Cyclin-dependent kinase inhibitors and basement membrane interact to regulate breast epithelial cell differentiation and acinar morphogenesis

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Abstract. *Objective:* The cyclin-dependent kinase inhibitors (CDKIs), p21^{CIP1} and p27^{KIP1} regulate growth and differentiation in diverse tissue types. We aimed to determine whether p21^{CIP1} or p27^{KIP1} could induce a terminally differentiated phenotype in breast cells, and to examine if CDKI expression is regulated by basement membrane interactions. *Materials and Methods:* Effects of increased CDKI expression on the phenotype of MCF-10A breast epithelial cells were examined by retroviral transduction of p21^{CIP1} or p27^{KIP1} cDNA. *Results:* Overexpression of p21^{CIP1} or p27^{KIP1} reduced MCF-10A growth rates in monolayer cultures, altered cellular morphology and stimulated accumulation of neutral lipid droplets, suggesting partial lactational differentiation. However, markers of luminal differentiation (oestrogen and progesterone receptors, α -lactalbumin, β -casein and adipophilin) were absent when examined by reverse transcriptase-polymerase chain reaction and immunohistochemistry. Cell-basement membrane contacts are known to be essential for full mammary epithelial cell differentiation and therefore parental MCF-10A cells were cultured on a basement membrane preparation (Matrigel) in which they form acini. Immunocytochemistry showed that Ki67, the cell proliferation marker, was initially expressed at high levels and as growth decreased p27^{KIP1} expression steadily increased. Surprisingly, p21^{CIP1} was highest at the early stages of acinus growth and was detected in proliferating cells, as demonstrated by colocalization in dual Ki67/p21^{CIP1} immunofluorescence. Overexpression of p21^{CIP1} or p27^{KIP1} impaired formation of acini, whereas their knockdown, using siRNA, increased acinus formation. *Conclusion:* We conclude that both p21^{CIP1} and p27^{KIP1} induce partial secretory differentiation of mammary cells in monolayer, but during acinus morphogenesis in 3D culture they have a highly regulated temporal expression pattern.

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INTRODUCTION

An early first full-term pregnancy has been demonstrated to decrease a woman's risk of breast cancer (Russo & Russo 1988; Kelsey *et al.* 1993). This suggests that factors that promote breast epithelial cell differentiation may be protective (Srivastava *et al.* 1997). One factor that may play a role in differentiation of normal breast is p27^{KIP1}, a cyclin-dependent kinase inhibitor (CDKI). Its role in terminal differentiation is demonstrated in p27^{KIP1} knockout mice where 1–2 additional cell divisions precede terminal differentiation, leading to an increased number of cells per body organ (Durand *et al.* 1998). p27^{KIP1} has been shown to be antitumorigenic in lymphomas, breast and prostate cancers (Ezhevsky *et al.* 1996; Campbell *et al.* 1997; Fredersdorf *et al.* 1997) and transduction of p27^{KIP1} using retroviral vectors causes reduction in tumour occurrence in mice (Craig *et al.* 1997). In breast luminal epithelium, p27^{KIP1}-positive cells also express steroid receptors, but not the Ki67 proliferation-associated antigen, supporting the hypothesis that p27^{KIP1} plays a role in terminal differentiation of normal human breast epithelium (Clarke *et al.* 2000). However, it is not clear whether p27^{KIP1} expression is a cause or consequence of cellular differentiation or expression of steroid receptors. Other related CDKIs such as p21^{CIP1} may also be involved in the differentiation process and their role in the normal human breast epithelium has yet to be defined.

The three known members of the Cip/Kip family; p21^{CIP1}, p27^{KIP1} and p57^{KIP2} all contain a cyclin-binding domain and share core homology around a C-terminal CDK-inhibitory domain (Matsuoka *et al.* 1995). The interactions between CDKIs, cyclin-dependent kinases (CDKs) and cyclins control entry of cells into the cell cycle. Extracellular signals that increase cyclin levels stimulate entry into the cell cycle by promoting association of cyclin D with CDK4/6, and cyclin E with CDK2. Activated cyclin/CDK complexes phosphorylate the cell cycle regulator retinoblastoma protein (pRb) (Buckley *et al.* 1997; Kato 1997), which in turn dissociates from the E2F family of transcription factors, allowing progression of the cell cycle. CDKIs stop entry into the cell cycle by inhibiting the kinase activity of cyclin D/CDK complexes. This maintains pRb in a hypophosphorylated inactive form that is bound to members of the E2F family (Grana *et al.* 1998). CDKIs are divided into two families: the Cip/Kip and INK4 families. The Cip/Kip CDKIs inhibit the activity of a broad range of CDKs, whereas the INK4 CDKIs are targeted specifically to CDK4 and CDK6.

Expression of p21^{CIP1} is induced by activation of the tumour suppressor, p53, to inhibit proliferation of cells with damaged DNA (Liu & Pelling 1995; Wu & Levine 1997). p21^{CIP1} is also involved in mediating exit from the cell cycle and promotion of terminal differentiation, as shown during the formation of muscle and alveoli in mice (Zhang *et al.* 1999), differentiation of myelomonocytic cells to monocytes and macrophages (Liu *et al.* 1996) and differentiation of neuroblastomas (Wainwright *et al.* 2001). However, this contradicts *in vivo* studies, where p21^{CIP1}-deficient mice develop normally (Deng *et al.* 1995) and exhibit limited resistance to radiation induced tumours (Martin-Caballero *et al.* 2001). p21^{CIP1} is able to inhibit the activity of a wide range of cyclin/CDK complexes, including cyclinD/CDK4, cyclinA/CDK2, cyclinE/CDK2, cyclinB/CDK1. Levels and activity of p27^{KIP1} are regulated by the abundance of p27^{KIP1} protein and subcellular distribution, which in turn is regulated by phosphorylation status of p27^{KIP1} (Fujita *et al.* 2003; Min *et al.* 2004). p27^{KIP1} levels vary throughout the cell cycle, increasing during G₁ phase to a maximum early in G₁. Decrease in p27^{KIP1} levels allows the cell to enter S phase. p27^{KIP1} levels act as a buffer to control the activity of the cyclin/CDK complex until the balance is disturbed, either by an increase in cyclin/CDK or a decrease in p27^{KIP1}. Up-regulation of p27^{KIP1} translation is stimulated by contact inhibition and cell density, whereas mitogenic factors cause a decrease in p27^{KIP1} mRNA translation and

increase in p27^{KIP1} degradation by the proteasome (Pagano *et al.* 1995; Montagnoli *et al.* 1999). We hypothesize that p21^{CIP1} and p27^{KIP1} may play roles in the terminal differentiation of normal breast epithelium, leading to a lactational phenotype and/or steroid receptor expression.

To investigate the roles of the Cip/Kip CDKIs in breast epithelial differentiation we used the non-cancerous breast epithelial cell line, MCF-10A. This cell line spontaneously immortalized following primary culture from a fibrocystic breast lesion (Soule *et al.* 1990). In common with other normal breast epithelial cells grown in monolayer culture, they are highly proliferative and do not express steroid receptors (Anderson *et al.* 1998). MCF-10A cells were treated with retroviral vectors containing CDKI transgenes and knockdown by short interfering RNA (siRNA) and we measured growth and phenotypic differentiation including steroid receptor and milk protein expression. We also examined CDKI expression in relation to growth in three-dimensional cultures of breast cells on basement membrane (BM) during formation of acinar structures that resemble the secretory units of the breast. This was carried out as the differentiation programme can be re-initiated in breast cells that are exposed to basement membrane signals in primary mouse mammary epithelial cells (Aggeler *et al.* 1991; Streuli *et al.* 1991).

MATERIALS AND METHODS

MCF-10A cells were obtained from the American Tissue Culture Collection. All cell culture media and sera were obtained from Invitrogen (Paisley, Scotland, UK). Supplements for culture media were supplied by Sigma (Poole, Dorset, UK). Unless otherwise stated, antibodies for immunohistochemistry were obtained from Dako (Ely, Cambridgeshire, UK). Matrigel and chamber slides were supplied by Fred Baker Scientific (Runcorn, Cheshire, UK). Unless stated otherwise, all other chemicals were supplied by Merck BDH (Poole, Dorset, UK).

Cell culture

MCF-10A human mammary epithelial cells were routinely grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 with 5% horse serum, epidermal growth factor (10 ng/mL), hydrocortisone (1 µg/mL), cholera toxin (1 µg/mL), insulin (10 µg/mL), penicillin (100 µg/mL) and streptomycin (100 µg/mL). 293T cells were cultured in DMEM with 10% foetal calf serum. Cells were passaged every 3–4 days upon achieving 80–90% confluence, by trypsinization, and were seeded at 1×10^6 cells per T75 flask.

Construction of CDKI/IRES-EGFP retroviral stocks

We used the Kat retroviral vector (Finer *et al.* 1994) to transduce genes into cells. This was originally supplied by Cell Genesys (Foster City, CA, USA) and was subsequently modified by introduction of an internal ribosome entry site (IRES) and enhanced green fluorescent protein (EGFP) sequence to form rKat.IRES.EGFP (Gilham *et al.* 2002).

Coding sequences for human p21^{CIP1} and p27^{KIP1} were ligated into the Kat vector, upstream of the IRES, so that expression of the transgene would correspond with EGFP expression. Direction of insertion of the genes and their sequence was verified by DNA sequencing performed on an ABI3100 capillary sequencer using Big Dye version 3 dye terminator chemistry (supplied by Applied Biosystems, Foster City, CA, USA). Transgene-containing plasmids were used to produce amphotropic retrovirus using a packaging cell line.

Production of amphotropic retrovirus

Retrovirus was produced as previously described (Gilham *et al.* 2002). Briefly, rKat vector DNA (13.2 µg) or 30 µg of pKatp21^{CIP1} or pKatp27^{KIP1} was transfected into 293T cells by a modified calcium phosphate transfection method. Serum-free DMEM containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid was adjusted to pH 7.1. DMEM with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 10% foetal calf serum was adjusted to pH 7.9. For transfection of 293T cells, DNA was added to 1 mL of pH 7.1 medium with 50 mM CaCl₂ and was incubated at room temperature for 10–30 min. 293T cells (at 50–60% confluence) cultured in a 10-cm culture dish were washed with serum free medium and 4 mL of pH 7.9 medium was added. The DNA/CaCl₂ mixture was added to the dish and left to incubate for 8–24 h. After removal of transfection medium, recombinant retrovirus was harvested at 48 and 72 h post-transfection and was then filtered through a 0.45-µm filter.

Retroviral transduction of MCF-10A cells and analysis fluorescence-activated cell sorting

Cells were trypsinized as described above, counted and plated at an approximate density of $1.3 \times 10^4/\text{cm}^2$ and were allowed to adhere overnight. Next day, medium was removed and cells were exposed to normal medium (untreated cells), rKat-GFP retrovirus (vector control) or rKat virus containing the coding sequence for p21^{CIP1} or p27^{KIP1} CDKIs. Virus was administered as a conditioned medium preparation containing 4 µg/mL hexadimethrine bromide (Sigma). Cells were exposed to virus overnight. Virus was then removed, and cells were washed with fresh medium and then were cultured for up to 3 days prior to fluorescence-activated cell sorting (FACS).

Retrovirally transduced cells were trypsinized and re-suspended in sterile phosphate-buffered saline (PBS) for either analysis or for sorting using a FACS Vantage cell sorter. The average percentage of GFP-positive cells in vector-treated cells was $72.9 \pm 5.1\%$. Transduction efficiency for the p21^{CIP1}/GFP construct was $22.9 \pm 6.8\%$ transduction efficiency for the p27^{KIP1}/GFP construct was $7.5 \pm 4.4\%$ ($n = 5$ for all sorts). GFP-positive cells were separated from untransduced cells and were plated into either chamber slides for immunohistochemistry, 96-well plates for growth assays or 35 mm dishes for culture prior to RNA extraction.

Analysis of p21^{CIP1} and p27^{KIP1} protein by Western blotting

Retrovirally transduced cells were FACS sorted for GFP positivity and in the case of p21^{CIP1}/p27^{KIP1} transduced cells were sorted into low and high GFP populations. Control (non-transduced cells) and GFP-positive transduced cells were lysed (50 mM Tris-HCl, 150 mM NaCl, 1% v/v non-idet P40, 2 mM ethylenediaminetetraacetic acid, 10 mM NaF with protease inhibitor cocktail; Roche, Nutley, NJ, USA), then 25 µg of total cellular protein was boiled in Laemmli sample buffer for 2 min and separated on a 10% (w/v) acrylamide gel. Proteins were transferred to nitrocellulose and were probed with a mouse monoclonal anti-p21^{WAF1} (clone HJ21, Neomarkers, Fremont, CA, USA), rabbit anti-p27^{KIP1} (Neomarkers) at the manufacturer's recommended dilutions. Bands were visualized by probing with appropriate horse radish peroxidase-coupled secondary antibodies (Dako, Ely Cambridgeshire) utilizing SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA). Images were acquired using an Epi Chem Darkroom (Jencons, Leighton Buzzard, Bedfordshire, UK) and Labworks 4 software (UVP, Upland, CA, USA).

Three-dimensional culture

Cells were trypsinized, FACS sorted in the case of retrovirally transduced cells, counted and plated onto a layer of low growth factor Matrigel in 8-well chamber slides at a density of 5000

cells/well, as previously described (Muthuswamy *et al.* 2001; Debnath *et al.* 2002). Cells were cultured for up to 12 days. At regular intervals, cells were processed for immunocytochemical studies. Media were removed from each well, the cell/Matrigel samples were washed twice in PBS, then fixed in 3.7% paraformaldehyde for 15 min at room temperature, or overnight at 4 °C and were washed twice in PBS, then permeabilised in methanol for 4 min at 4 °C. Cells were washed twice in PBS and the cell/Matrigel sample was cut from the slide and embedded in 2% high-melting-point agarose. Embedded samples were mounted in wax blocks and 3 µm sections were cut for immunocytochemistry.

Immunocytochemistry

Sections cut from samples mounted in wax blocks were dewaxed in xylene and were re-hydrated through graded alcohols to PBS; antigens were retrieved by pressure cooking for 100 s at full pressure in 10 mM citric acid (pH 6.0). For whole cell staining in chamber slides, cells were washed with PBS then fixed in 3.7% paraformaldehyde for 15 minutes at room temperature, followed by 4 min in methanol at -20 °C and 2 min in acetone at -20 °C. Samples were blocked with 10% non-immune rabbit serum, then were incubated with primary monoclonal antibodies against p21^{CIP1} (1 : 25 dilution, clone SX118), p27^{KIP1} (1 : 20 dilution, clone SX53G8), Ki67 (1 : 50 dilution, clone MIB-1), ERα (1 : 33 dilution, clone 1D5), PR (1 : 33 dilution, clone PGR636) or adipophilin (1 : 20 dilution, clone AP125; Progen Biotechnik GmbH, Heidelberg, Germany) for 60 min. Negative controls were carried out for each antigen using non-immune mouse serum at the same dilution as the antibody used. Samples were then probed with a biotinylated rabbit or mouse antibody (1 : 300 dilution) for 40 min, followed by streptavidin-peroxidase complex (Standard ABC Elite, Vector Laboratories, Peterborough, UK) for 35 min. Staining was visualized by using the chromogenic substrate diaminobenzidine. After washing, tissues were counterstained with haematoxylin, dehydrated and mounted in XAM neutral mounting medium.

Dual immunofluorescence

Sections were initially treated as described above for immunohistochemistry but they were incubated with a rabbit polyclonal primary antibody against one antigen after blocking with 10% non-immune goat serum. The first antigen was visualized using a FITC-conjugated goat antirabbit secondary antibody. Sections were then blocked a second time with 10% non-immune goat serum and then were incubated with a monoclonal antibody against the second antigen. The second antigen was visualized with a TRITC-conjugated goat antimouse secondary antibody. Sections were mounted in Vectashield mounting medium for fluorescence microscopy (Vector Laboratories). Primary antibodies used were polyclonal rabbit antihuman Ki67 (1 : 125 dilution, Novocastra, Newcastle upon Tyne, UK), monoclonal mouse antihuman p21^{CIP1} (1 : 25 dilution, clone SX118) and p27^{KIP1} (1 : 125 dilution, clone SX53G8). Secondary antibodies used were FITC-goat antirabbit (Sigma F-9887), diluted 1 : 25 and TRITC-conjugated goat antimouse IgG1 (Santa Cruz sc-2084, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), diluted 1 : 100. Several high power microscope fields were used to quantify the colocalization of p21^{CIP1} and Ki67 at days 1 and 3 of acini developments by counting the number of cells that were single and double stained by each antibody.

Lipid staining

FACS-sorted transduced cells were plated into chamber slides and were allowed to adhere overnight, prior to staining for lipids with the neutral lipid specific Oil-Red-O staining technique. After fixation in 4% (w/v) formalin for 15 min, the cells were washed and stained

with 0.3% (w/v) Oil Red O in 60% isopropanol for 10 min. Colour was developed with 60% isopropanol, counterstained with haematoxylin and eosin and specimens were mounted in an aqueous mountant. To calculate the average number of droplets/cell, droplets larger than $\sim 1 \mu\text{m}$ were counted in a representative high power field of control or transduced cells. Increased expression was assessed by greater than 20 droplets ($> \sim 1 \mu\text{m}$) being present in more than half of the cells in each culture chamber.

Sulphorhodamine B growth assay

Cells were transduced with retrovirus and were FACS sorted as described above, then were plated into 96-well plates in triplicate at a density of 1000 cells/well and were cultured for up to 12 days in serum free culture medium (DMEM/F12 with epithelial growth factor (10 ng/mL), hydrocortisone (1 $\mu\text{g/mL}$), cholera toxin (1 $\mu\text{g/mL}$), insulin (10 $\mu\text{g/mL}$), penicillin (100 $\mu\text{g/mL}$) and streptomycin (100 $\mu\text{g/mL}$). Total cell protein was analysed at days 1, 3, 6, 9 and 12 by sulphorhodamine B assay as previously described (Robson *et al.* 1998).

Reverse transcriptase-polymerase chain reaction

GFP-positive FACS-sorted cells were plated into 35 mm dishes and were cultured for 2 days in the presence or absence of 3 $\mu\text{g/mL}$ ovine prolactin. They were then treated with TRIzol reagent (Gibco BRL, Grand Island, NY, USA) to extract RNA, according to the manufacturer's instructions. RNA was quantified by spectrophotometry at 260 nm. Before reverse transcription, 1 μg of total RNA from each sample was DNase treated in a 10- μL reaction for 30 min at 37 °C using a DNA-freeTM kit (Ambion, Huntingdon, Cambridgeshire, UK). DNase treated RNA was then divided into two tubes, each containing approximately 500 ng of RNA and reverse transcribed using TaqMan[®] reverse transcription kit (Applied Biosystems, Branchburg, NJ, USA) in a 20- μL reaction. One tube was incubated without *Taq* as a negative control for each sample.

cDNA produced by the reverse transcription was amplified by PCR, using forward and reverse primers specific to the sequence. Primers for reverse transcriptase-polymerase chain reaction (RT-PCR) were designed against coding sequences for human α -lactalbumin; human β -casein; human oestrogen receptor α (ER); human progesterone receptor (PR) and the house-keeping gene, human acidic ribosomal phosphopeptide p0 (ARPP0) all were synthesized by MWG Biotech AG (Ebersberg, Germany). The primers were tested for sequence specificity using plasmids containing the appropriate coding sequences. For amplification of α -lactalbumin cDNA, the forward primer 5'-AAAATGAGGTTCTTTGTC-3' and reverse primer 5'-CATAT-TCCTGTGCTTTCATTG-3' were used. For β -casein RT-PCR, the forward primer 5'-GCAG-CAAGGAGAGGATGA-3' and reverse primer 5'-GGTATCGTTGGAGATTAAAG-3' were used. For ER α RT-PCR, the forward primer was 5'-CCCTACTGCATCAGATCCAA-3' and reverse primer was 5'-CCTGGCGTCGATTATCTGAA-3'. For PR RT-PCR, the forward primer was 5'-TTTCATCCACGTGCCTATCC-3' and reverse primer was 5'-TCCGCTTTGTACAGGAT-GCA-3'. For ARPP0 RT-PCR, the forward primer was 5'-TGGAAGTCCAACTACTTCT-3' and reverse primer was 5'-TGGAAAAAGGAGGTCTTCTC-3'. Twenty-five microlitres amplification reactions were carried out using the JumpStartTM REDTaqTM ReadyMixTM PCR reagent (Sigma) following the manufacturers instructions. RT-PCR was also carried out to verify expression knockdown of p21^{CIP1} and p27^{KIP1} by siRNA using the following primers. For p21^{CIP1}, the forward primer was 5'-GACTGTGATGCGCTAATGG-3' and reverse primer was 5'-CTTCCTCTTGGAGAAGATCA-3'. For p27^{KIP1}, the forward primer was 5'-AACGTGCGAGTGTCTAACG-3' and reverse primer was 5'-TCCGACGGAT-CAGTCTTTG-3'.

Assessing the effects of CDKI overexpression and knockdown on acinus formation

Cells were retrovirally transduced with either p21^{CIP1} or p27^{KIP1}, sorted for GFP by FACS and GFP-positive cells plated onto Matrigel. The number and size of acini formed were measured at regular intervals. To achieve knockdown of gene expression, we screened six commercially available siRNA duplexes for each target gene using the siPort transfection reagent (Ambion). Pilot experiments were performed in MCF-7 cells grown in monolayer to optimize transfection conditions for p21^{CIP1} and p27^{KIP1} directed siRNA, with varying concentrations of siRNA and siPort transfection reagent. We chose MCF-7 cells for optimization of siRNA dosing as they express endogenous p21^{CIP1} and p27^{KIP1} in monolayer, whereas MCF-10A cells only expressed p21^{CIP1} and p27^{KIP1} when cultured on Matrigel. Successful and specific knockdown was assessed by RT-PCR for both CDKIs and a house-keeping gene (as described above). p21^{CIP1} and p27^{KIP1} siRNAs knocked down the expression of p21^{CIP1} and p27^{KIP1}, respectively, with no effects on each other or the house-keeping gene ARPP0. Protein levels were predicted to be reduced as both p21^{CIP1} and p27^{KIP1} have a rapid turnover, p21^{CIP1} being regulated transcriptionally and p27^{KIP1} being regulated mainly by proteolysis. To examine the effects of siRNA targeting p21^{CIP1} or p27^{KIP1}, we transfected MCF-10A cells grown in monolayer using concentrations of the most effective siRNA duplex that caused specific knockdown in MCF-7 cells. siRNA concentrations used were 30 nM (6 : 1 ratio with siPort) for p21^{CIP1} and 30 nM (12 : 2 ratio with siPort) for p27^{KIP1}. Cells were then trypsinized and plated onto Matrigel as described above. Diameter and number of acini formed was measured every 3 days prior to re-application of fresh siRNA to maintain exposure of cells to siRNA duplexes for prolonged knockdown of CDKI expression.

Statistical analysis

Measurements are shown as the mean $\pm$ the standard error of the mean (SEM) and were compared using a two-tailed Student's *t*-test.

RESULTS

Virally transduced cells express functional CDKIs

Western blotting of rKat-p21^{CIP1} showed increased p21^{CIP1} protein levels (Fig. 1a) and cells transduced with rKat-p27^{KIP1} had highly elevated p27^{KIP1} levels (Fig. 1b) while in untransfected or rKat-GFP-transduced cells there was no detectable p21^{CIP1} or p27^{KIP1} protein. Immunocytochemical analysis showed nuclear staining for p21^{CIP1} in cells transduced with rKat-p21^{CIP1} (Fig. 1d) while cells transduced with rKat-p27^{KIP1} and stained for p27^{KIP1} immunoreactivity showed very strong p27^{KIP1} staining, both in the nucleus and the cytoplasm (Fig. 1f). The staining intensity for p27^{KIP1} in rKat-p27^{KIP1}-transduced cells mirrors the levels of protein detected by Western blotting and a flattened cell morphology with a large nucleus was observed. rKat-GFP-transduced cells were completely negative for p21^{CIP1} or p27^{KIP1} immunoreactivity (Fig. 1c,e, respectively). Transduction with p21^{CIP1} had no effect on p27^{KIP1} immunoreactivity and *vice versa* (results not shown).

To assess functionality of exogenous CDKIs, transduced MCF-10A cells were sorted for GFP expression by FACS, were plated into 96-well plates and were cultured for up to 12 days. Control cells and cells transduced with GFP alone (empty vector) exhibited normal growth, entering log phase at day 3 and reaching a plateau by day 9. Cells transduced with p21^{CIP1} or p27^{KIP1} showed significantly retarded growth compared to GFP-transduced cells at day 6

Figure 1. Ectopic CDKI expression decreases proliferation. Proteins were extracted from FACS-sorted cells. Twenty-five micrograms of total proteins were separated on a 10% acrylamide gel and stained for either p21^{CIP1} (a) or p27^{KIP1} (b) immunoreactivity. Lanes are: non-transduced cells (lane C), GFP-vector transduced cells (lanes 1 and 3), p21^{CIP1}/GFP-transduced cells (lane 2) and p27^{KIP1}/GFP-transduced cells (lane 4). Cells were transduced with either empty vector (c and e), pKat-p21^{CIP1} (d) or pKat-p27^{KIP1} (f) and stained for either p21^{CIP1} (c and d) or p27^{KIP1} (e and f) immunoreactivity. (g) FACS-sorted transduced cells were plated into 96-well plates and cultured for up to 12 days. Cell protein was stained with sulforhodamine B and optical density measured at 540 nm p21^{CIP1} (▲) and p27^{KIP1} (■) transduced cells showed retarded growth compared to control cells (◆) or vector treated cells (●). Results are expressed as average $\pm$ SEM.

(p21^{CIP1} versus GFP, $P = 0.043$; p27^{KIP1} versus GFP, $P = 0.014$) suggesting that GFP-positive CDKI-transduced cells expressed functional CDKIs. Cell number for CDKI-transduced cells eventually reached a plateau by day 12 (Fig. 1g). Expression of p21^{CIP1} or p27^{KIP1} would usually be associated with a complete block on entry into the cell cycle. However, we were still able to observe cell proliferation at a lower rate than by wild-type cells. This lead us to believe that there were some non-transduced cells contaminating the FACS-sorted cells, or that the sorted cells

were a heterogeneous population containing cells that express CDKIs at variable levels, leading to a spectrum of growth-regulatory effects. Transduced and FACS-sorted cells were therefore scored for GFP content after 9 days in culture using the conditions from the growth assays. In empty vector treated cells, the majority examined were GFP-positive ($84 \pm 7.6\%$). In p21^{CIP1}- and p27^{KIP1}-transduced cells, GFP positivity was much lower ($45 \pm 20\%$ and $34 \pm 5.6\%$, respectively), indicating that contamination by a small population of GFP-negative cells could be responsible for the growth rate observed. This would also explain the low transduction rates observed.

Transduction with CDKIs has no effect on steroid receptors or on milk protein RNA expression

Cells transduced with rKat-p21^{CIP1} or rKat-p27^{KIP1} were ER and PR-negative by immunocytochemistry (results not shown). Therefore, to verify the absence of any steroid receptors, we used semiquantitative RT-PCR to try to detect any induction of ER or PR (Fig. 2a). However, there was no change in mRNA levels in any of the virally transduced cells. Lack of induction of a functional ER protein in transduced cells was verified by the complete lack of mRNA for PR (Fig. 2a) as expression of PR is completely dependent on the presence of a functional ER (Clarke *et al.* 1997a,b).

To determine whether transduction with CDKIs caused the cells to develop a lactational phenotype, we used RT-PCR to detect mRNA for the milk proteins α -lactalbumin and β -casein in transduced cells that had been cultured in the presence or absence of prolactin ($3 \mu\text{g/mL}$). No induction of α -lactalbumin or β -casein expression by CDKI transduction was detected (Fig. 2b), even in the presence of prolactin. Normal breast was used as a positive control sample.

CDKI transduction stimulates accumulation of neutral lipids

Staining of neutral lipid droplets with Oil Red O showed little staining in control cells (mean 1.4 droplets/cell; Fig. 2c) or cells transduced with empty vector (mean 2.1 droplets/cell; Fig. 2d). In CDKI-transduced cells sorted for GFP, exogenous p21^{CIP1} (mean 14.8 droplets/cell; Fig. 2e) and p27^{KIP1} (mean 21.8 droplets/cell; Fig. 2f) stimulated a marked accumulation of lipid droplets. The effects of different levels of p27^{KIP1} expression were visible on lipid staining, where transduced cells sorted for low or high GFP (result not shown) showed increasing lipid content as p27^{KIP1} increased. Cells were also stained with an antibody to adipophilin, a protein that is associated with lipid droplets in lactating mammary epithelium (Heid *et al.* 1998; Schwertfeger *et al.* 2003). However, compared to normal breast, no adipophilin immunoreactivity could be seen in CDKI-transduced cells (data not shown).

Temporal regulation of p21^{CIP1} and p27^{KIP1} expression in three-dimensional cultures of MCF-10A cells on BM

A variety of studies has shown that BM plays a crucial role in regulation of gene expression by breast epithelial cells (Streuli *et al.* 1991; Novaro *et al.* 2003). Thus, we examined the effects of culturing untransduced MCF-10A cells on Matrigel. A single cell suspension of MCF-10A cells seeded onto Matrigel-formed multicellular acini over a period of 12 days. The acini grew from single cells and over the course of 12 days formed large spherical structures over $100 \mu\text{m}$ across. By day 12, acini contained a hollow lumen (indicated by * in Fig. 3a) surrounded by a single cell epithelial layer. Immunostaining of these structures showed tightly regulated expression of CDKIs during development of acini. This is in contrast to monolayer culture, where little expression was seen. The proliferating cell marker, Ki67, was present at high levels, $49.95 \pm 33.35\%$ on day 1, gradually decreasing until days 9 and 12 where they reached an equilibrium

Figure 2. Ectopic CDKIs do not induce lactation-related genes. (a) RNA was extracted from non-transduced MCF-10A cells (Con), FACS-sorted cells transduced with GFP, p21^{CIP1} or p27^{KIP1}. Normal breast tissue was used as a positive control. RNA was reverse transcribed and the cDNA amplified by PCR using probes for human oestrogen receptor- α (ER); human progesterone receptor (PR) and human acidic ribosomal phosphoprotein p0 (ARPP0) as a loading control. (b) RNA was extracted from non-transduced MCF-10A cells (Con), FACS-sorted cells transduced with GFP, p21^{CIP1} or p27^{KIP1}, after culture in the presence (+PRL) or absence of ovine prolactin (-PRL) (3 μ g/mL) for 2 days. Normal breast tissue was used as a positive control. RNA was reverse transcribed and the cDNA amplified by PCR using probes for human α -lactalbumin and human β -casein. Human acidic ribosomal phosphoprotein p0 (ARPP0) was used as a loading control. Untreated cells (c), vector-treated cells (d), p21^{CIP1}-transduced cells (e) or high pKat-p27^{KIP1}-transduced cells (f) were FACS-sorted, plated into chamber slides and cultured. Neutral lipids were stained with Oil Red O.

Figure 3. Basement membrane induces tightly controlled CDKI expression. (a) Untransduced MCF-10A cells were cultured on Matrigel for up to 12 days formed acini. These structures were fixed and sectioned at regular intervals and examined for p27^{KIP1}, Ki67 or p21^{CIP1} immunoreactivity. One thousand cells were scored for staining positivity in triplicate and results expressed as average percentage of positive cells $\pm$ SEM. Results are p27^{KIP1} (■) (b), Ki67 (■) and p21^{CIP1} (▲) (c). (d) Representative photomicrographs showing colocalization of Ki67 and p21^{CIP1} MCF-10A cells were cultured on Matrigel, fixed and sectioned as described in Materials and Methods. Sections were subjected to dual immunofluorescence for p21^{CIP1} (red) and Ki67 (green). Nuclei were counterstained with DAPI (blue). Dual-labelled cells are indicated by arrows.

Figure 4. Ectopic CDK1 expression inhibits acinus formation. FACS-sorted MCF-10A cells transduced with GFP-containing vector (solid bar), p21^{CIP1} (shaded bar) or p27^{KIP1} (open bar) were cultured on Matrigel as described in Materials and Methods. The number (a) and diameter (b) of acini formed were assessed at days 6 and 12 during culture. Results are expressed as percentage GFP control $\pm$ SEM. Statistical significance was determined by Student's *t*-test, with significance being indicated by a *P*-value (*P* = 0.05). Significance was determined between GFP transduced and p21^{CIP1} and p27^{KIP1} (indicated by *) or p21^{CIP1} and p27^{KIP1} (indicated by †).

of 6.3% to 3.45% (Fig. 3a,c). This is in contrast with p27^{KIP1} immunoreactivity, where p27^{KIP1} was initially undetectable and was only observed from day 3 onwards at low levels ($2.29 \pm 1.91\%$) with a gradual increase up to day 12 ($13.87 \pm 11.85\%$) (Fig. 3a,b). Therefore, as acinus growth decreased, Ki67 staining decreased and p27^{KIP1} increased. Unexpectedly, p21^{CIP1} levels were initially high, being expressed in $44.65 \pm 17.85\%$ of cells at day 1, although by day 6, this had decreased to $17.55 \pm 0.25\%$. After day 6, p21^{CIP1} immunoreactivity remained at a relatively constant level of 4.15% to 2.15% (Fig. 3a,c). Dual immunofluorescence for p21^{CIP1} and Ki67 showed colocalization of the two antigens at days 1 and 3 (marked with arrows), after which only Ki67 fluorescence was visible (Fig. 3d). On average, at days 1 and 3, 43% of p21^{CIP1}-positive cells were proliferative (Ki67-positive) and 75% of proliferative cells contained p21^{CIP1}.

FACS-purified rKat-GFP, rKat-p21^{CIP1} and rKat-p27^{KIP1} cells were also plated onto Matrigel to assess whether overexpression of CDKIs throughout formation of acini would have any effect on acinus formation. At days 6 and 12 of culture, the number of acini formed and the diameter of acini was measured in nine randomly selected fields of view in three separate experiments. By day 6, a statistically significant decrease in the number of acini formed was seen with both p21^{CIP1} (43% reduction) and p27^{KIP1} (57% reduction) (Fig. 4a), compared to vector-transduced cells. This reduction remained at similar levels (46% and 63%, respectively) at day 12, indicating that overexpression of p21^{CIP1} or p27^{KIP1} decreases the ability of MCF-10A cells to form acini. Additionally, transduction with p21^{CIP1} or p27^{KIP1} caused a statistically significant decrease in the size of acini formed by MCF-10A cells compared to vector-transduced cells (Fig. 4b). At day

6, the difference between p21^{CIP1} and p27^{KIP1} was statistically significant ($P = 0.003$), with p27^{KIP1} causing a greater decrease in acinar diameter. However, by day 12, p21^{CIP1} and p27^{KIP1} caused a similar decrease in acinar diameter to each other (41% and 44%, respectively). This suggests that p27^{KIP1} overexpression is able to cause an earlier arrest of cells undergoing acinar development compared to p21^{CIP1}.

Effects of knockdown of p21^{CIP1} and p27^{KIP1} on acinus formation

Transfection conditions for CDKI siRNAs were optimized using the MCF-7 cell line, which, in contrast to MCF-10A cells grown in monolayer (see Fig. 1c,d), expresses endogenous p21^{CIP1} and p27^{KIP1} protein. Cells were exposed to 10, 30 or 60 nM siRNA with the manufacturer's recommended concentration of siPort. Specific knockdown was seen at concentrations from 10 to 60 nM for p21^{CIP1} and 30–60 nM for p27^{KIP1} knockdown (Fig. 5a). Transfection efficiency was assessed using a FITC-tagged scrambled siRNA sequence, which indicated that up to 90% of both MCF-7 and MCF-10A cells were transfected (results not shown). MCF-10A cells were transfected with either p21^{CIP1} or p27^{KIP1} siRNA to cause persistent knockdown of CDKIs, plated onto Matrigel and size and number of acini measured at 3-day intervals. At day 3, cells transfected with p21^{CIP1} or p27^{KIP1} siRNA showed an increase in the percentage acinus formation after knockdown by siRNA compared to the scrambled control (Fig. 5b,c, respectively). As the experiment progressed (day 9), the percentage acinus formation after p21^{CIP1} knockdown returned to control levels (Fig. 5b). However, the cells with p27^{KIP1} knockdown showed increased acinus formation (Fig. 5c). When measuring the size of acini formed, cells treated with p21^{CIP1} siRNA showed an initial increase in the size of acini formed that was statistically significant (Fig. 5d). p27^{KIP1} knockdown had no effect on acinus size during the early stages of formation, as the experiment progressed, the acini formed showed a statistically significant decrease in size compared to control (Fig. 5e).

DISCUSSION

MCF-10A cells were retrovirally transduced to overexpress p21^{CIP1} and p27^{KIP1}. Transduced cells were purified by FACS for GFP and functionality of transduced CDKIs was demonstrated by reduced growth rates compared to control cells. CDKI expression, particularly p27^{KIP1}, increased nuclear size and caused a granular appearance of the cytoplasm. Expression of CDKIs induced a partial lactational differentiation as shown by accumulation of neutral lipid droplets. However, there was no induction of milk proteins in transduced cells, even when cultured in the presence of prolactin. Untransduced MCF-10A cells cultured on BM rich in fibronectin and laminin showed tightly regulated time-dependent expression of p21^{CIP1} and p27^{KIP1} during formation of acinar structures. For example, Ki67 was prevalent during the early phases of acinus formation while p27^{KIP1} staining increased at later stages as acini increased in size and slowed in their growth. Surprisingly, p21^{CIP1} was highest in the earliest stages of acinus growth, and was present in proliferating cells as demonstrated by colocalization in dual Ki67/p21^{CIP1} immunofluorescence. Overexpression of p21^{CIP1} or p27^{KIP1} combined with BM decreased the ability of MCF-10A cells to form acini, and any acini formed were significantly smaller than those formed by vector-treated cells. In contrast, knockdown of CDKIs by siRNA increased the number of acini formed, but had little effect on size.

The lag in growth rates of CDKI-transduced cells grown as monolayer is most likely due to functional expression of CDKIs. However, this inhibition of growth appears to be lost by day

Figure 5. siRNA-mediated knockdown of CDKIs alters acinus formation. (a) Optimization of siRNA knockdown using MCF-7 cells. Cells were treated overnight with increasing concentrations of p21^{CIP1} siRNA or p27^{KIP1} siRNA in the presence of siPort transfection reagent (Ambion) at a ratio of 6 : 1 (ng siPort: ng siRNA). RNA was extracted from cells and subject to RT-PCR for either p21^{CIP1}, p27^{KIP1} or the house-keeping gene ARPP0 as a loading and specificity control. Specific knockdown of both p21^{CIP1} and p27^{KIP1} could be observed using 30–60 nM siRNA (b–d). Effects of CDKI directed siRNAs on acinus formation by MCF-10A cells. Cells were transfected with control siRNA (solid bars) or 60 nM siRNA for p21^{CIP1} (b and d) or p27^{KIP1} (c and e) and plated onto Matrigel as described in Materials and Methods. The number (b and c) and diameter (d and e) of acini formed were assessed at days 3 and 9 during culture. Results are expressed as percentage control siRNA transfected cells $\pm$ SEM. Statistical significance was determined by Student's *t*-test, with significance being indicated by a *P*-value (*P* = 0.05) (indicated by *). Significance was determined between control siRNA transfected and p21^{CIP1} or p27^{KIP1} siRNA.

12 of culture. Fluorescence microscopy showed the presence of GFP-negative cells in the FACS-sorted population used for growth assays. Therefore, transduced and FACS-sorted cells were scored for GFP positivity after 9 days in culture, revealing the presence of a high percentage of GFP-negative cells that had outgrown the transduced ones. Therefore, the apparent loss of CDKI action, with respect to growth inhibition in CDKI-transduced MCF-10A cells, may be due to outgrowth of a contaminating population of untransduced cells. It has also been suggested that CDKIs may have cell cycle-independent roles including regulation of apoptosis, cell migration and acting as transcriptional co-factors (Bastians *et al.* 1998; Steinman *et al.* 2001) any of which may account, at least partially, for the changes observed. However, results obtained from cell growth assays and acinus-formation assays suggest that ectopic expression of CDKIs does have an inhibitory effect on the cell cycle.

Transduced cells showed no induction of ER expression, although mRNA for ER was seen at the same level of expression in all MCF-10A samples, including non-transduced cells, despite exhibiting no ER immunoreactivity. The expression of PR is dependent on the action of oestrogen (Cho *et al.* 1991; Clarke *et al.* 1997a) and the complete absence of PR expression in the presence of ER message suggests that a functional ER protein is not induced by CDKI expression. However, it has been reported that a subclone of MCF-10A cells exhibits spontaneous expression of ER with an increase in p21^{CIP1} when cultured under certain conditions (Lane *et al.* 1999), which may explain the presence of ER mRNA. The absence of ER protein may be due to a lack of BM, as primary cells grown on tissue culture-treated plastic ware rapidly lose the ability to express steroid receptors, whereas cells grown on BM continue to express ER and PR (Xie & Haslam 1997; Novaro *et al.* 2003). The lack of induction of differentiation markers may also be due to the effects of long-term cell culture on the MCF-10A cell line, possibly causing genetic alterations, such as loss of p16^{INK4a} (Huschtscha *et al.* 1998). This may cause a selective pressure, favouring the growth of a population of differentiation resistant cells.

Basement membrane also plays a key role in regulating the lactational differentiation of breast epithelial cells (Streuli *et al.* 1991; Streuli *et al.* 1995; Edwards *et al.* 1998). Therefore, we examined the effects of the commercially available BM, Matrigel, which is rich in laminin and fibronectin as is the BM of mammary terminal duct lobular units. A single-cell suspension of MCF-10A cells cultured at clonal density in Matrigel forms hollow spherical structures analogous to secretory alveoli or acini (Soule *et al.* 1990; Tait *et al.* 1990; Muthuswamy *et al.* 2001; Debnath *et al.* 2002). When MCF-10A acinus sections were subject to immunocytochemical analysis for Ki67 (growth), p21^{CIP1} and p27^{KIP1} expression, we found tightly regulated expression of CDKIs. During the early phases of acinus formation, Ki67 levels were high, indicating that cells were undergoing proliferation. As the size of acini increased the proliferation decreased to a low, relatively constant level. This has been previously described for Ki67 in MCF-10A Matrigel cultures (Debnath *et al.* 2002), where after 15 days in culture, the acini reach an optimum size that is maintained by a constant turnover of cells. This is due to a constant low level of proliferation, balanced by apoptosis. As expected for a cell cycle inhibitor, p27^{KIP1} was undetectable during periods of cell proliferation with no detectable immunoreactivity before day 3. Thereafter, p27^{KIP1} levels increase as Ki67 immunoreactivity decreases. This increase in p27^{KIP1} levels may play an anti-apoptotic role, promoting cell survival in growth-arrested cells as demonstrated by the role of p16^{INK4a}, p21^{CIP1} and p27^{KIP1} in anoikis resistance (Collins *et al.* 2005).

Surprisingly, p21^{CIP1} expression was initially high and colocalized with the proliferative cell marker, Ki67. Both antigens decreased in levels with a similar time-course. This may be explained by several reports (LaBaer *et al.* 1997; Sherr & Roberts 1999; Weiss *et al.* 2000),

which suggest that p21^{CIP1} may act as an assembly factor for cyclin/CDK complexes, facilitating the activation of CDKs and allowing for entry into the cell cycle. The ability of p21^{CIP1} to act as both an inhibitor and initiator of the cell cycle seems paradoxical. It has been suggested that this may be due to phosphorylation of p21^{CIP1} by Akt (Rossig *et al.* 2001). Phosphorylation of p21^{CIP1} at threonine 145 prevents proliferating cell nuclear antigen binding to p21^{CIP1}, attenuating CDK-p21^{CIP1} complex formation and promoting proliferation. It should also be noted that p21^{CIP1} and Ki67 immunoreactivity in these acini never totally disappears. The presence of heterogeneous p21^{CIP1} staining within the nucleus may be due to its possible role in regulating gene expression (Coqueret 2003).

We find that p21^{CIP1} is present in proliferating cells and we suggest that these cells may be functioning as a progenitor-like subpopulation of MCF-10A cells. These cells may undergo regular divisions to replace cells lost by apoptosis. These proliferative p21^{CIP1}-positive cells may be analogous to stem-like or progenitor cells that have been demonstrated in cell lines (Singh *et al.* 2003). p21^{CIP1} has been used as a marker for stem cells (Topley *et al.* 1999; Cheng *et al.* 2000b; Clarke *et al.* 2005), which adds weight to the theory that the p21^{CIP1}-containing MCF-10A cells in acini may be stem-like cells. It would also be interesting to investigate whether p16^{INK4a} levels are elevated at any point during acinus formation, as it has been shown in fibroblasts that senescence induces a transient increase in p21^{CIP1} followed by an increase in p27^{KIP1} (Alcorta *et al.* 1996), which is a similar temporal pattern to that observed during culture on Matrigel.

To demonstrate the importance of the timing of CDKI expression we transduced p21^{CIP1} or p27^{KIP1} into cells and cultured them on Matrigel where seeding of single-cell suspensions led to the growth of clonal colonies, forming acini. We observed that there was a reduced rate of acinus formation in transduced cells, coupled with a decrease in diameter of acini that could be observed throughout the culture period. This effect was greater with exogenous p27^{KIP1} than p21^{CIP1}, suggesting that expression levels of CDKIs is critical for controlling the formation of acini. In wild-type cells, the timing of CDKI expression is strictly regulated during development of acini; however, in retrovirally treated cells, expression of CDKIs is continuous and will therefore be able to inhibit cell proliferation from the outset of acinus formation, which may in turn lead to impaired formation of acini. Conversely, siRNA-mediated knockdown of p21^{CIP1} throughout the experiment may cause an initial increase in the potential of cells to form acini. This may be due to the removal of the check on proliferation of the progenitor population within the MCF-10A cell line. This effect may be similar to the effects seen in keratinocytes that have lost p21^{CIP1}, where growth conditions favour a subpopulation of cells that is more proliferative, multipotent and exhibits clonal growth (Topley *et al.* 1999). Knockdown of p27^{KIP1} throughout the experiment caused an increase in acinus formation; however, immunohistochemistry demonstrated the absence of p27^{KIP1} immunoreactivity during the early stages of acinus formation in untransduced cells. This apparent increase in the number of acini formed may be due to an increase in progenitor cells derived from stem-like cells as described in p27^{KIP1} deficient haematopoietic cells (Cheng *et al.* 2000a). In this report, p21^{CIP1} regulates stem cell proliferation while p27^{KIP1} regulates the proliferation of precursor cells. Once the inhibitory effect of p27^{KIP1} is removed by siRNA transfection these cells are able to proliferate and form acini, increasing the efficiency of acini formation. This highlights the importance of precise control of CDKI expression during formation of acini by MCF-10A cells. Our findings contradict those in p27^{KIP1}(-/-) mice (Muraoka *et al.* 2001) that show hypoplasia, low proliferation, decreased ductal branching and impaired lobuloalveolar differentiation of mouse mammary gland and those that show no noticeable effect of p27^{KIP1} deficiency on breast development (Davison *et al.* 2003).

We conclude that retroviral transduction of the CIP/KIP CDKIs, p21^{CIP1} and p27^{KIP1} into MCF-10A monolayer cultures is unable to induce a differentiated, secretory, luminal epithelial phenotype. However, CDKIs are able to stimulate an accumulation of neutral lipids, indicating a step towards lactational differentiation. We demonstrated that BM plays an important role in controlling endogenous expression of p21^{CIP1} and p27^{KIP1}, although it is possible that the changed regulation is simply a function of being grown in three-dimension rather than two-dimension. MCF-10A cells cultured on Matrigel demonstrated very specific temporal control of p21^{CIP1} and p27^{KIP1} expression during acinar structure formation. As demonstrated by expression of exogenous CDKIs or siRNA-mediated knockdown, altering levels or timing of expression can affect formation of acini. This suggests an important role for CDKIs during formation of the mature human mammary gland during periods of mammary epithelial growth, such as puberty, menstrual cycles and pregnancy. These results serve to highlight the important role that BM signalling plays in the control of cell proliferation and differentiation by altering expression of cell-cycle regulatory proteins.

Increased expression of p21^{CIP1} or p27^{KIP1} is able to induce partial secretory differentiation of MCF-10A cells cultured as a monolayer. When cultured on BM they form acini and exhibit a tightly regulated temporal expression pattern, where p21^{CIP1} may be a marker for proliferative progenitor cells and p27^{KIP1} is expressed in more terminally differentiated cells. The timing and expression levels are crucial for normal development of acini and may be important in promoting terminal differentiation of normal breast tissue.

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