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Journal of Cellular Biochemistry 110:1234–1243 (2010)

Journal of Cellular Biochemistry

The Transcriptional Programme of Contact-Inhibition

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ABSTRACT

Proliferation of non-transformed cells is regulated by cell–cell contacts, which are referred to as contact-inhibition. Vice versa, transformed cells are characterised by a loss of contact-inhibition. Despite its generally accepted importance for cell-cycle control, little is known about the intracellular signalling pathways involved in contact-inhibition. Unravelling the molecular mechanisms of contact-inhibition and its loss during tumourigenesis will be an important step towards the identification of novel target genes in tumour diagnosis and treatment. To better understand the underlying molecular mechanisms we identified the transcriptional programme of contact-inhibition in NIH3T3 fibroblast using high-density microarrays. Setting the cut off: $\geq$1.5-fold, $P \leq 0.05$, 853 genes and 73 cDNA sequences were differentially expressed in confluent compared to exponentially growing cultures. Importing these data into GenMAPP software revealed a comprehensive list of cell-cycle regulatory genes mediating G0/G1 arrest, which was confirmed by RT-PCR and Western blot. In a narrow analysis (cut off: $\geq$2-fold, $P \leq 0.002$), we found 110 transcripts to be differentially expressed representing 107 genes and 3 cDNA sequences involved, for example, in proliferation, signal transduction, transcriptional regulation, cell adhesion and communication. Interestingly, the majority of genes was upregulated indicating that contact-inhibition is not a passive state, but actively induced. Furthermore, we confirmed differential expression of eight genes by semi-quantitative RT-PCR and identified the potential tumour suppressor transforming growth factor-β (TGF-β)-1-induced clone 22 (TSC-22; tgfb1i4) as a novel protein to be induced in contact-inhibited cells. J. Cell. Biochem. 110: 1234–1243, 2010.

KEY WORDS: CONTACT-INHIBITION; CELL-CYCLE REGULATION; GENE EXPRESSION PROFILING; FIBROBLASTS

Proliferation of non-transformed cells is controlled by mitogenic and antimitogenic signals. The receptors and downstream effectors of proliferative stimuli exerted by growth factors or growth hormones have been extensively studied during the last decades. It is generally accepted that important anti-proliferative signals are mediated by cell–cell contacts, a cellular mechanism which is referred to as contact-dependent inhibition of growth or contact-inhibition [Eagle and Levine, 1967; Holley and Kiernan, 1968]. In adult tissues, contact-inhibition is thought to be continuously active, playing a critical role in the repression of somatic cell proliferation and probably organ size control [Zeng and Hong, 2008]. Release from contact-inhibition is associated with uncontrolled cellular proliferation, that is, cell transformation, tumour promotion and progression [Abercrombie, 1979; Fagotto and Gumbiner, 1996]. In vitro, contact-inhibition becomes apparent by the fact that adherent, non-transformed cells stop proliferating at a critical cell density forming a confluent monolayer. In contrast, transformed cells are characterised by loss of contact-inhibition manifested by a higher saturation density and the emergence of multilayered foci. Despite its importance for cell-cycle control, knowledge about the intracellular signalling cascade mediating contact-inhibition is still scarce. However, understanding the molecular mechanisms of contact-inhibition and its loss during tumour development will be an important step towards the identification of novel target genes for tumour diagnosis and treatment. In an attempt to identify novel tumour-related genes, Kim

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Stiftung Rheinland Pfalz für Innovation; Grant number: 8312-386261/530; Grant sponsor: Forschungsfond Johannes Gutenberg-University.

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Received 30 November 2009; Accepted 31 March 2010 • DOI 10.1002/jcb.22638 • © 2010 Wiley-Liss, Inc.
Published online 17 May 2010 in Wiley InterScience (www.interscience.wiley.com).

et al. [2004], for instance, have identified *ING4* as a candidate tumour suppressor gene in human cancer by a search for genes that suppress loss of contact-inhibition.

We previously demonstrated that the tumour suppressor $p16^{INK}$ mediates contact-inhibition in human fibroblasts by increased association with Cdk4 [Wieser et al., 1999]. It is also known that $p27^{KIP1}$ accumulates in confluent cells thereby inhibiting the cyclin E/Cdk2 complex [Polyak et al., 1994; Dietrich et al., 1997]. As a consequence, the retinoblastoma gene product (pRB) remains in the hypophosphorylated state thus inhibiting progression into S-phase [Polyak et al., 1994; Dietrich et al., 1997; Wieser et al., 1999]. Furthermore, the activation and nuclear translocation of PKCδ is involved in contact-inhibition in human and murine fibroblasts [Heit et al., 2001]. Although it is very likely that reorganisation of the actin cytoskeleton is one major target of PKCδ, its precise role in contact-inhibition and linkage to the cell-cycle machinery has to be resolved. Recently, we have revealed that prolonged activation of p38α MAPK is required for contact-inhibition with $p27^{KIP1}$ being one important downstream target of p38α MAPK [Faust et al., 2005], which presents a novel link between prolonged p38α MAPK-activation and the cell-cycle machinery [Swat et al., 2009].

To enlighten the molecular mechanisms of contact-inhibition, we analysed the transcriptional programme of contact-inhibition in mouse fibroblasts. To this end we compared gene expression in proliferating and confluent NIH3T3 fibroblasts by using the Affymetrix technology (GeneChip® Mouse Expression Array 430A) with each chip containing more than 22,000 probe sets representing more than 14,000 genes and identified the potential tumour suppressor transforming growth factor-β (TGF-β)-1-induced clone 22 (TSC-22; also known as TGF-β-1-induced transcript 4, tgfb1i4) as a novel protein to be induced in contact-inhibited cells.

MATERIALS AND METHODS

CELL CULTURE

Non-transformed NIH3T3 cells were cultured in Dulbecco's modified Eagle medium (DMEM; PAA) supplemented with 10% fetal calf serum (FCS) (PAA), 4 mM glutamine, penicillin and streptomycin (100 U/ml). Cells were either seeded to a low density of $2.44 \times 10^4/cm^2$ (60% confluence) or to a high density of $1.81 \times 10^5/cm^2$ (100% confluence) and harvested after 24 h except otherwise stated.

FLOW CYTOMETRY

Cells were trypsinised and washed twice with phosphate-buffered saline (PBS). $1–2 \times 10^6$ cells were vortexed in 200 μl of PBS and fixed with 2 ml of ice-cold 70% ethanol for 30 min at 4°C. Cells were then permeabilised by incubation with 1 ml of 0.2% Tween-20/PBS for 15 min at 37°C. Cells were resuspended in 2% FCS/PBS in the presence of RNAse A (11.25 kU/sample) and incubated with propidium iodide (50 μg/sample, Applichem) for 30 min at room temperature in the dark. Finally, the cells were resuspended in 800 μl of PBS and flow cytometric analysis was performed by a FACSCalibur (Becton Dickinson).

WESTERN BLOT

Total cell extracts were prepared by lysing the cells in hot Laemmli sample buffer and protein concentration was determined according to Smith et al. [1985]. Equal amounts of protein (20–50 μg protein/lane) were separated by SDS–PAGE (7.5–10%) and electroblotted overnight onto Immobilon membrane (Millipore). The membranes were blocked for 1 h with 5% low-fat milk powder in TBS (50 mM Tris–HCl, pH 7.5, 150 mM NaCl) containing 0.05% Tween-20 and then incubated for 1.5 h at room temperature with anti-pRb (1:1,000, BD Pharmingen), anti-TSC 22 (1:200, Santa Cruz), anti-Cdc25A, anti-Skp2, anti-Plk1 (1:300, Santa Cruz), anti-cyclin A (1:1,000, Santa Cruz) or anti-cyclin D1 antibodies (1:2,000, Cell Signaling) followed by incubation with horseradish-peroxidase-conjugated secondary antibody and ECL-detection according to the manufacturer's instructions or with anti-p27 antibody (1:1,000, Santa Cruz), followed by detection with alkaline phosphatase. To control for equal loading, the blots were stripped and reprobed with anti-p38α antibody (1:1,000, Cell Signaling) followed by ECL-detection.

SEMI-QUANTITATIVE RT-PCR

Total RNA was reverse transcribed using the Advantage RT-for-PCR Kit (BD Clontech) according to the manufacturer's instructions. The following primers were used for PCR: cyclin A: sense 5′-GCCTGCCTTCACCATTCATGTG-3′, antisense 5′-GTCTGGTGAAGGTC-CACAAGAC-3′; cyclin D1: sense 5′-CTGTGCGCCCTCCGTATCTTA-3′, antisense 5′-GGCGGCCAGGTTCCACTTGAG-3′; itm2b: sense 5′-GGAATTCAATGCCACAGCAT-3′, antisense 5′-GGTCCCTTTAACA-TATGCGA-3′; ncam: sense 5′-CTCCCTGCCTCCAACCATCATC-3′, antisense 5′-TCTCTCATCTTCCTCCTCGTTCTC-3′; pRB: sense 5′-CAACCCCCCCAAACCACTGA-3′, antisense 5′-CCAGATGTAGGG-GGTCAGGA-3′; TSC-22: sense 5′-CGATGGATCTAGGAGTTTACC-3′, antisense 5′-GCAATGAAATGGGTGACTGTG-3′; tle-2: sense 5′-CGTGAGGAAGACAAGAGTGAC-3′, antisense 5′-CAGACACGTG-GAAGGAGTAAG-3′; tle-6: sense 5′-CTGGAGAGGAACAAGAT-GAG-3′, antisense 5′-C TAGAAGAGCTGAGGGAGAC-3′; skp2: sense 5′-CAAGCATTCAAAACTCCTGAA-3′, antisense 5′-CACAGT-CACGTCTGGGTGCAGATT-3′; cdc25a: sense 5′-CGGATGTCAACGT-CACACTT-3′, antisense 5′-GTTAAGAGTCATCCACGAGG-3′; plk1: sense 5′-CTCCCTGGAGCTGCACAAGAGGAGGAA-3′, antisense 5′-TCTGTCTGAAGCATCTTCTGGATGAG-3′; dusp9: sense 5′-CTCCAT-GAACGATACCTACG-3′, antisense 5′-CAGCATCCCACTCTTCTTTG-3′; suv39H1: sense 5′-GATCCGTGAGCAGGAGTATTAC-3′, antisense 5′-GATGCCTTTCTGGACTACAC-3′; aurora kinase B: sense 5′-TTGA-CAACTTTGAGATTGGG-3′, antisense 5′-GCTGGTCGTAGAAGTAG-TTGT-3′; β-actin: sense 5′-TGCGTGACATCAAAGAGAAG-3′, antisense 5′-CGGATGTCAACGTCACACTT-3′. PCR conditions are available upon request.

AFFYMETRIX MICROARRAY HYBRIDISATION

Total RNA was extracted by RNAWiz (Ambion) according to the manufacturer's instructions and purified using RNeasy mini columns (Qiagen). The quality of the RNA was tested using photometry, agarose gel electrophoresis and Bioanalyzer Technology (Agilent). Total RNA (10 μg) was reverse transcribed and the resulting double-stranded cDNA was transformed into biotin-labelled antisense cRNA by an in vitro transcription step and quality

controlled by gel electrophoresis and photometry. The biotinylated cRNA targets were cleaned up and fragmented (approximately 100–200 bp). The quality of the labelled cRNA was tested using a GeneChip Test3 array (Affymetrix) before hybridisation (15 μg of cRNA for 16 h) to GeneChip Mouse Expression Array 430A (Affymetrix). The chips were stained with streptavidin–phycoerythrin and finally scanned using the Scanner 2500 (MAS 5.0 software). Experiments were performed in duplicates. The data preprocessing steps, background-adjustment, normalisation and computation of GCRMA gene expression measures according to Wu and coworkers [Wu et al., 2004] were carried out using the *gcrma* software library of the BioConductor Project (www.bioconductor.org). Statistical analysis was performed by empirical Bayes inference applying a moderated *t*-statistic for paired data [Smyth et al., 2003]. Clustering of genes involved in contact-inhibition (discriminators: P-value ≤ 0.002, $|\log 2 \text{ ratio}| \geq 1$) was analysed by using GO mining tool [Cheng et al., 2004]. Pathway analysis was performed by importing the gene expression data (discriminators: P-value ≤ 0.05, $|\log 2 \text{ ratio}| \geq 0.6$) into the GenMAPP software [Dahlquist et al., 2002; http://www.genmapp.org].

RESULTS

NIH3T3 fibroblasts were either sparsely seeded or seeded to confluence in the presence of FCS and cultured for 24 h. Flow cytometric analysis revealed that nearly 90% of the cells were arrested in G0/G1-phase in confluent cultures (Fig. 1A). As surrogate markers for G0/G1 arrest we further analysed expression of known cell-cycle regulatory proteins by Western blotting and RT-PCR [Polyak et al., 1994; Dietrich et al., 1997, 2002; Wieser et al., 1999]. G0/G1 arrest was reflected by the phosphorylation status of pRB: while the hyperphosphorylated species of pRB (116 kDa band) was predominant in proliferating cultures, only hypophosphorylated pRB, represented by the 107 kDa band [Mittnacht and Weinberg, 1991], was detected in confluent cultures (Fig. 1B). In accordance, cyclin D1—the regulatory subunit of Cdk4—was downregulated and p27Kip—a Cdk2/cyclin E inhibitor—was upregulated in confluent NIH3T3 cultures (Fig. 1C). Finally, cell-density-dependent decrease in cyclin D1-mRNA was shown by semi-quantitative RT-PCR (Fig. 1D).

Total RNA of the samples was then processed and used for gene array hybridisation. The validity of our data was confirmed by the fact that individual genes or proteins previously described to be differentially expressed in confluent fibroblasts, such as Gas1 [Del Sal et al., 1992], Egr1 [Gos et al., 2005], Slc25a5 (Ant2) [Barath et al., 1999] and Cdc25A [Afrakhte et al., 1998] were similarly regulated according to our microarray experiments (Gas1: fold change 1.7 [exp. 1] and 1.9 [exp. 2], $P = 0.005$; Egr1: fold change 1.6 [exp. 1] and 7.7 [exp. 2], $P = 0.086$; Slc25a5: fold change 0.8 [exp. 1] and 0.6 [exp. 2], $P = 0.05$; Cdc25A: fold change 0.7 [exp. 1] and 0.4 [exp. 2], $P = 0.03$).

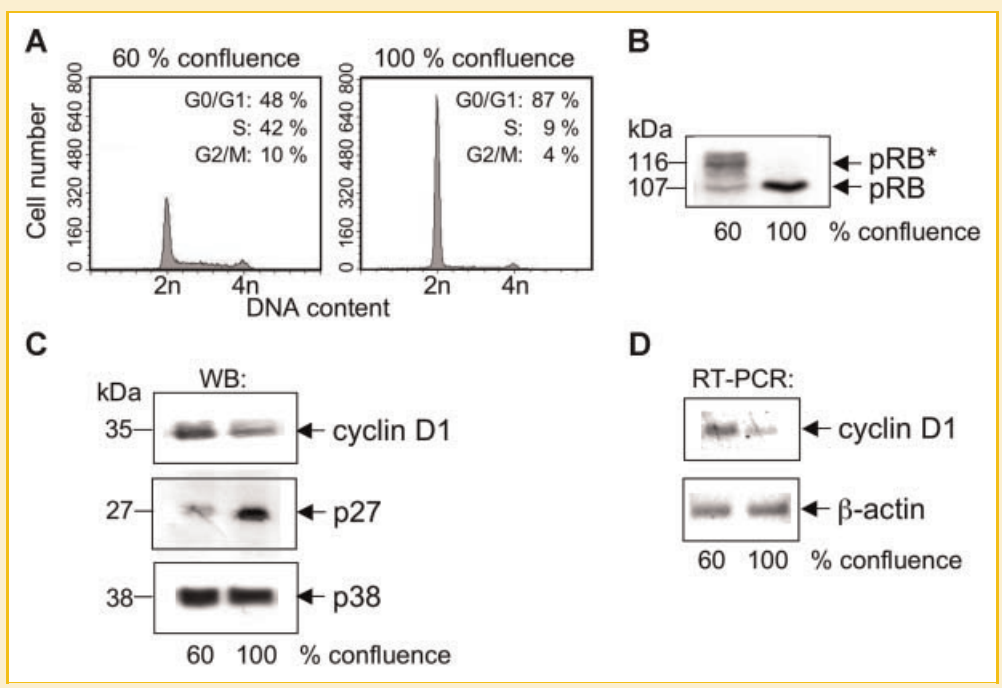

Fig. 1. Cell-cycle arrest in confluent NIH3T3 cultures. A–D: NIH3T3 mouse fibroblasts were either sparsely seeded (60% confluence) or to confluence (100% confluence) and cultured for 24 h. A: Cell-cycle distribution was determined after propidium iodide staining by flow cytometry. B: Total cellular extracts were subjected to immunoblotting using anti-pRb antibody. pRB* = hyperphosphorylated pRB. C: Total cellular extracts were subjected to Western blotting using anti-cyclin D1 or anti-p27 antibodies. To control for equal loading, the blots were stripped and reprobed with anti-p38α antibody. D: Semi-quantitative RT-PCR was performed using primers for cyclin D1. The figures represent one out of three independent experiments each leading to similar results.

Since it is a general drawback in microarray analysis that the calculated differences in gene expression are often smaller than those obtained by other experimental approaches, such as RT-PCR and Northern blot, so that changes in gene expression in microarray analysis may be underestimated [Irizarry et al., 2003; Cope et al., 2004], we started with a relatively broad data analysis. Setting the cut off at $\geq$1.5-fold and $P \leq 0.05$, 1,174 transcripts representing 853 genes and 73 cDNA sequences were differentially expressed in contact-inhibition (information available upon request). Importing the data into the GenMAPP software (see the Materials and Methods Section) led to identification of a comprehensive list of cell-cycle regulatory genes mediating cell-cycle arrest in contact-inhibition (Fig. 2A) as well as of genes involved in DNA synthesis (see Fig. S1, Supplementary Data). The decrease in cyclin D1 was not significant in the array analysis, although a tendency was detected (down-regulation 0.88 [exp. 1], 0.86 [exp. 2], $P = 0.13$) but was shown by RT-PCR and Western blot (Fig. 1). Differential expression of Skp2, Cdc25A, cyclin A and Polo-like kinase1 (Plk1) was confirmed by semi-quantitative RT-PCR and Western blot (Fig. 2B). Upregulation of pRB was shown by RT-PCR (Fig. 2B) and although quantification

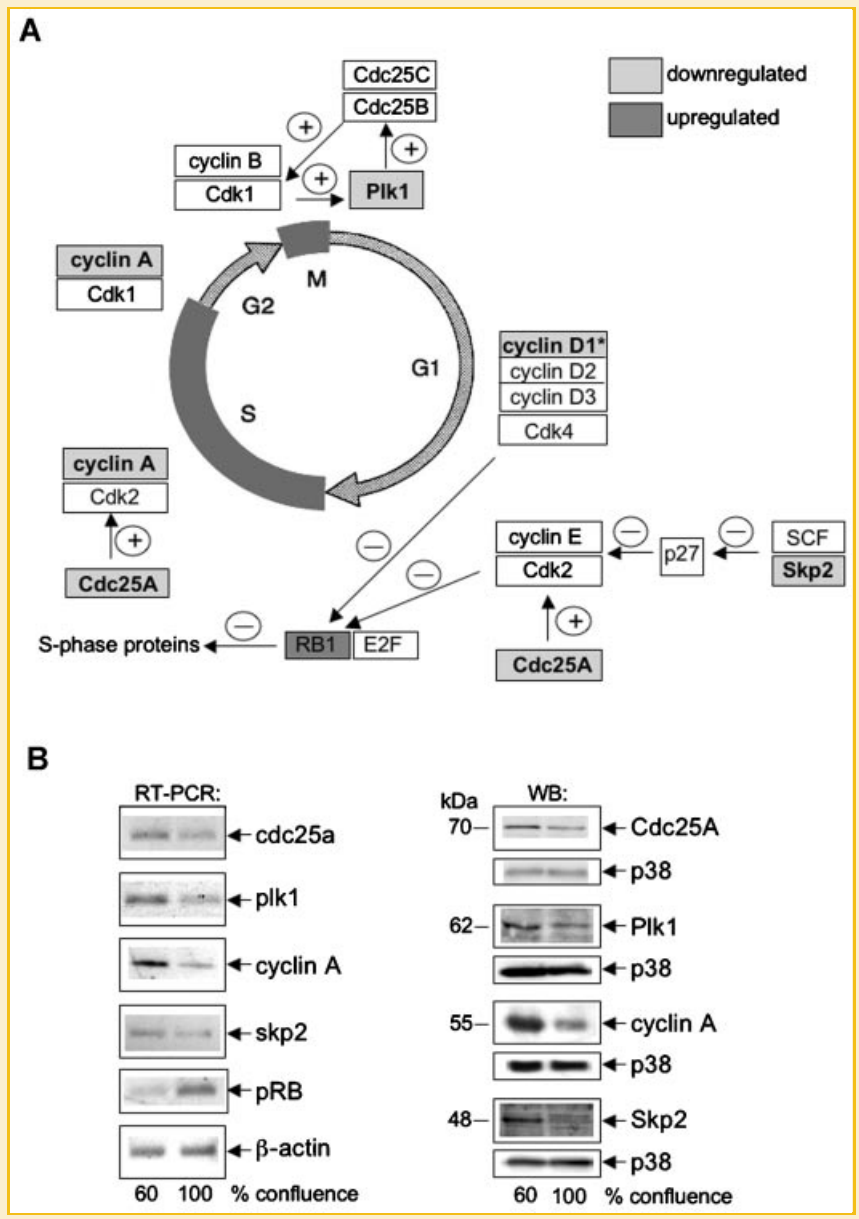

Fig. 2. Differential expression of genes involved in cell-cycle regulation. A: Expression data (cut off: $\geq$1.5-fold, $P \leq 0.05$) were integrated into GenMAPP. Bright grey indicates a lower, dark grey a higher gene expression in confluent cultures. *Not significant on the array, see Figure 1. Verification of differential gene expression by RT-PCR (B) and Western blot (C). Cells were seeded to 60% or 100% confluence. B: mRNA was isolated after 24 h. RT-PCR was performed using the indicated primers. C: Total cell extracts were prepared after 24 h and subjected to Western blotting. The figures represent one out of three independent experiments each leading to similar results.

of the Western blot is difficult due to differently migrating species of phosphorylated pRB, the data also suggest an increase in pRB at the protein level (Fig. 1B).

For a narrow analysis we set the cut off: $\geq$2-fold, $P \leq 0.002$. By this approach, we found 110 transcripts to be differentially expressed in contact-inhibited cultures representing 107 known genes and 3 cDNA sequences with hitherto unknown functions (Fig. 3A and Table I). Interestingly, the majority of genes was upregulated in confluent cultures indicating that contact-inhibition is not a passive state due to a lack of proliferative genes, but rather actively induced. Gene ontology revealed that most of these genes (85%) are involved in proliferation, signal transduction, transcriptional regulation, cell adhesion and communication, development, cell death, cellular transport, immune/inflammatory response or cellular metabolism (Fig. 3B and Table I).

Furthermore, we confirmed differential expression of eight genes with hitherto limited or lacking evidence for a role in contact-inhibition, such as the cell membrane proteins itm2b and ncam, the transcriptional corepressors transducin-like enhancer of split-2 and -6 (tle-2, tle-6), the dual phosphatase dusp9, the histone methyltransferase suv39H1, the mitotic protein aurora B kinase which regulates chromosomal segregation as well as the potential tumour suppressor TGF-β-1-induced clone 22 (TSC-22) (Fig. 4A,B). Together with the observation that TSC-22 is decreased in a variety of tumours and downregulation of TSC-22 enhances proliferation in vitro and in vivo, we hypothesised that TSC-22 might be involved in the signalling pathway of contact-inhibition and therefore analysed the expression of TSC-22 protein. Indeed, a threefold induction of TSC-22 was detected in confluent cultures by Western blot analysis (Fig. 4C). These data show for the first time that protein expression of the potential tumour suppressor TSC-22 is increased during contact-inhibition and suggests that TSC-22 is involved in cell-cycle regulation induced by cell–cell contacts.

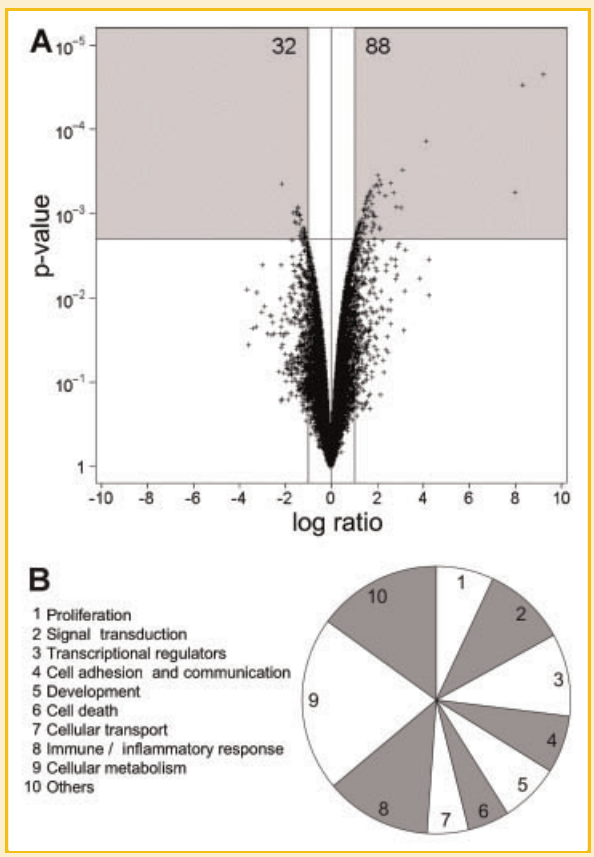

Fig. 3. Differential gene expression in confluent versus proliferating cultures. A: Volcano plot. For each of the 22,690 transcripts on the GeneChip Mouse Expression Array 430A, the magnitude of the mean effect (log 2 ratio) as well as the *P*-value of the corresponding moderated *t*-statistic is shown. Vertical and horizontal lines indicate discriminators used (P-value ≤ 0.002, $|\log 2 \text{ ratio}| \geq 1$). Genes within the grey area are considered to be differentially expressed. B: Analysis of differential gene expression by GO mining tool.

DISCUSSION

Contact-inhibition is a crucial mechanism of regulating proliferation in vivo and in vitro. To better understand the molecular mechanisms of contact-inhibition we analysed the transcriptional programme of contact-inhibition by using high-density microarrays. Broad data analysis (cut off: $\geq$1.5-fold, $P \leq 0.05$) combined with RT-PCR and Western blot analysis led to the identification of a comprehensive list of cell-cycle regulatory proteins mediating cell-cycle arrest, such as Skp2, Cdc25A, pRB and Polo-like kinase1. These results indicate that G0/G1 arrest in confluent NIH3T3 cells is governed by inhibition of the cyclin/Cdk–pRB axis by multiple mechanisms. It is generally accepted that during G1-phase, cyclin D/Cdk4 and downstream cyclin E/Cdk2 phosphorylate pRB which then dissociate from the transcription factor E2F thereby allowing transcription of S-phase-specific genes, such as cyclin A, and thereby entry into S-phase [Cobrinik, 2005; Malumbres and Barbacid, 2005]. In contrast, in confluent cultures pRB remains hypophosphorylated due to inhibition of cyclin D1/Cdk4—and cyclin E/Cdk2—activity thereby blocking transcriptional activity of E2F [Polyak et al., 1994; Dietrich et al., 1997 and present work]. In line with its pivotal role as a G1–S gatekeeper, we detected upregulation of pRB mRNA in confluent NIH3T3 cells. Upstream, the observed downregulation of the catalytic subunit cyclin D1 will lead to inhibition of Cdk4 activity [Malumbres and Barbacid, 2005]. (Even though downregulation of cyclin D1 was not statistically significant when analysing the array data, a tendency was detected and was independently shown by RT-PCR and Western blot.) Although downregulation of cyclin D1 in confluent cultures is common in many cell types [Dietrich et al., 2002; Baba et al., 2003], it is nevertheless cell-type specific, since it is not observed, for example, in human fibroblasts [Dietrich et al., 1997]. Downregulation of the phosphatase Cdc25A as well by upregulation of the Cdk-inhibitor p27 will lead to a decrease in cyclin E/Cdk2 activity. Cdc25A is known to dephosphorylate Cdk2 (Thr14 and Tyr15), which is required for Cdk2-activation. Hence, downregulation of Cdc25A leads to an increase in inhibitory phosphorylations of Cdk2.

An important inhibitor of the cyclin E/Cdk2 complex is p27. A reasonable explanation for the observed p27 upregulation is the decrease in Skp2. Skp2 belongs to the family of F-box proteins and is known to be a crucial component of the SCF complex [for review, see

TABLE I. Differentially Expressed Genes in Contact-Inhibition

Gene name	Symbol	GenBank accession no.	Fold-change Exp. 1	Fold-change Exp. 2	*P*-value
Cellular metabolism					
Arachidonate 5-lipoxygenase activating protein	Alox5ap	NM_009663	4.9	4.2	0.0005
Cytoplasmic polyadenylation element binding protein 4	Cpeb4	NM_026252	3.4	3.2	0.0006
N-acetylglucosamine kinase	Nagk	NM_019542	3.0	3.0	0.0006
Sumo3 (suppressor of mif two, 3) homologue 1 (yeast)	Smt3h1	NM_019929	3.2	3.7	0.0007
Enoyl coenzyme A hydratase 1, peroxisomal	Ech1	NM_016772	3.3	2.9	0.0009
Microsomal glutathione-*S*-transferase 2	Mgst2	NM_174995	3.9	3.2	0.001
TSPY-like 4	Tspyl4	NM_030203	4.0	3.1	0.001
Paternally expressed 3	Peg3	NM_008817	2.2	2.2	0.001
Praja 2, RING-H2 motif containing	Pja2	NM_144859	2.6	2.3	0.002
Aldo keto reductase family 1, member B10	Akr1b10	NM_172398	2.1	2.1	0.002
Dehydrogenase/reductase (SDR family) member 7	Dhrs7	NM_025522	2.0	2.0	0.002
Valyl-tRNA synthetase 2	Vars2	NM_011690	−2.7	−2.7	0.0008
Splicing factor 3a, subunit 2	Sf3a2	NM_013651	−2.7	−2.7	0.0009
Mannosidase 1, alpha	Man1a	NM_008548	−2.5	−2.6	0.001
Glutamate-cysteine ligase, modifier subunit	Gclm	NM_008129	−2.9	−2.6	0.001
Carbamoyl-phosphate synthetase 2	Cad	NM_023525	−3.2	−2.7	0.001
Thioredoxin reductase 1	Txnrd1	NM_015762	−2.6	−3.1	0.001
Tripartite motif protein 27	Trim27	NM_009054	−2.7	−2.5	0.001
GCN5 general control of amino acid synthesis-like 2	Gcn5l2	NM_020004	−2.2	−2.2	0.001
Phosphoglycolate phosphatase	Pgp	NM_025954	−2.2	−2.2	0.002
Deoxycytidine kinase	Dck	NM_007832	−2.1	−2.4	0.002
Cell proliferation					
Peripheral myelin protein 22	Pmp22	NM_008885	3.6	3.9	0.0005
Neural precursor cell expressed, developmentally down-reg.9	Nedd9	NM_017464	3.0	3.6	0.0009
Sestrin 3	Sesn3	NM_030261	4.1	3.3	0.001
LOH, 11, chromosomal region-2, gene A homologue (human)	Loh11cr2a	NM_172767	3.2	4.4	0.002
SAC3 domain containing 1	Sac3d1	NM_133678	−2.9	−2.6	0.0009
CTF 18, chromosome transmission fidelity factor 18 homologue	Chtf18	NM_145409	−2.6	−2.5	0.001
Aurora kinase B	Aurkb	NM_011496	−2.5	−2.3	0.002
Peroxisome proliferator activated receptor binding protein	Pparbp	NM_013634	−2.2	−2.1	0.002
Signal transduction					
Platelet-derived growth factor receptor, beta polypeptide	Pdgfrb	NM_008809	9.9	7.7	0.0003
Prostaglandin E receptor 4 (subtype EP4)	Ptger4	NM_008965	4.1	4.7	0.0004
Dimethylarginine dimethylaminohydrolase 2	Ddah2	NM_016765	3.9	4.4	0.0005
Tribbles homologue 2 (*Drosophila*)	Trib2	NM_144551	4.6	5.8	0.0006
Ras homologue gene family, member C	Rhoc	NM_007484	2.1	2.3	0.002
MARCKS-like protein	Mlp	NM_010807	2.8	2.4	0.002
Dual specificity phosphatase 9	Dusp9	NM_029352	−4.6	−4.1	0.0004
Hepatoma-derived growth factor	Hdgf	NM_008231	−2.7	−2.9	0.0009
Tyrosine kinase, non-receptor, 2	Tnk2	NM_016788	−2.6	−2.5	0.001
G protein-coupled receptor kinase 6	Gprk6	NM_011938	−2.3	−2.6	0.002
Transcriptional regulators					
Transducin-like enhancer of split 2	Tle2	NM_019725	7.6	5.7	0.0005
Transforming growth factor beta 1 induced transcript 4	Tgfb1i4	NM_009366	3.1	3.0	0.0006
Basic helix-loop-helix domain containing, class B2	Bhlhb2	NM_011498	3.2	3.9	0.0009
Regucalcin gene promoter region related protein	Rgpr	NM_033354	2.4	2.4	0.001
Transcription factor 4	Tcf4	NM_013685	2.3	2.3	0.001
cAMP responsive element modulator	Crem	NM_013498	2.2	2.3	0.002
Nuclear factor of activated T-cells 5	Nfat5	NM_018823	2.1	2.2	0.002
Forkhead box M1	Foxm1	NM_008021	−2.3	−2.7	0.002
Suppressor of variegation 3-9 homologue 1 (*Drosophila*)	Suv39h1	NM_011514	−2.3	−2.1	0.002
Coactivator-associated arginine methyltransferase 1	Carm1	NM_021531	−2.3	−2.1	0.002
Cell adhesion and communication					
Integrin, alpha 6	Itga6	NM_008397	3.0	3.3	0.0007
LIM domain containing preferred translocation partner in lipoma	Lpp	NM_178665	3.0	2.9	0.0007
LIM domain and actin binding 1	Lima1	NM_023063	2.6	2.6	0.0009
Integral membrane protein 2B	Itm2b	NM_008410	2.3	2.5	0.001
Neural cell adhesion molecule 1	Ncam1	NM_010875	2.9	2.5	0.002
Integrin, beta-like 1	Itgbl1	NM_145467	2.2	2.5	0.002
Tight junction protein 2	Tjp2	NM_011597	−2.3	−2.2	0.002
Development					
Matrix gamma-carboxyglutamate (gla) protein	Mglap	NM_008597	4.7	4.0	0.0006
Calmodulin-like 4	Calml4	NM_138304	3.4	3.8	0.0006
Carnitine deficiency-associated gene expressed in ventricle 1	Cdv1	NM_009879	3.0	2.9	0.0007
Semaphorin 5a	Sema5a	NM_009154	3.1	3.9	0.001
Selenoprotein P, plasma, 1	Sepp1	NM_009155	3.3	4.3	0.001
Zinc finger proliferation 1	Zipro1	NM_011757	2.2	2.2	0.002
Cell death					
BCL2-like 11 (apoptosis facilitator)	Bcl2l11	NM_009754	3.3	3.4	0.0005
Nuclear receptor subfamily4, group A, member 1	Nr4a1	NM_052975	2.7	2.9	0.0009
Programmed cell death 4	Pdcd4	NM_019752	3.7	5.1	0.002
Tumour necrosis factor receptor superfamily, member 21	Tnfrsf21	NM_178589	−2.9	−3.3	0.0009

TABLE I. (*Continued*)

Gene name	Symbol	GenBank accession no.	Fold-change Exp. 1	Fold-change Exp. 2	*P*-value
Protease, serine, 25	Prss25	NM_019752	−2.2	−2.3	0.001
Transport					
Ceruloplasmin	Cp	NM_007752	20.1	15.7	0.0001
Selenium binding protein 1	Selenbp1	NM_009150	10.3	6.8	0.0008
Elastin microfibril interfacer 1	Emilin1	NM_133918	3.4	2.9	0.001
Tumour necrosis factor, alpha-induced protein 1 (endothelial)	Tnfaip1	NM_009395	−2.3	−2.2	0.001
Treacher Collins Franceschetti syndrome 1, homologue	Tcofl	NM_011552	−2.3	−2.7	0.002
Immune/inflammatory response					
Lymphocyte antigen 6 complex, locus C	Ly6c	NM_010741	4.0	4.3	0.0003
Chemokine (C-X3-C motif) ligand 1	Cx3cl1	NM_009142	5.5	6.9	0.0004
Tumour necrosis factor (ligand) superfamily, member 13	Tnfsf13	NM_023517	4.2	3.8	0.0004
Guanylate nucleotide binding protein 2	Gbp2	NM_010260	3.2	3.3	0.0005
Interleukin 18	IL18	NM_008360	2.8	2.9	0.0007
Tumour necrosis factor receptor superfamily, member 9	Tnfrsf9	NM_011612	2.5	2.9	0.001
Major histocompatibility complex, class I-related	Mr1	NM_008209	3.7	5.1	0.001
Fc receptor, IgG, alpha chain transporter	Fcgrt	NM_010189	2.7	3.2	0.001
Toll-like receptor 2	Tlr2	NM_011905	2.3	2.4	0.001
Toll-like receptor 1	Tlr1	NM_030682	2.2	2.3	0.002
Lymphocyte antigen 6 complex, locus A	Ly6a	NM_010738	2.2	2.3	0.002
Toll-interleukin 1 receptor domain-containing adaptor protein	Tirap	NM_054096	−2.3	−2.6	0.002
Others					
Transmembrane protein 119	Tmem119	NM_146162	8.7	6.0	0.008
Transmembrane protein 205	Tmem205	NM_178577	2.9	2.5	0.001
Zinc finger protein 292	Zfp292	NM_013889	2.7	3.1	0.001
Yippee-like 5 (*Drosophila*)	Ypel5	NM_027166	2.6	3.0	0.001
LUC7-like 2 (*S. cerevisiae*)	Luc7l2	NM_138680	2.4	2.6	0.001
Transmembrane protein 218	Tmem218	NM_025464	2.4	2.3	0.001
Stomatin	Stom	NM_013515	2.3	2.4	0.001
Histidine triad nucleotide binding protein 3	Hint3	NM_025798	2.3	2.4	0.001
Autophagy 10-like (*S. cerevisiae*)	Apg10l	NM_025770	2.3	2.3	0.001
Mbt domain containing 1	Mbtd1	NM_134012	2.3	2.4	0.001
Down syndrome critical region homologue 5 (human)	Dscr5	NM_019543	2.3	2.2	0.001
Family with sequence similarity 13, member c	Fam13c	NM_024244	3.9	3.0	0.002
Musculoskeletal, embryonic nuclear protein 1	Mustnl	NM_181390	2.5	3.0	0.002
Zinc finger protein 260	Zfp260	NM_011981	2.3	2.5	0.002
Biglycan	Bgn	NM_007542	2.2	2.2	0.002
G protein-coupled receptor 177	Gpr177	NM_026582	2.1	2.2	0.002
Transmembrane protein 106a	Tmem106	NM_144830	2.2	2.1	0.002
CD164 sialomucin-like 1	Cd164l1	NM_054042	2.2	2.1	0.002
Yolk sac gene 2	Ysg2	NM_011734	2.1	2.0	0.002
Nurim (nuclear envelope membrane protein)	Nrm	NM_134122	−2.2	−2.4	0.002
Translin	Tsn	NM_011650	−2.2	−2.2	0.002
LSM2 homologue, U6 small nuclear RNA associated	Lsm2	NM_030597	−2.1	−2.1	0.002
Nicalin homologue (zebrafish)	Ncln	NM_134009	−2.1	−2.3	0.002
RIKEN cDNA 2310061J03 gene	2310061J03Rik	NM_133677	2.8	3.0	0.0009
RIKEN cDNA A930001N09 gene	A930001N09Rik	NM_029870	3.4	4.4	0.001
RIKEN cDNA 2010315L10 gene	2010315L10Rik	NM_025917	2.3	2.4	0.001

Nakayama and Nakayama, 2005]. The SCF complex, an E3 ubiquitin ligase, mediates ubiquitination of p27 [Carrano et al., 1999] thereby regulating proteasomal degradation of p27 during G1 phase [Pagano et al., 1995]. Several mechanisms are known to regulate transcription of Skp2. Positive regulators are, for example, the transcription factors E2F and FoxM1 [Wang et al., 2005; Assoian and Yung, 2008], as well as the Ser/Thr kinase Akt [Ecker and Hengst, 2009], whereas the tumour suppressor PTEN inhibits Skp2 expression [Hershko, 2008]. As outlined above, the transcriptional activity of E2F is inhibited in confluent cultures due to hypophosphorylation of pRB. In addition, we detected a decreased expression of the transcription factor FoxM1 (see Table I; unpublished observations). We conclude that Skp2 transcription via E2F and FoxM1 is blocked in confluent cultures.

We have previously shown that p38α MAPK is an important regulator of p27 protein accumulation in contact-inhibition [Faust et al., 2005]. Swat et al. [2009] then revealed that prolonged activation of p38α uncouples cell proliferation from mitogenic stimulation by inducing EGFR degradation through downregulation of the EGFR-stabilising protein Sprouty 2. This mechanism provides a further possible explanation for the observed transcriptional inhibition of Skp2: one consequence of attenuation of the EGFR pathway is very likely a decrease in Akt activity. In addition, we observed upregulation of PTEN protein levels in confluent NIH3T3 cultures (unpublished observation), which may also contribute to transcriptional inhibition of Skp2.

The observed decrease in cyclin A and Plk1 expression is very likely also the consequence of downregulated E2F activity and FoxM1 expression. Transcription of both proteins is known to be regulated via E2F and FoxM1.

To narrow data analysis we then set the cut off: $\geq$2-fold, $P \leq 0.002$ and revealed that 110 transcripts are differentially expressed in confluent compared to exponentially growing cultures representing 107 genes and 3 cDNA sequences involved, for example, in proliferation, signal transduction, transcriptional regulation, cell adhesion and communication. Differential expression of eight interesting genes was confirmed by RT-PCR, that is, ncam, itm2b, transducin-like enhancer of split-2 and -6, suv39H1,

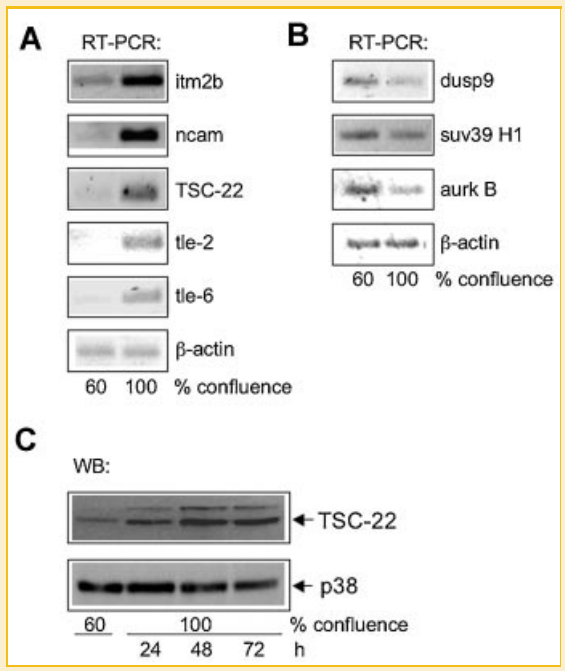

Fig. 4. Verification of differentially expressed genes in contact-inhibition. A,B: NIH3T3 mouse fibroblasts were either sparsely seeded (60% confluence) or to confluence (100% confluence) and cultured for 24 h. Semi-quantitative RT-PCR was performed to confirm expression of upregulated (A) or down-regulated (B) genes. C: NIH3T3 mouse fibroblasts were either sparsely seeded (60% confluence) and cultured for 24 h or to confluence (100% confluence) and cultured for 24, 48 or 72 h. Total cellular extracts were subjected to Western blotting using an anti-TSC22 antibody. To control for equal loading, the blots were stripped and reprobed with anti-p38α antibody. The figures represent one out of three independent experiments each leading to similar results.

aurora kinase B as well as TGF-β-1-induced clone 22 (TSC-22). Ncam (neural cell adhesion molecule) is known to be a glycoprotein-forming homophilic interactions especially in neurons [Rutishauser et al., 1988]. However, a single report described Ncam as a mediator of contact-inhibition in a mouse fibroblast cell line [Aoki et al., 1991], but its function in contact-inhibition is not known to date. Differential expression of the cell membrane protein itm2b, the transcriptional corepressors transducin-like enhancer of split-2 and -6 (tle-2, tle-6), the dual phosphatase dusp9 and the histone methyltransferase suv39H1 in confluent cultures has not been described so far and their role in the signalling cascade of contact-inhibition remains to be elucidated.

TSC-22 is known as a potential tumour suppressor with hitherto unknown functions. It was originally identified as a gene upregulated in response to TGF-β or peroxisome proliferator-activated receptor γ (PPARγ), characterised as a transcriptional repressor [Kester et al., 1999, 2000; Gupta et al., 2003] and is the founding member of the TSC-22/Dip/Bun family of leucine zipper transcription factors. Increasing evidence suggests that TSC-22 plays a role in cell-cycle regulation and differentiation. For instance, overexpression of TSC-22 inhibits proliferation in some leukaemia as well as colon carcinoma cells [Gupta et al., 2003; Yu et al., 2009]. In line with these observations, TSC-22 mRNA increases in postmitotic colon cells in vivo. Interestingly, treatment of tumour cell lines with vesnarinone, a kalium channel blocker, induces transcriptional activation of TSC-22 leading to cell-cycle arrest [Kawamata et al., 2003]. Vice versa, downregulation of TSC-22 enhances cellular proliferation in human salivary gland cancer cell lines in vitro and in vivo [Kawamata et al., 1998; Nakashiro et al., 1998]. Accordingly, a decrease in TSC-22 is observed in several tumours such as large granular lymphocyte leukaemia, glioblastomas, salivary gland and prostate carcinomas [Shostak et al., 2003; Rentsch et al., 2006; Doi et al., 2008; Yu et al., 2009] as well as during chemically induced liver carcinogenesis [Iida et al., 2005] and was identified as an acute molecular marker of non-genotoxic rodent hepatocarcinogenesis [Michel et al., 2005; Fielden et al., 2007]. In line with an inhibitory role of TSC-22 in cell proliferation, we detected a threefold induction of TSC-22 protein in confluent NIH3T3 cells. Kawamata et al. [1998] detected accumulation of TSC-22 mRNA with increasing confluence in salivary cancer cells. Together with our observation of an increase in TSC-22 protein in NIH3T3 cells, we conclude that TSC-22 plays a role in contact-inhibition. Interestingly, in *Xenopus*, xTSC-22 associates with p27 potentially increasing its nuclear activity [Hashiguchi et al., 2007]. Whether TSC-22 also associates with p27 in mammalian cells is not known to date and the functional role of TSC-22 in contact-inhibition remains to be determined.

In summary, our data indicate that contact-inhibition is not a passive state, but rather is actively induced. This arrest is maintained by affecting different target proteins in the cell cycle. Finally, we detected the transcriptional regulator TSC-22 as a novel protein induced in contact-inhibition. Although TSC-22 is suggested to be a potential tumour suppressor, its role in cell-cycle control is totally unclear. However, our studies suggest that TSC-22 is involved in the signalling cascade of contact-inhibition. Further analysis of its precise role in contact-inhibition shall enlighten its function and help to design specific activators for cancer treatment.

ACKNOWLEDGMENTS

This work was supported by the Stiftung Rheinland-Pfalz für Innovation (8312-386261/530) and Forschungsfond of the Johannes Gutenberg-University and is part of the PhD Thesis of M.K.

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