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p63 contributes to cell invasion and migration in squamous cell carcinoma of the head and neck

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Abstract

The transcription factor p63 is commonly over-expressed in squamous cell carcinomas of the head and neck (SCCHN). By microarray analysis of p63-siRNA-treated SCCHN cells we identified 127 genes whose expression relies on over-expression of p63. More than 20% of these genes are involved in cell motility. Chromatin immunoprecipitation and reporter assay revealed PAI-1 and AQP3 as direct p63 transcriptional targets. In addition to PAI-1, most of the key cell motility-related molecules are up-regulated by p63, such as MMP14 and LGALS1. Our findings indicate a contribution by p63 in cell invasion and migration, supporting an oncogenic role for p63 in SCCHN.

Keywords: p63; Invasion; Migration; SCCHN

1. Introduction

Squamous cell carcinoma of the head and neck (SCCHN) is the sixth most common cancer worldwide. SCCHN arises within basal cells of squamous epithelium and has complex epidemiology and pathogenesis [1]. One common finding in SCCHN and some other squamous carcinomas is over-expression of the p63 protein, [2,3] which associates with amplification of the p63 locus at chromosome 3q27-29 [4,5]. The *p63* gene is a member of the p53 transcription factor family and can produce six different proteins from two promoters and the use of differential splicing. The TAp63 isoforms are similar to p53 in transactivation capacity, whilst the ΔNp63 isoforms act as inhibitors of transcription by occupying DNA binding sites without transactivating [6]. These original observations were, however, an over-simplification, as a second TA domain exists in the N-terminal region and an inhibitory domain has been identified in the C-terminally extended p63α proteins [7,8]. Expression of p63 is required for the proper formation of limbs, epidermis and other epithelial tissues including breast and prostate, as evidenced by the phenotypes of p63-null mice [9,10] and the malformations seen in people that inherit mutations in the *p63* gene [11].

The predominant p63 isoform expressed in normal squamous epithelia and squamous carcinomas including SCCHN is ΔNp63α [2–4,12–14]. Various

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functions have been ascribed to ΔNp63α in squamous epithelia and cancers and it is now well accepted that p63 plays an important role in cell fate specification, cell proliferation, differentiation, senescence and adhesion [15,16]. It has been reported that high p63 expression is associated with a more aggressive phenotype and poor prognosis in oral squamous cell carcinoma (OSCC) [17]. However, it has also been shown that high ΔNp63 protein levels in primary tumours accurately predict response to platinum based chemotherapy and a favorable outcome in head and neck cancer patients [18]. Similarly, Takahashi et al. found that lower p63 expression was associated with poor prognosis in esophageal squamous cell carcinoma [19]. In a recent report, Oliveira et al. found no significant association between p63 protein expression and survival, recurrence or metastasis by examining 106 OSCC patients [20]. In summary, the role(s) of p63 in tumourigenesis is still not clear.

Here, we investigated the role(s) of p63 in SCCHN by using siRNA-mediated inhibition of p63 to identify genes that rely on endogenous p63 expression in FaDu cells. This cell line is derived from a primary human SCCHN, has a similar expression profile of p63 to primary human tumours [3,13] and relies on elevated p63 expression for survival, [21] similar to other human SCCHN cell lines [22,23]. By microarray analysis we could show that p63 modulates multiple aspects of cell adhesion and migration in SCCHN.

2. Materials and methods

2.1. Cell culture and siRNA transfection

The human cell line FaDu originating from a human SCCHN of the hypopharynx (American Tissue Culture Collection, ATCC) was cultured in DMEM containing 10% FCS (Invitrogen, Grand Island, NY, USA). These cells express mutant p53 protein and high levels of p63 similar to primary human SCCHN tumours. At RNA level both N-terminal variants (TA and ΔN) as well as the three C-terminal splice variants (α, β and γ) can be detected, whereas only the ΔN isoform is detected at protein level (own unpublished data). Very low levels of the TAp63 isoforms were detected at mRNA level only. As reported previously, four complementary siRNA oligonucleotides targeting p63 (Dharmacon, Lafayette, CO, USA) were assessed. The p63siRNA3 duplex (5′-CACACAUGGUAUCCAGA UGTT-3′; 5′-CAUCUGGAUACCAUGUGUGTT-3′) targeting all p63 isoforms provided the most efficient inhibition and was used for all experiments. A control siRNA targeting luciferase served as a negative control [21]. Cells were transfected with the oligonucleotide duplexes (100 nM) premixed with Oligofectamine (Invitrogen) in serum-free media for 4 h and the efficiency of inhibition was determined by collecting cells 48 h later for p63 protein and mRNA expression.

2.2. Microarray sample preparation

1.5×10^6 FaDu cells plated 24 h previously were transfected with p63siRNA3 or control siRNA. Total RNA was extracted with Trizol (Invitrogen) 48 h after transfection. Three independent transfections were carried out with p63siRNA3 and two with control siRNA. For each culture, 20 μg total RNA was used for preparation of double-stranded cDNAs and biotinylated cRNAs were hybridized to HG-U133A chips (Affymetrix, Santa Clara, CA, USA) according to standard protocols in the Department of Clinical Microbiology at Umeå University.

2.3. Microarray data analysis

Expression values were calculated with either the RMA method available with the Affy package of BioConductor, [24] or with dCHIP software [25] using the default setting (PM-MM model). Similar results were obtained with both analyses and data presented is derived from dCHIP analysis. Arrays were quality assessed by array images, RNA degradation profiles, histograms and box plots pre- and post-normalisation in BioConductor, and calculation of percentage outliers in dCHIP. Expression values from dCHIP were truncated to 0 and probe sets that had high variance or were not changed were removed. By comparing the two controls with three independent p63 siRNA-treated samples, genes were identified that had a fold change of at least 1.3-fold at 90% confidence, a mean expression level of at least 30 in either treated or control arrays and a difference of at least 10.

2.4. Semi-quantitative and auantitative RT-PCR analysis

The same cDNA for microarray analysis was used for microarry data confirmation. Semi-quantitative RT-PCR analysis was performed for p63 using AmpliTag Gold DNA polymerase (Applied Biosystems, Branchburg, NJ, USA) in a GeneAmp 9600 thermal cycler (Perkin-Elmer). Quantitative RT-PCR was then performed for 14 selected genes using the Light Cycler (Roche Diagnostics Corp) and the LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics Cort) according to the manufacturer's instructions. Primer sequences are listed in Table 1.

2.5. Western blotting

Total proteins extracted from FaDu cells were separated on 10% or 15% SDS–polyacrylamide gels. The following antibodies were used: 4A4 mouse monoclonal

Table 1
Primers for microarray validation

Gene symbol	Primer sets	
ANXA1	Forward: GGTCTACAGAGAGGAACTGAAGA	Reverse: GTCACCCTTAGCAAGAGAAAGC
AQP3	Forward: CATCTACACCCTGGCACAGA	Reverse: TCCAGAGGG GTAGG TAGCAA
FABP5	Forward: AAGAAACCACAGCTGATGGC	Reverse: CATGACACACTC CACCACTA
IGFBP3	Forward: ACAGCCAGCGCTACAAAGTT	Reverse: CTG GG AC TCAG CACATTGAG
LYN	Forward: TCCCTGTATCAGCGACATGA	Reverse: AGTTCCTGGCTTCAGGGTTT
MMP1	Forward: TGGATCCAGGTTATCCCAAA	Reverse: TCCTGCAGTTGAACCAGCTA
MMP10	Forward: CATGCCTACCCACCTGGAC	Reverse: GAGCAG CAACGAGG AATAAATTG
MMP14	Forward: GAAGCCTGGCTACAGCAATATG	Reverse: CCGTAAAACTTCTGCATGGCA
PVRL1	Forward: GGCTTGACCGCATTCTTCCT	Reverse: TGCAGTGCAGAACCACGTC
RAB38	Forward: TGCACCAGAACTTCTCTTCG	Reverse: GCACCCATAGCTTCTCGGTA
SERPINE1	Forward: TGGAGAGAGCCAGATTCATCA	Reverse: AG TAG AGGG CATTCACCAG CA
SERPINI1	Forward: CTGCTGCTGTCTCAGGAATG	Reverse: TCAGGATGCATGACTCGTCC
TRAF4	Forward: GCCTGGCTTCGACTACAAGT	Reverse: GGATAGG CAGGCC CAATACT
UBE2E3	Forward: AAGGTTACTTTCCGCACCAGA	Reverse: AATAGTCAAAGCGGGACTCCA
p63	Forward: CCAGACTCAATTTAGTGAGC	Reverse: ACTTGCCAGATCATCCATGG
P-actin	Forward: ACCATGGATGATGATATCGC	Reverse: TTGCTGATCCACATCTGCTG

against p63 (diluted 1:2000) annexin A1 (1:5000), PAI-1 (1:200) (all from Abcam, Cambridge, UK), FABP5 (1:20000) (BioVendor, Brno, Czech Republic), Lyn (1:250) (BD Transduction Laboratories, San Jose, CA, USA), PVRL1 (1:500) (Invitrogen) and β-actin (1:20000) (Chemicon International, Temecula, CA, USA). Horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit immunoglobulins (Pierce, Rockford, IL, USA) were used as secondary antibodies and signals detected with enhanced chemiluminescence (Amersham Biosciences, UK).

2.6. Formaldehyde crosslinking and chromatin immunoprecipitation (ChIP)

One kilobyte regions upstream of exon 1 of the validated potential p63 target genes were examined for presence of putative p53-type response elements (p53-REs): two or more tandem repeats of RRR CWWGYYY separated by less than 13 bp. Known p53 consensus sequences located in AQP3, IGFBP3 and PAI-1 genes were used directly (IGFBP3 was used as the positive control for the assays). ChIP experiments were performed using the EZ ChIP™ chromatin immunoprecipitation kit (Upstate, Lake Placid, NY, USA) according to the manufacturer's protocol. A polyclonal antibody that specifically recognizes ΔNp63 isoforms [14] was used for immunoprecipitation. Preimmune sera was used as negative control. Eluted DNA was PCR amplified with primers specific for the regulatory regions of the candidate genes. PCR was performed with AmpliTag Gold DNA polymerase (Applied Biosystems) for 35 cycles of 94 °C for 30 s, annealing at 54–61 °C for 30 s and 72 °C for 30 s. Experiments were performed three times. Primer sequences for ChIP are listed in Table 2.

2.7. Plasmids and luciferase assay

The promoter region containing p53-REs was PCR-amplified, cloned into pGEM-T vector (Promega, Madison, WI, USA), followed by cloning into the KpnI and XhoI site of the pGL3-Basic reporter plasmid (Promega). For luciferase assays, Saos2 cells were seeded into 12-well plates (1×10^5 cells/well) 24 h prior to transfection. Saos2 cells lack p53 as well as all sioforms of p63 both RNA and protein level (own unpublished data). Cells were cotransfected with 250 ng of the reporter plasmid containing promoter response elements, 3 ng of pRL-TK encoding *Renilla* luciferase cDNA and 500 ng of the pcDNA3 vector either empty as control or containing p53 or p63 isoforms, using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA). The total amount of DNA transfected was kept constant (1 μg) with parental pcDNA3. At 48 h after transfection, luciferase activity was measured by a dual luciferase reporter assay system (Promega) and the transfection efficiency standardized against *Renilla* luciferase activity. The value obtained by control transfection was arbitrarily set at 1. Data shown are the fold change in luciferase activity of experimental cells versus control cells. Means and 95% confidence interval were calculated after at least 3 independent transfections.

3. Results

3.1. Microarray analysis of p63 regulated genes

FaDu cells were transfected with siRNAs targeting all p63 isoforms or luciferase and harvested 48 h later. Three separate replicates of p63siRNA-treated cells and two replicates for the control siRNA were used to synthesize biotinylated cRNA for hybridization on Hgu133A arrays. Microarray data showed a 1.84-fold down-regulation of

Table 2
Primers for ChIP analysis

Gene symbol	Primer sets	
ANXA1	Forward: GGTATTAGGATTGGGGCAGA	Reverse: AAAGGAAGCCACACCTAGCA
AQP3	Forward: GGGTAAGTCAGATGGGAGAGG	Reverse: GTGTCTACACATGGCGGATG
IGFBP3	Forward: GTGAGTGGGACTTTGGCATT	Reverse: TCCAGCTCAGATGGGAAAAC
LGALS1	Forward: AAAGGACAGGGTGCACAGAG	Reverse: CTCCTCGGGAAGGCTAAAGA
MMP14	Forward: TCTCCCTCTGCAGGTCTCAT	Reverse: GGATGTGGGAGACTTTGTCC
PAI-1	Forward: CAGAGGGCAGAAAGGTCAAG	Reverse: CTCTGGGAGTCCGTCTGAAC
PVRL1	Forward: CATGGACGCCTGCAAGTT	Reverse: CACGAGTCATGCCCCTTC
SERPINI1	Forward: TACCAGCAACTGAGGCACTG	Reverse: ACGAGTCCCCATAAGCCTCT

p63 mRNA after transfection with p63 siRNA. Down-regulation of p63 mRNA and protein was demonstrated by RT-PCR and Western blotting (Fig. 1A and B). In addition to p63, 129 probe sets representing 127 genes were identified as differentially expressed (64 up-regulated and 63 down-regulated). These genes code for proteins involved in diverse cellular functions and include protein kinases and phosphatases, metabolic and detoxifying enzymes, solute and water carriers, cellular adaptor proteins, proteins involved in ubiquitin metabolism and proteins involved in transcription (Table 3). Within this list, 27 genes (more than 20% of the identified genes) are related to migration, adhesion and invasion (Table 4). Quantitative RT-PCR for 14 genes and Western blotting for 5 genes confirmed that silencing of p63 alters the expression of each of these genes in FaDu cells (Fig. 1C and D).

3.2. Chromatin immunoprecipitation (ChIP)

To investigate the potential for direct regulation of gene expression, we searched for putative p63 binding sequences similar to the canonical p53 responsive element RRRCWWGYYY. Binding of ΔNp63 was then assessed by chromatin immunoprecipitation (ChIP) for 8 genes including IGFBP3 as a positive control [22]. We demonstrated binding of ΔNp63 to 4 genes: AQP3, IGFBP3, PAI-1 and SERPINI1 (Fig. 2). No binding was seen to putative sites in the ANXA1, LGALS1, MMP14 or PVRL1 genes. The lack of p63 binding to these genes indicates that they could be indirectly regulated by p63, or that other p63-binding sites, so far not identified, are operating.

3.3. Luciferase assay

Since AQP3 and PAI-1 have been shown to be transcriptional targets of p73 and p53, we further cloned the promoter sequence of AQP3 and PAI-1 containing p63 binding sites into a luciferase reporter vector pGL3-basic separately, in order to examine whether *in vivo* binding of p63 confers p63-dependent transcription. Results of dual luciferase reporter assays (Fig. 3) showed that in accordance with previous studies, ectopic expression of p53 transactivated the promoters of AQP3 [26] and PAI-1 [27]. In addition, we found that the PAI-1 promoter could be activated by all p63 isoforms, preferably TAp63γ, whereas for the AQP3 promoter, only TAp63β and TAP63γ isoforms promoted transcriptional activity. These results confirmed a direct role for p63 in the regulation of AQP3 and PAI-1 transcription.

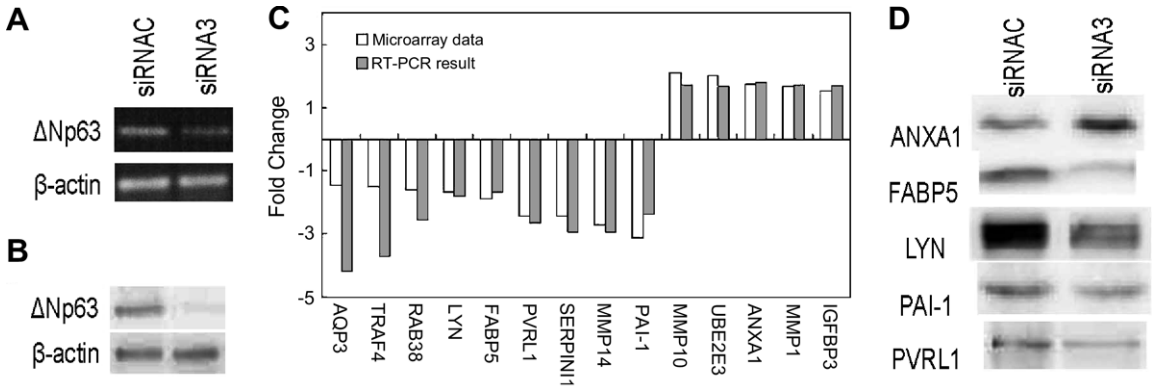

Fig. 1. Gene expression confirmed by RT-PCR and Western blot analysis. (A and B) Semi-quantitative RT-PCR and western blot was performed to confirm down-regulation of p63 by siRNA. β-actin was used as a normalization control. (C) A selection of 14 genes identified as differentially expressed by microarray were analyzed by quantitative RT-PCR. (D) Western blot analysis showed the predicted up-regulation of ANXA1 and down-regulation of FABP5, LYN, PAI-1 and PVRL1 in parallel with p63 down-regulation in FaDu cells.

Table 3
p63 regulated genes revealed by p63 knock down in FaDu cells

Accession	Gene	FC
R37427	TEM8	−8.82
NM_003906.1	MCM3	−3.26
NM_000602.1	PAI-1	−3.11
AU149305	MMP14	−2.73
NM_018175.1	Unknown	−2.6
NM_005025.1	SERPINI1	−2.45
AF110314.1	PVRL1	−2.44
AI814527	ADAM8	−2.39
NM_003246.1	THBS1	−2.19
NM_001447.1	FAT2	−2.18
U78168.1	cAMP-GEFl	−2.09
J04964.1	STS	−2.01
NM_006158.1	NEFL	−2.01
NM_004455.1	EXTL1	−1.98
BE904333	FLJ14251	−1.97
AU148057	DKK3	−1.96
AB007941.1	KIAA0472	−1.95
NM_014355.1	EN01B	−1.93
NM_001169.1	AQP8	−1.91
AV693216	PLXNB1	−1.91
NM_020958.1	KIAA1622	−1.89
NM_001444.1	FABP5	−1.87
AF091627.1	TP63	−1.84
AI753659	Sec23A	−1.84
NM_004925.2	AQP3	−1.83
NM_002081.1	GPC1	−1.82
NM_002083.1	GPX2	−1.82
BF978872	DLAT	−1.78
NM_014778.1	NUPL1	−1.77
NM_014516.1	CNOT3	−1.76
M 33376.1	AKR1C1	−1.76
X93921.1	DUSP7/Pyst2	−1.76
AL021707	UNC84B	−1.71
AW612574	ANP32E	−1.71
AI356412	LYN	−1.68
NM_003282.1	TTNI2	−1.68
BE299456	C16orf45	−1.66
NM_001353.2	AKR1C1	−1.65
AC004770	FADS 3	−1.64
NM_022337.1	RAB38	−1.62
NM_001549.1	IFIT4	−1.61
NM_022898.1	BCL11B	−1.6
BC005043.1	BC005043	−1.57
U15172	BNIP1	−1.55
AA081084	TAZ	−1.55
AL133052.1	Clorf37	−1.55
AB014530.1	HIPK1	−1.55
NM_002372.1	MAN2A1	−1.54
U05598.1	AKR1C2	−1.53
AF082185.1	TRAF4	−1.52
AU121431	KIAA1164	−1.52
AA853175	SLC16A3	−1.5
BC003560.1	RPN2	−1.49
NM_020119.1	ZAP	−1.49
NM_005886.1	KATNB1	−1.47
NM_017913.1	CDC37L1	−1.47
AB001325	AQP3	−1.45

Table 3 (*continued*)

Accession	Gene	FC
AL568804	SPTLC1	−1.45
AI613483	KFZp586J0720	−1.45
AL048503	DUSP3	−1.44
NM_000435.1	NOTCH3	−1.44
NM_004403.1	DFNA5	−1.42
AF133425.1	TSPAN1	−1.42
NM_022338.1	Cllorf24	−1.41
AV733308	ITGA6	−1.39
NM_002305.2	Galectin 1	−1.34
AK022426.1	CACNB2	11.87
S72848.1	IL6R	11.03
NM_002222.1	ITPR1	3.43
BC005314.1	ALDOB	3.26
NM_005568.1	LHX1	2.64
NM_016102.1	TRIM17	2.44
U82277.1	LILRA2	2.28
NM_001897.1	CSPG4	2.27
NM_021038.1	MBNL1	2.24
AW972855	Unknown	2.2
NM_022823.1	FNDC4	2.14
NM_002425.1	MMP10	2.09
NM_006874.1	ELF2	2.04
AB017644.1	UBE2E3	2.03
NM_025099.1	FLJ22170	2.03
AA502643	YWHAE	2.02
AF317549.1	ZNF268	1.97
NM_000753.1	PDE3B	1.96
NM_024331.1	C20orfl21	1.95
NM_003051.1	SLC16A1	1.92
NM_000104.2	CYP1B1	1.92
NM_003528.1	H2BFQ	1.89
NM_003856.1	IL1RL1	1.88
AL024509	Unknown	1.86
AF257501.1	SS18	1.85
U47635.1	MTMR6	1.83
AL359763	Unknown	1.83
NM_018132.1	C6orfl39	1.81
AF152475.1	PCDHA1	1.76
AA131826	SPTBN1	1.76
NM_000700.1	ANXA1	1.74
AL049301.1	DKFZp564P073	1.74
AF243424.1	STRN3	1.69
AB012305.1	CDK2	1.67
AI478592	CIB2	1.66
NM_002421.2	MMP1	1.65
K03193.1	EGFR	1.64
NM_016448.1	DTL	1.64
NM_004815.1	PARG1	1.61
NM_005757.2	MBLL39	1.6
NM_003092.1	SNRPB2	1.57
NM_015920.1	RPS27L	1.57
NM_030577.1	MGC10993	1.57
U26710.1	CBLB	1.56
AF119814.1	ZNF291	1.56
AI671049	CCNE1	1.55
M31159.1	IGFBP3	1.54
AF131748.1	SCS	1.54
NM_014928.1	KIAA1046	1.52

Table 3 (*continued*)

Accession	Gene	FC
AU132789	ZNF273	1.52
NM_013262.2	MIR	1.52
AA890010	SEC22L1	1.51
BC002387.1	NAP1L1	1.5
AI700518	NFIB	1.5
AA156240	RAIG1	1.5
AU144378	CDH11	1.5
NM_006420.1	ARFGEF2	1.5
NM_005754.1	G3BP	1.48
NM_018156.1	FLJ10619	1.48
AF113514.1	MYST4	1.47
NM_002293.2	LAMC1	1.46
NM_006754.1	SYPL	1.46
NM_005625.1	SDCBP	1.43
NM_030757.1	MKRN4	1.38

4. Discussion

SCCHN has an increasing incidence in developing countries, whereas survival rates for SCCHN have not improved over the last twenty years [1] High local recurrence rate after therapy and distant metastasis are major causes of death in patients with SCCHN, prompting substantial efforts in identifying molecular biomarkers that could predict patients at risk for disease recurrence and metastasis. It is obvious that even though various studies have demonstrated abnormalities of p63 expression in SCCHN, the role(s) of p63 in SCCHN are still unclear and the prognostic value of p63 in SCCHN is controversial. Though any prognostic marker system based on one single factor will be problematic, the uncertainty of the role of p63 in SCCHN might partly be due to the existence of the six different isoforms of p63, the large number of p63 targets involved in proliferation, differentiation, apoptosis and adhesion [15,28] or the different SCCHN primary sites studied by different groups [29]. Here, we have inhibited expression of all p63 isoforms in SCCHN cells that express similar quantities of p63 as primary human SCCHN in order to identify genes that rely on endogenous p63 expression in a relevant cellular context. The cells used, FaDu, have a mutation in codon 248 of p53, a residue shown to be involved in the interaction of p53 with ΔNp63. A mutation here abolishes this interaction and thus also the p53 mediated degradation of ΔNp63 [30].

Table 4
List of 27 cellular movement associated genes that are differentially expressed following inhibition of p63

Gene symbol	Description	Fold change
ADAM8	ADAM metallopeptidase domain 8	−2.39
ANXA1	Annexin A1	1.74
AQP3	Aquaporin 3	−1.45
CDH11	Cadherin-11	1.5
CSPG4	Chondroitin sulfate proteoglycan 4 (melanoma-associated)	2.27
DKK3	Dickkopf homolog 3	−1.96
EGFR	Epidermal growth factor receptor	1.64
FABP5	Fatty acid binding protein 5 (psoriasis-associated)	−1.87
FAT2	FAT tumor suppressor homolog 2 (Drosophila)	−2.18
GPC1	Glypican 1	−1.82
IGFBP3	Insulin-like growth factor binding protein 3	1.54
ITGA6	Integrin, alpha 6	−1.39
LAMC1	Laminin, gamma 1 (formerly LAMB2)	1.46
LGALS1	Lectin, galactoside-binding, soluble, 1 (galectin 1)	−1.34
MAN2A1	Mannosidase, alpha, class 2A, member 1	−1.54
MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	1.65
MMP10	Matrix metallopeptidase 10 (stromelysin 2)	2.09
MMP14	Matrix metallopeptidase 14 (membrane)	−2.73
PCDHA1	Protocadherin alpha 1	1.76
PLXNB1	Plexin B1	−1.91
PVRL1	Poliovirus receptor-related 1 (herpesvirus entry mediator C; nectin)	−2.44
RAPGEF3	Rap guanine nucleotide exchange factor (GEF) 3	−2.09
SDCBP	Syndecan binding protein (syntenin)	1.43
SERPINE1	Serpin peptidase inhibitor, clade E (Nexin, plasminogen activator inhibitor type 1), member 1	−3.11
SERPINI1	Serpin peptidase inhibitor, clade I (neuroserpin), member 1	−2.45
THBS1	Thrombospondin 1	−2.19
TSPAN1	Tetraspanin 1	−1.42

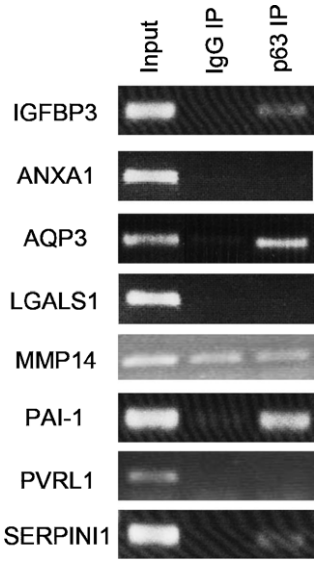

Fig. 2. *In vivo* binding of p63 to the regulatory regions of target genes. Formaldehyde cross-linked chromatin from FaDu cells was immunoprecipitated with antibodies against ΔNp63 or preimmune sera and analysed by PCR with specific primer pairs flanking the indicated regulatory regions. IGFBP3 Box A [22] amplification was used as a positive control. One representative result of three independent experiments is shown. Input, non-immunoprecipitated crosslinked chromatin.

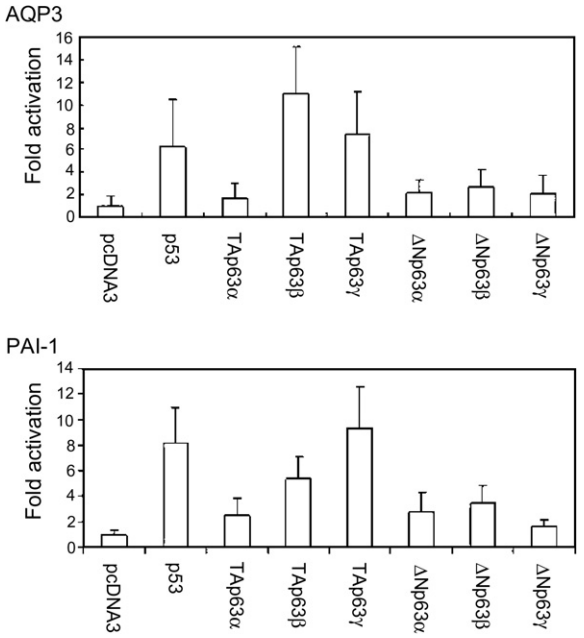

Fig. 3. Promoters of AQP3 and PAI-1 are activated by p53 and p63. Saos2 cells, lacking both p53 and all p63 isoforms, were transfected with reporter plasmid containing the indicated promoter region and cotransfected with p53 or p63 expression plasmids. Cells were lysed after 48 h and luciferase activity measured by a dual luciferase reporter assay system. The value obtained by control transfection was set at 1. Data is shown as fold activation in luciferase activity of experimental cells relative to control cells. Means and 95% confidence interval were calculated after at least 3 independent transfection triplicates.

A major set of genes identified in this study are involved in cellular migration, adhesion and/or invasion and our observations are supported by recent reports that also have identified p63 regulation of adhesion-related genes using microarrays [31–33]. We extended these observations by demonstrating binding of ΔNp63 to regulatory elements within the PAI-1, AQP3 and SERPINI1 genes and by demonstrating that PAI-1 and AQP3 are direct transcriptional targets of p63. In a previous study, we have shown that inhibiting p63 expression in FaDu cells does not affect cell proliferation or expression of epithelial differentiation markers, but results in significant decreased survival for these cells [21] This decreased survival might be the consequence of decrease in adhesiveness when endogenous p63 was knocked down. In the same way, *in vitro* cell migration assays showed that loss of p63 in squamous carcinoma cell lines leads to increased cell migration [32]. However, *in vivo* regulation of adhesion and migration by p63 is far more complex and remains to be further elucidated.

It is well known that altered expression of integrins and other cell adhesion molecules, matrix metalloproteases (MMPs), other proteases and protease inhibitors are closely linked with tumor growth and are major determinants of metastasis [34]. Two main extracellular protease systems play a crucial role in degrading the extracellular matrix for cancer cell invasion and metastasis: the plasminogen-activating systems and the matrix metalloproteinase system. The role of PAI-1 in promoting cancer invasiveness and metastatic spreading has been well studied and the prognostic value of PAI-1 in head and neck squamous cell carcinoma has been examined, showing high levels of PAI-1 associated with a significantly shorter disease-free survival [35,36]. MMPs were thought to be essential for basement-membrane penetration during metastasis and up-regulation of MMPs in cancer has been linked to acquisition of an invasive phenotype. MMP14 is present in several head and neck squamous cell carcinoma cell lines and is frequently over-expressed in aggressive, metastatic neoplasms, such as SCCHN [37]. Numerous studies have shown MMP14 as a key enzyme for tumour invasion and metastasis through direct extracellular matrix degradation [38–40] and also controlling tumour-

induced angiogenesis [37]. Therefore, by regulating the expression of PAI-1 and MMP14, over-expression of p63 in some SCCHN cases might confer tumors with a more aggressive phenotype. Interestingly, we also found that MMP1 and MMP10, other matrix metalloproteinases up-regulated in SCCHN, were down-regulated by p63 in FaDu cells. As cells need proper adhesion regulation for survival and for migration, these results suggest a delicate role of p63 in modulating cell adhesiveness and motility.

Another new p63 target found in this study was a water channel protein AQP3. AQP3 plays a major role in fluid homeostasis and dysregulation of AQP3 was found in some neoplastic lung tissues [41]. In addition to being water channels, aquaporins are also involved in swelling, cell migration and signal transduction [42]. Recently, it was found that AQP3 facilitated cell migration in corneal re-epithelialization [43]. As a p63-regulated gene in SCCHN shown in this study, AQP3 might play an important role in SCCHN by regulating cell migration.

In addition to PAI-1, MMP14 and AQP3, our affymetrix data showed that many of the cell adhesion and motility-related molecules are up-regulated by p63, such as LGALS1, FABP5, PVRL1, SERPINI1 and THBS1. LGALS1 [44], FABP5 [45], PVRL1 [46] and THBS1 [47] have been shown to be up-regulated in SCCHN and up-regulation of SERPINI1 was found in prostate cancer [48] In particular, LGALS1 is a potential cancer target involved in different events of tumor growth and metastasis [49]. Over-expression of LGALS1 at the invasive front of tumors is associated with poor prognosis in early-stage oral squamous cell carcinoma [44]. Among the few p63 down-regulated adhesion-related genes, ANXA1 has been shown to be down-regulated in SCCHN [50].

In conclusion, the use of siRNA to inhibit endogenous p63 expression in SCCHN cells with a p63-expression pattern similar to primary tumors has identified p63-regulated genes that function in diverse pathways of relevance to human squamous cancers. Strikingly, when combined with previous studies, these data indicate that over-expression of p63 in human cancer influences cell adhesion at multiple points. Thus, like p53, p63 can be considered a central node in the network of pathways responsible for neoplastic transformation of epithelial cells and for tumour progression. These data provide a molecular framework for understanding the role of p63 in human cancer and for explaining the associations of high level ΔNp63α expression in SCCHN tumors with aggressive behavior and poor prognosis *in vivo* [17].

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