmed by TaqMan analysis (Fig. 5C). NGAL (Neutrophil Gelatinase Associated Lipocalin), the human homolog of mouse lipocalin, is covalently bound to MMP-9 and protects MMP-9 from degradation [49,50]. Lipocalins are transcriptionally regulated by C/EBPβ [51], which is likely suppressed by decreased expression of C/EBPδ (Fig. 2C) and increased expression of GADD153 (Fig. 2D). Moreover, increased expression of MMP-9 [52] and lipocalins [53] is associated with tumor cell growth and invasion, which correlates with a role for NHE1 activity in these processes [14,15].

Conclusions

In summary, global profiling revealed genes regulated by loss of NHE1 activity and decreased pH_i. A number of the differentially regulated genes involved in growth factor signaling, cell cycle progression, and cytoskeleton and extracellular matrix remodeling are consistent with previously established roles of NHE1 activity and pH_i in mitogenic responses, cell proliferation, and tumor metastasis and invasion. In contrast, some genes, including those regulating carbohydrate metabolism and microtubule dynamics, have previously not been linked to NHE1 activity. An important future direction is to determine primary and secondary effects of gene regulation by NHE1 and of particular interest is whether promoters within the genes differentially regulated in LAPE cells are pH-responsive.

Methods

Cell culture and RNA preparation

The generation of LAPN and LAPE cells was as previously described [5]. In brief, NHE1-null LAP1 cells developed from parental Ltk-mouse muscle fibroblasts [16] were used for stable expression of wild-type NHE1 (LAPN cells) or expression of NHE1-E266I containing a single point substitution of glutamate266 for isoleucine that results in complete loss of ion translocation activity (LAPE) [5,6]. Cells were maintained in DMEM supplemented with 10% FCS in the presence of 25 mM $NaHCO_3$ and 5% CO_2. Total RNA was prepared from cells plated for 48 h by using Qiagen's RNeasy® midi kit.RNA. RNA was collected from five independent cell platings and used for five separate DNA array hybridizations.

cDNA synthesis and microarray hybridization

Total RNA was converted to double-stranded cDNA using the SuperScript Choice system (Gibco BRL), except that HPLC-purified T7-$(dT)_{24}$ oliomer (5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG – $(dT)_{24}$) was used instead of the oligo (dT) or random primers provided with the SuperScript Choice kit. Double-stranded cDNA was collected by ethanol precipitation. Biotinylated cRNA was then generated from the cDNA by an in vitro transcription (IVT) reaction using the ENZO BioArray™ HighYield™ RNA Transcript Labeling Kit. IVT products (cRNA) were collected by using Qiagen's RNeasyR mini kit, then ethanol-precipitated and quantitated. The cRNA was fragmented by alkaline treatment and hybridized to a GeneChip probe array from Affymetrix (Santa Clara, CA). The Affymetrix murine oligonucleotide array (Mu11KSubB) is complementary to ~6,500 murine genes and expressed sequence tags (ESTs). Each gene or EST is represented on the array by 16 – 20 feature pairs. Each feature pair contains a 25-bp oligonucleotide sequence, which is either a perfect match to the gene or a single central-base hommomeric mismatch control.

Microarray hybridization analysis

Affymetrix GeneChip analysis was performed using standard procedures [54]. The expression level of any particular transcript was calculated by subtracting he difference between the fluorescence intensities of the perfect match and mismatch feature pairs and then averaging over the entire probe set (Avg Diff). The Avg Diff value for each transcript was averaged over the five experiments for LAPN and LAPE cells; these average values obtained from five independent hybridizations were then used to calculate fold changes in LAPE cells relative to LAPN cells for each transcript. We did not use comparison algorithms supplied with the Affymetrix software.

Immunoblot analysis

Proteins from total cell lysates were separated by SDS-PAGE as previously described [5] and transferred to PVD nitrocellulose membranes for immunoblotting. Antibodies for immunoblotting included Fyn (NeoMarkers), PLCγ-1 and Erk 1–2 (Cell Signaling), GADD45, FEN1, and cyclin B1 (Santa Cruz Biotechnology), and actin (Sigma).

TaqMan™ analysis

Confirmation of GeneChip data was accomplished using TaqMan™ chemistry with the ABI 7700 Prism real-time PCR instrument (ABI, Foster City CA). Custom primers specific to the genes of interest were synthesized by Life Technologies and TaqMan™ probes for each gene were synthesized by Integrated DNA Technologies, Inc. The forward and reverse primers for mouse GADD153 (GenBank accession no. X67083) were 5'-GAAACGAAGAGGAAGAATCAAAAAC-3' and 5'-ATCTGGAGAGCGAGGGCTTT-3', respectively, and the probe was 5'-FAM/ACCCTGCGTCCCTAGCTTGGCTGAC/TAM-3', corresponding to an amplicon of 122 bp. The forward and reverse primers for mouse Wee1 kinase (GenBank accession no. NM_009516) were 5'-TTGCTCTTGCTCTCACAGTCGT-3' and 5'-TGGGAAAGCACTTGTGGGAT-3', respectively, and the probe was 5'-FAM/CCTTCCCAGAAATGGAGAGCACTGGC/TAM-3', corresponding to an amplicon of 118 bp. The forward and reverse primers for mouse Glut4 (GenBank accession no. NM_009204) were 5'-TGGCCATCTTCTCTGTGGGT-3' and 5'-ATTGGCTAGGCCCATGAGG-3', respectively, and the probe was 5'-FAM/TATGCTGGCCAACAATGTCTTGGCC/TAM-3', corresponding to an amplicon of 138 bp. The forward and reverse primers for mouse 24p3 (GenBank accession no. W13166) were 5'-GGCAGCTTTACGATGTACAGCA-3' and 5'-TCTGATCCAGTAGCGACAGCC-3', respectively, and the probe was 5'-FAM/CATCCTGGTCAGGGACCAGGACCAG/TAM-3', corresponding to an amplicon of 111 bp. For each gene, PCR was conducted in triplicate with 50 μl reaction volumes of 1x PCR buffer A (Applied Biosystems, Foster City, CA), 2.5 mM $MgCl_2$, 0.4 μM each primer, 200 μM each dNTP, 100 nM probe and 0.025 u/μl Taq Gold (ABI, Foster City CA). For each experiment, a large master mix of the above components was made and aliquoted into each optical reaction tube. Each primer/probe set (5 – 10 μl) was then added, and PCR conducted using the following cycle parameters: 95°C 12 min × 1 cycle, (95°C 20 sec, 60°C 1 min) × 40 cycles. Data analysis was carried out using sequence detection software that calculates the threshold cycle (Ct) for each reaction which is used to quantitate the amount of starting template in the reaction. A difference in Ct values (ΔCt) was calculated for each gene by taking triplicate Ct values from three reactions and subtracting the mean Ct of the triplicates for the control gene, GAPDH, for each cDNA sample at the same

concentration. An additional difference in Ct values (ΔCt) was calculated for each gene by taking the triplicate ΔCt values for each gene in the mutant LAPN1-E266I cells and subtracting the mean ΔCt of the triplicates for the wild-type LAPN cells. The relative expression levels were calculated as = $2^{-\Delta\Delta Ct}$ [55].

Quantitative RT-PCR analysis

Relative quantitative RT-PCR was preformed using QuantumRNA™ 18S internal standards from Ambion, Inc. (Austin TX) that included 18S Primers and Competimers™. By optimizing the assay and choosing an appropriate18S Primer:Competimer ratio for each sample, the 18S signal was reduced to the same linear range as that identified empirically for the gene specific product. The amplicon for the 18S primers was 315 bp. Custom primers specific to the genes of interest were synthesized by Life Technologies. The forward and reverse primers for calpactin I light chain (GenBank accession no. M16465) were 5'-GTGGACAAAATAATGAAGGAC-3' and 3'-ACAAGAAGCAGTGGGGCAGAT-5', respectively, corresponding to an amplicon of 222 bp. The forward and reverse primers for CEBPδ (GenBank accession no. NM_007679.1) were 5'-ATACCTCAGACCCCGACAGCG-3' and 3'-CAAAAGTCTGTCGGAAATGTC-5', respectively, corresponding to an amplicon of 220 bp. Total RNA was isolated from LAPN1 and LAPN1-E266I cells in four separate experiments, using Qiagen's RNeasy® midi kit. Reverse transcription of total RNA from each sample was carried out using random decamers and the RETROscript™ kit from Ambion. RT reactions were then subjected to PCR using the gene specific primers above (final concentration of 0.4 uM each), the appropriate 18S

Primer:Competimer ratio and 10uCi/ul [α-^{32}P]dCTP for labeling. PCR was conducted using the following cycle parameters: (94°C 30 sec, 57°C 30 sec, 72°C 30 sec) × 21 cycles for CEBPδ and 19 cycles for calpactin I light chain. Empirically derived 18S Primer:Competimer ratios were 1:18 for CEBPδ and 2:8 for calpactin I light chain.

Zymography

Activity of type IV collagenase (MMP9) was determined by zymography, as previously described [56].

Abbreviations

Ezrin, radixin, moesin (ERM); Intracellular pH (pH_i), Na-H exchanger type 1 (NHE1); NGAL (Neutrophil Gelatinase Associated Lipocalin); type IV collagenase (MMP-9).

Authors' contributions

LP participated in the design of the study, prepared and analyzed samples for array analysis, conducted analyses for protein and RNA expression, and performed the statistical analysis. DB conceived of the study, conducted analyses for protein expression, and participated in the design and coordination of the study. LP and DB prepared the manuscript.

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