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In Vitro Differentiation of HT-29 M6 Mucus-Secreting Colon Cancer Cells Involves a Trychostatin A and $p27^{KIP1}$-Inducible Transcriptional Program of Gene Expression

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Tumor cell dedifferentiation—such as the loss of cell-to-cell adhesion in epithelial tumors—is associated with tumor progression. To better understand the mechanisms that maintain carcinoma cells in a differentiated state, we have dissected in vitro differentiation pathways in the mucus-secretor HT-29 M6 colon cancer cell line, which spontaneously differentiates in postconfluent cultures. By lowering the extracellular calcium concentration to levels that prevent intercellular adhesion and epithelial polarization, our results reveal that differentiation is calcium-dependent and involves: (i) a process of cell cycle exit to G_0 and (ii) the induction of a transcriptional program of differentiation gene expression (i.e., mucins MUC1 and MUC5AC, and the apical membrane peptidase DPPIV).
In calcium-deprived, non-differentiated postconfluent cultures, differentiation gene promoters are repressed by a trichostatin A (TSA)-sensitive mechanism, indicating that loss of gene expression by dedifferentiation is driven by histone deacetylases (HDAC). Since TSA treatment or extracellular calcium restoration allow gene promoter activation to similar levels, we suggest that induction of differentiation is one mechanism of HDAC inhibitor antitumor action. Moreover, transcriptional de-repression can also be induced in non-differentiating culture conditions by overexpressing the cyclin-dependent kinase inhibitor $p27^{KIP1}$, which is normally induced during spontaneous differentiation. Since $p27^{KIP1}$ downregulation in colon cancer is associated with poor prognosis independently of tumor cell division rates, we propose that $p27^{KIP1}$ may prevent tumor progression by, at least in part, enhancing the expression of some differentiation genes. Therefore, the HT-29 M6 model allows the identification of some basic mechanisms of cancer cell differentiation control, so far revealing HDAC and $p27^{KIP1}$ as key regulatory factors of differentiation gene expression.
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In the intestine, epithelial stem cells are located at the base of the Lieberkühn crypts and give rise to several differentiation cell types by means of a process of migration upwards the villi. In the colon, a similar process of cell differentiation appears to take place. One of the earliest events of differentiation is the inhibition of cell proliferation by exit from the stem cell microenvironment of the crypts. Several transcription factors have been found involved in defining the diverse differentiated cell lineages of the epithelium (reviewed in Sancho et al., 2003). The transcription factor TCF-4, which forms heterodimers with its coactivator β-catenin, is required to promote the expression of genes essential for the maintenance of the stem cell compartment (Korinek et al., 1998; Sancho et al., 2003). In turn, β-catenin also participates in E-cadherin-mediated homophylic cell-to-cell adhesion by regulating its interaction with the actin cytoskeleton (Nelson and Nusse, 2004). The exit from the stem cell compartment implies the activation of genes coding for differentiation proteins such as brush border-associated enzymes and mucins, which are repressed by β-catenin/TCF-4 in the stem cell compartment (van de Wetering et al., 2002).
Loss of epithelial cell differentiation characteristics is associated with tumor progression. Particularly, epithelial-to-mesenchymal transition (EMT) is a process involved in the emergence of more invasive and metastatic tumor cell variants (Thiery, 2002). Reactivation of the β-catenin/TCF-4-dependent gene expression program in colon tumors—that is, dedifferentiation into a stem cell-like phenotype—is thought to participate in EMT and invasiveness (Morin et al., 1997). Moreover, the alteration of certain

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adhesion molecules appears as a determinant event during epithelial tumor dedifferentiation and EMT (Savagner, 2001). For example, E-cadherin membrane expression is commonly lost in cancer cells acquiring invasive properties (Christofori and Semb, 1999). Moreover, both in vitro and in vivo evidence point to E-cadherin as a pivotal mediator of cell cycle exit in differentiating epithelial cells (Hermiston et al., 1996; St. Croix et al., 1998). The antiproliferative effects of E-cadherin may, in part, be explained by its capacity to form complexes with β-catenin, thus competing with TCF-4 and interfering with β-catenin nuclear function (Orsulic et al., 1999; Gottardi et al., 2001; Stockinger et al., 2001). $p21^{CIP1}$ and cyclin D1, as targets of the β-catenin-TCF4 pathway (van de Wetering et al., 2002; Wu et al., 2003), might be among the proteins ultimately affected by E-cadherin to restrain cell division. Moreover, the cyclin-dependent kinase inhibitor $p27^{KIP1}$ has also been shown to be a major cell cycle target activated by E-cadherin in HT-29 colon cancer cells, a pathway likely mediated by PI3k (St. Croix et al., 1998). It is thus plausible that both cell cycle inhibitors, $p21^{CIP1}$ and $p27^{KIP1}$, contribute to the cell cycle exit in the differentiating epithelium in response to differentiation signals.

The relationship between cell-to-cell adhesion—and, in general, the process of epithelialization—and the differentiation gene expression programs is an issue largely unexplored. An in vitro model of cancer cells that has provided important clues on the mechanisms required for the differentiation of cancer cells is that of the HT-29 colon cancer cell line. While HT-29 cells display limited differentiated features in culture—particularly, they lack cell polarization in membrane domains and only express low levels of some intestinal markers—cell populations isolated by their capacity of survival to several types of stress reproducibly activate differentiation programs of the enterocytic or mucus-secretor phenotypes. Among the best characterized models, acquired resistance to the antimetabolite drug methotrexate yields HT-29 cell populations that form polarized columnar-like cell monolayers after achieving high density culture confluency (Lesuffleur et al., 1990, 1991, 1998). Specifically, cell selection by low doses of methotrexate, such as 1 μM, gives rise to the emergence of mucus-secretor populations while step-wise increases in drug concentration to 0.1 or 1 mM lead to the selection of enterocytic populations. To date, the mechanistic relationship between cell survival to drug-induced stress and the capacity to activate differentiation programs remains unknown.

We have focused on the in vitro differentiation of HT-29 M6 cells, previously isolated by their resistance to 1 μM methotrexate. These cells display a mucus secreting phenotype characterized by the synthesis of several brush-border associated enzymes (dipeptidyl-peptidase IV, APN), secreted mucins (MUC5AC, MUC6), and a brush-border-associated MUC1 transmembrane mucin form (Lesuffleur et al., 1990, 1993; Fabre et al., 1999; Gouyer et al., 2001; Leteurtre et al., 2004). Differentiated HT-29 M6 cells also display polarized apical and baso-lateral membrane domains and distinct intercellular junctional complexes. By using the method of extracellular calcium shift to synchronize cell-to-cell adhesion, we have characterized the kinetics of epithelialization and differentiation gene expression and dissected some of the regulatory pathways involved in the activation of the differentiation program. First, cell-to-cell adhesion was strongly associated with phenotypic features of differentiation except for the formation of mucus vesicles, which appeared as cell adhesion-independent. Dedifferentiation by calcium deprivation did not involve the activation of the β-catenin/TCF-4 pathway but was mediated, at least in part, by a trichostatin A (TSA)-sensitive repressive mechanism on differentiation gene promoters. Second, the results also revealed a closed molecular link between cell cycle regulation and differentiation, such that, despite alterations of the cancer cell cycle, retention of $p27^{KIP1}$ inducibility during epithelialization was causally associated with the induction of the transcriptional program of differentiation gene expression. Further analysis of this model should lead to a better comprehension of the cellular functions required to maintain the differentiation status of cancer cells.

Materials and Methods

Cell culture

HT-29 M6 cells were obtained by step-wise treatment of HT-29 colon carcinoma cells with 0.1 and 1.0 μM methotrexate (Lesuffleur et al., 1990). Cells were routinely passaged every 2–3 days by seeding at 2×10^5 cells/cm^2. These cultures reached culture confluence after 3 days of seeding, approximately. Cultures were maintained in D-MEM (Gibco—Invitrogen S.A., Prat de Llobregat, Spain) supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin. To culture cells in the presence of low Ca^{2+} concentrations, calcium-free D-MEM was supplemented with FBS partially depleted of Ca^{2+} by the use of the cationic exchange Chelex® resin according to manufacturer's instructions (Amersham—GE Healthcare, Buckinghamshire, England). Trichostatin A (Sigma, St. Louis, MO) was diluted in ethanol and added to the cells at a concentration of 5 μM overnight when indicated.

Cell cycle analysis

Cell suspensions were obtained by treating cell cultures with trypsin. Flow cytometry analysis of the DNA content per cell was performed by staining ethanol-fixed cell suspensions with propidium iodide. To label the fraction of cells replicating DNA, 5-bromo-2′-deoxyuridine (BrdU) was added to cultures at a concentration of 10 μM as a 1 h pulse (except for Fig. 2F, as detailed therein) and detected by inmunofluorescence on ethanol-fixed cell suspensions using the monoclonal antibody clone 3D4 (Pharmingen—BD Biosciences, Franklin Lakes, NJ).

Western blotting

Cell extracts were obtained by scraping PBS-washed cultures, pelleting cells, and resuspending in lysis buffer (50 mM Tris-Cl pH 7.4, 5 mM EDTA, 250 mM NaCl, 0.1% Triton X-100, 50 mM NaF, 0.1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin). Pocket proteins were resolved by long run SDS–polyacrylamide gel electrophoresis (SDS–PAGE) to optimize resolution of phosphorylated forms as described (Mayol et al., 1995). Proteins were electro-transferred to nitrocellulose membranes, and immunoblots performed using the following antibodies: monoclonal anti-$p27^{KIP1}$ clone 57 (Transduction—BD Biosciences, Franklin Lakes, NJ), monoclonal anti-$p21^{CIP1}$ clone Ab-1 (Calbiochem—EMD Biosciences, San Diego, CA), monoclonal anti-pRB clone G3-245 (BD Pharmingen), and rabbit polyclonal anti-p130 (Santa Cruz Biotechnologies, Santa cruz, CA). Immunolabeled membranes were revealed by a horseradish peroxidase-conjugated second antibody and the enzyme chemiluminescence ECL kit (Amersham Pharmacia Biotech).

Northern blotting

Total RNA was isolated using acid guanidinium thiocyanate–phenol–chloroform (Chomczynski and Sacchi, 1987). RNA was fractionated by agarose gel electrophoresis and transferred to nylon membranes using standard procedures. Membranes were prehybridized using ExpressHyb Hybridization solution (Clontech—BD Biosciences, Franklin Lakes, NJ), and α-^{32}P-dCTP-labeled probes added to this solution for 1 h at 68°C. Probes were obtained as 300–400 bp fragments from restriction enzyme digestions of the human cDNAs coding for MUC1, MUC5AC, or DPPIV.

DNA constructs

All reporter constructs were subcloned in the pGL3 vector (Promega, Madison, WI) that contains the firefly luciferase gene as a reporter. Fragments −756/+49 of the human MUC1 promoter (Guaita et al., 2002) and −4,000/+68 of the human MUC5AC promoter (Li et al., 1998) were donated by J. Baulida and F. Ulloa, respectively. Fragment −307/+14 of the human DPPIV promoter (Erickson et al., 1999) was obtained by PCR amplification of HT-29 M6 genomic DNA using the sense 5′-TCA GAG CTC GGC CGG GAT

GCC AGT G-3′ and antisense 5′-CCG CTC GAG CGG CAC TCA CCT TCA T-3′ primers. The 2,400 base-pair $p21^{CIP1}$ gene promoter subcloned in pGL3 (Prabhu et al., 1997) was given by A. Rodolosse.

Transfections and luciferase reporter analysis

Cells were cultured in 24-well plates for variable periods of time as indicated in the text and transfected with 1 μg of DNA using the Lipofectamine reagent according to manufacturer's instructions (Gibco—Invitrogen S.A., Prat de Llobregat, Spain). Cells were harvested and lysed for subsequent analysis with the luciferase reporter analysis kit according to manufacturer's instructions (Promega). pGL3-firefly luciferase activities were normalized to renilla luciferase activity from pRSV added as 10 ng/sample in each transfection. Transfections were performed in duplicates, and results shown are the average ± mean error of at least three independent experiments.

Results

HT-29 M6 in vitro cell differentiation is dependent on extracellular calcium

HT-29 M6 cells spontaneously differentiate to acquire an epithelial mucus-secreting phenotype at culture postconfluence (Lesuffleur et al., 1990). To characterize this in vitro differentiation process, we have used a high cell density seeding procedure that allows the synchronous induction of differentiation, as reported previously (Navarro et al., 1999). Transmission electron microscopy analysis of differentiating cultures revealed that a cell multilayer was formed after 9 days of seeding (Fig. 1A), with the upper cell layer displaying some differentiation features (junctional complexes and mucus vesicles at the apical cytoplasm) (Fig. 1B,C). We have defined this condition as an early stage of in vitro differentiation. By extending cell culture to 2–3 weeks, scattered areas of this multilayer re-organized into a more compact columnar cell monolayer, and these areas progressively spread to encompass the entire culture (Fig. 1D).

Since epithelialization (i.e., development of intercellular junctions and cellular polarization) is dependent on extracellular calcium-mediated adhesion (Gumbiner et al., 1988; Grindstaff et al., 1998), we assessed the effects of partially depleting extracellular calcium on the differentiation phenotype of HT-29 M6 cells. As reported in other epithelial cell culture models (Duden and Franke, 1988), we found that depletion of calcium in medium to approximately 100 μM—which will be referred to as low-calcium medium (LCM) in contrast to the standard medium containing 2 mM calcium—suppressed intercellular adhesion as seen by the isolated appearance of cells and by the absence of junctional complexes at the ultrastructural level (Fig. 1E,F). The normal basolateral membrane distribution of E-cadherin and β-catenin was also lost by LCM culture (Fig. 1G). These effects were reversible since shifting the cultures to standard medium led to a membrane re-distribution of E-cadherin and β-catenin within 24 h (Fig. 1G). Together, these results illustrate that the early

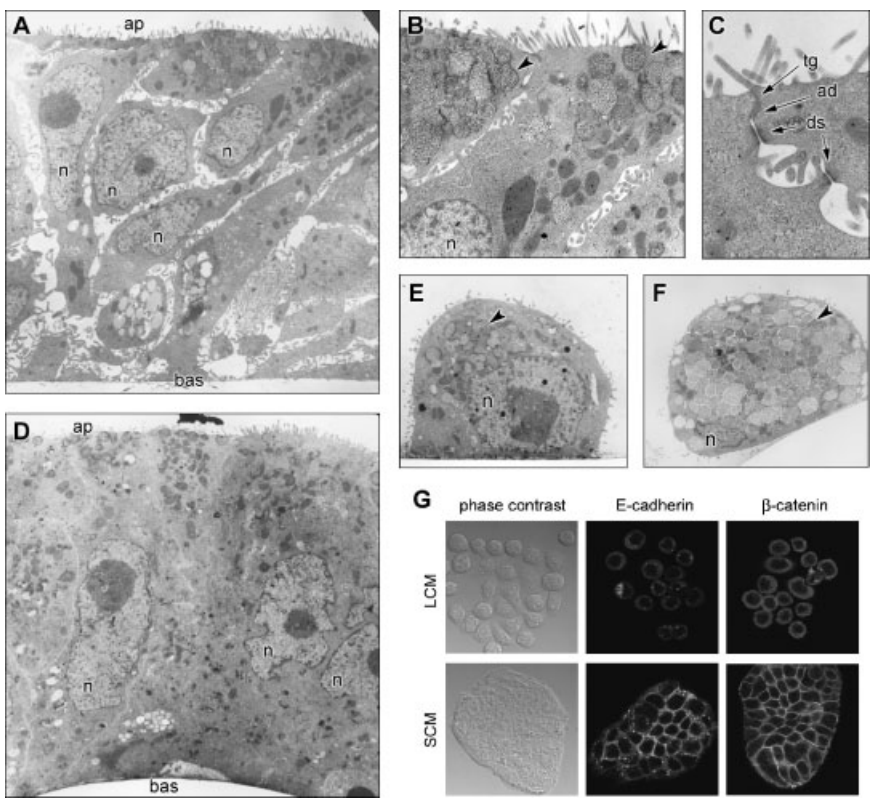

Fig. 1. Early and late stages of HT-29 M6 in vitro differentiation are suppressed by LCM culture conditions. Electron microscopy analysis of postconfluent HT-29 M6 cell layers perpendicular to the substrate plane was performed on samples taken at 9 (A, B, and C) or 21 (D) days after seeding in standard medium, and at 9 (E) or 21 (F) days after seeding under LCM medium. Note upper cells of the cell multilayer shown in A displaying differentiated features, which include mucus vesicles in the apical cytoplasm (B) and intercellular junctional complexes (C). In D, differentiation has proceeded to the formation of a columnar cell monolayer with closer lateral contacts between cells. Intercellular adhesion and membrane domain polarization are lost under LCM culture conditions (E and F). *ap*, apical; *bas*, basal; *n*, nucleus; *tg*, tight junction; *ad*, adhesion junction; *ds*, desmosome. G: phase contrast microscopy and inmunofluorescence for the detection of E-cadherin or β-catenin in HT-29 M6 cell colonies grown under LCM conditions (LCM) and after 24 h of a calcium-shift to SCM conditions (SCM).

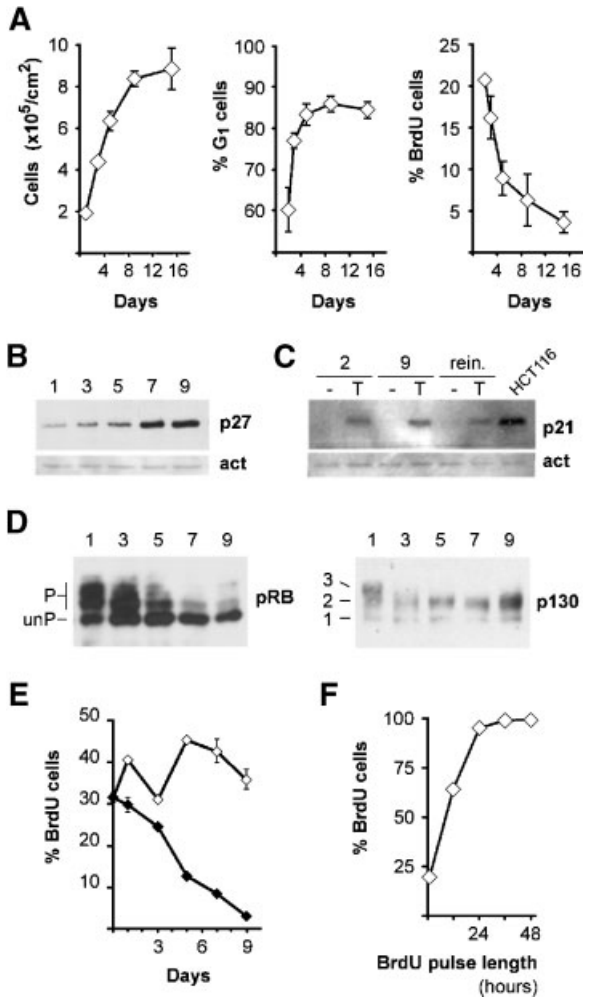

Fig. 2. In vitro differentiation involves cell cycle exit to G_0, which is suppressed by inhibition of epithelialization. A: cell proliferation kinetics of SCM cultures seeded at 50% confluence and growing to postconfluence during the early stage of differentiation; cell suspensions were obtained at the indicated days of culture and analyzed to calculate the cell density by cell counting (left), the percentage of cells with a G_0/G_1 DNA content (center), and the percentage of cells labeled by a 1 h BrdU pulse prior harvesting (right). B: Western blot analysis on samples taken during the time course experiment shown in A revealed that the CDK inhibitor $p27^{KIP1}$ was induced during differentiation (*p27*) with respect to normalization using actin antibodies (*act*). C: On the contrary, Western blot with $p21^{CIP1}$ antibodies (*p21*) showed that its basal expression was almost undetectable and was not induced during differentiation; cultures were grown for the indicated days and treated overnight (*T*) or not (−) with 5 μM TSA before harvesting; *rein.*, HT-29 M6 cells reinitiated in the cell cycle by low density seeding after culture confluence; these results demonstrated that $p21^{CIP1}$ was strongly responsive to its known activator TSA at any stage of the differentiation process, but was unable to be induced spontaneously; a lane containing a cell lysate from a confluent culture of the non-differentiating HCT116 colon cancer cell line was included as an instance of $p21^{CIP1}$ basal expression under conditions of cell quiescence (*HCT116*). D: As in *B*, using antibodies that detect all the phosphorylated forms of p130 and pRB; inhibition of cell proliferation by SCM culture involved protein phosphorylation patterns characteristic of cell cycle exit to G_0 as observed by the disappearance of p130 phosphorylated form 3 (3) and the accumulation of p130 form 2 (2), while the phosphorylated forms of pRB (*P*) in proliferating cultures gave rise to mostly unphosphorylated pRB (*unP*) in 9-day differentiated cultures. E: Time course of 1 h-BrdU pulse labeling during culture under LCM conditions (*white diamonds*) or after shifting the cultures to standard culture conditions (*black diamonds*); time *0* refers to LCM cultures grown to confluence for 5 days. F: Cumulative BrdU labeling in LCM cultures under the continuous presence of BrdU up to 48 h, showing that almost 100% of the cells are continuously cycling in the absence of epithelialization.

stage of HT-29 M6 differentiation involves the formation of intercellular junctions and membrane polarization, which are dependent on the presence of extracellular calcium. It should be noted that, despite the suppression of epithelialization, LCM culture did not completely abrogate the formation of cytoplasmic mucus vesicles (Fig. 1E). These vesicles were still detected in some LCM-cultured cells, although they were less abundant than in standard cultures, and accumulated evenly distributed in the cytoplasm to become a common feature in long-term LCM cultures (Fig. 1F). Therefore, part of the differentiation program of HT-29 M6 can proceed in the absence of epithelialization.

HT-29 M6 in vitro cell differentiation involves cell cycle exit to G_0

We found that the growth rate of HT-29 M6 cells decreased progressively during the early stage of in vitro differentiation, reaching a plateau after 9 days of seeding (Fig. 2A). Growth arrest involved the accumulation of cells with a G_0/G_1 DNA content and a decrease in the percentage of cells incorporating BrdU (Fig. 2A). Moreover, cell growth arrest was characterized by the induction of the cyclin-dependent kinase inhibitor $p27^{KIP1}$ (Fig. 2B) and by hypophosphorylation of pRB and accumulation of p130 forms 1 and 2 (Fig. 2D), which are the pocket protein phosphorylation patterns characteristic of exit to G_0 (Grana et al., 1998). Therefore, the early stage of HT-29 M6 differentiation involves cell cycle exit apparently to the G_0 quiescence state. It is worth noting that $p21^{CIP1}$, another CDK inhibitor, was not induced during HT-29 M6 differentiation although it is typically induced in the differentiating epithelia of the intestine and colon (Fig. 2C).

In agreement with the known role of intercellular adhesion on contact inhibition, cell cycle exit was suppressed by LCM culture as observed by a steadily elevated fraction of cells incorporating BrdU in the postconfluence phase of culture (Fig. 2E). Noteworthy, suppression of intercellular adhesion permitted the whole cell population to cycle continuously since almost 100% of the cells incorporated BrdU within 36 h of cumulative labeling (Fig. 2F). Comparison of cell cycle synchronization under standard and LCM culture did not reveal any change in the cell division time nor any sign of loss of cell viability by the deprivation of calcium (data not shown). As with intercellular adhesion and polarization, shifting the culture conditions to standard culture restored cell cycle exit (Fig. 2E). Therefore, cell cycle exit to G_0 is closely related with the calcium-dependent differentiation program.

Differentiation marker gene expression is triggered by the calcium-dependent differentiation program

Differentiated HT-29 M6 cells express a set of genes characteristic of an epithelial, mucus-secreting phenotype (Lesuffleur et al., 1990, 1993; Fabre et al., 1999; Gouyer et al., 2001). We thus analyzed the expression kinetics of differentiation markers during the early stage of differentiation. The mRNA levels of MUC1 and DPPIV, two apical membrane proteins, and the secreted mucin MUC5AC were upregulated 7–9 days after seeding at high cell density (Fig. 3A). By contrast, undifferentiated HT-29 cells expressed only low levels of DPP-IV mRNA and undetectable levels of MUC1 and MUC5AC transcripts (Fig. 3A). Therefore, the gene expression program specific of HT-29 M6 is induced during the early stage of in vitro differentiation.

We also analyzed whether the induction of these differentiation markers depended on extracellular calcium. Northern blot

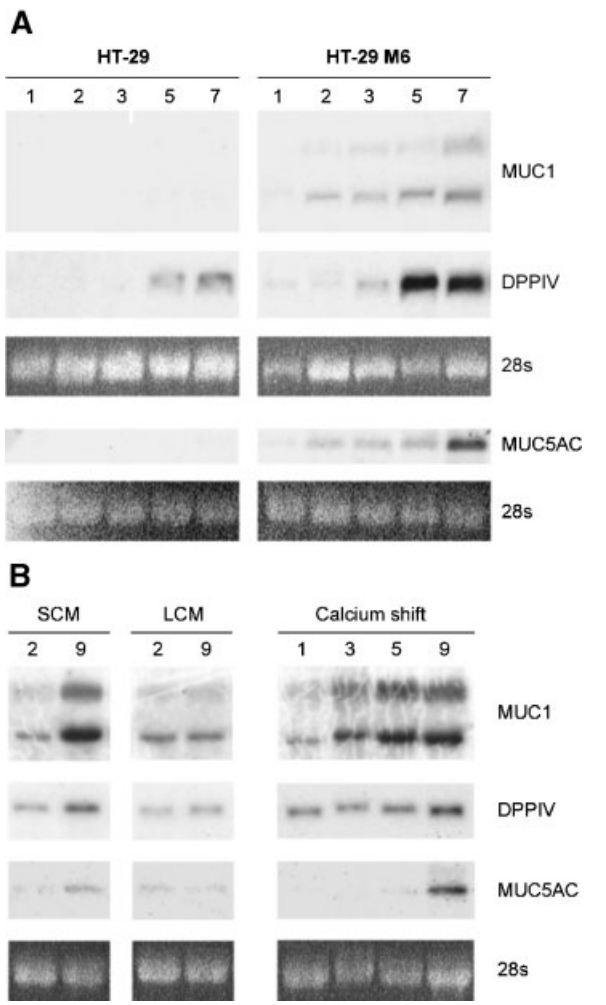

Fig. 3. Differentiation gene expression is induced during the early stage of differentiation and is suppressed by LCM culture. A: Total RNA was isolated during the experiment shown in Figure 1A (days 1, 2, 3, 5, and 7), including undifferentiated parental HT-29 cells and differentiating HT-29 M6 cells, and Northern blot analysis was performed using cDNA probes for the detection of the differentiation genes MUC1, DPPIV, and MUC5AC as indicated. B: Northern blot analysis, as in A, was performed from HT-29 M6 cell lysates at 2 and 9 days after seeding (2 and 9) and cultured under LCM or standard culture conditions (LCM and SCM, left parts); confluent LCM cultures were treated with 2 mM calcium chloride the fifth day after seeding (Day 0), and Northern blot was performed on samples harvested throughout the following days (1, 3, 5, and 9; *calcium shift*, right part). Ethidium bromide-stained 28s rRNA transferred on the nitrocellulose membranes is shown as a normalizer.

analysis showed that the mRNA induction observed during the early stage of HT-29 M6 differentiation was suppressed when cell growth to culture confluence was performed under LCM conditions (Fig. 3B). Again, LCM effects were reversible as restoring standard culture conditions in LCM cultures led to a marked upregulation of MUC1 and MUC5AC mRNA levels (Fig. 3B). DDPIV mRNA levels were induced to a lesser extent, possibly due to the presence of its higher basal expression levels prior the calcium shift. Therefore, the induction of differentiation gene expression is also dependent on extracellular calcium, but the constitutive basal expression of DPPIV appears to be calcium-independent.

The calcium-dependent differentiation gene expression program is regulated at the transcriptional level

To characterize the mechanisms regulating the induction of differentiation and their dependence on extracellular calcium, we first assessed whether the HT-29 M6 differentiation gene expression program was regulated at the transcriptional level. The results shown above provided a synchronous method to induce the calcium-dependent differentiation program of HT-29 M6 cells by shifting a homogeneously proliferating LCM culture to standard medium. This is in contrast with prior studies in which a detailed analysis of the activation of the differentiation program was not possible due to asynchrony in the establishment of cell–cell contacts. In this manner, cells cultured to confluence for 5 days under LCM conditions (i.e., proliferating and undifferentiated) were transfected with plasmids containing the luciferase reporter gene under the control of the MUC1, MUC5AC, or DPPIV gene promoter fragments previously reported to drive the expression of their respective genes in epithelial cells (Kovarik et al., 1993, 1996; Erickson et al., 1999; Gaemers et al., 2001; Perrais et al., 2002). Afterwards, cell cultures were either maintained undifferentiated by culture in LCM or induced to differentiate by shifting to standard medium. The results showed that gene promoter activity did no vary substantially throughout the days following transfection when cells were grown under LCM conditions (Fig. 4A–C). On the contrary, the calcium-shift induced a gradual three- to sevenfold increase in transcriptional activity that peaked after 9 days of transfection (Fig. 4A–C). These increases in transcriptional activity were abolished when cycloheximide or actinomycin D were added to the cells for 48 h prior to harvesting (data not shown), indicating that reporter induction was due to increased promoter activity rather than to accumulation of either luciferase protein or mRNA, respectively. Therefore, differentiation gene expression is, at least in part, triggered by the transcriptional activation of differentiation gene promoters. It should be noted that, despite being analyzed by transient transfection, transcriptional activity assayed by the luciferase reporters could still be detected several days after transfection with reproducible results indicating that it is a method suitable for long-term kinetic studies on gene expression (see below).

Gene expression is repressed by a TSA-sensitive mechanism in the absence of calcium-dependent differentiation

To investigate the mechanism(s) responsible for the lack of promoter activation observed under LCM culture conditions, LCM cultures were treated with 5 μM TSA, an inhibitor of histone deacetylases (HDAC), for 15 h prior cell harvesting on day 9 after transfection. TSA provoked an increase in the activity of the three gene promoters in LCM cultures to levels similar to those reached after induction of promoter activity by the calcium-shift (Fig. 4D–F). Therefore, the HT-29 M6 gene expression program is silenced in the absence of calcium-dependent differentiation by HDAC-dependent transcriptional repression. It is worth noting that cells transiently transfected with the transcriptional reporters were still responsive to induction by TSA even several days after transfection, again enforcing the suitability of the luciferase reporter method in the study of gene expression kinetics.

$p27^{KIP1}$ is sufficient to induce the HT-29 M6 mucus-secreting gene expression program in the absence of calcium-dependent differentiation

The induction of p27 in the early stage of in vitro differentiation (Fig. 2B) paralleled the induction of HT-29 M6 gene expression, suggesting a coordinated regulation of cell cycle exit and expression of differentiation markers. In contrast, $p21^{CIP1}$ was

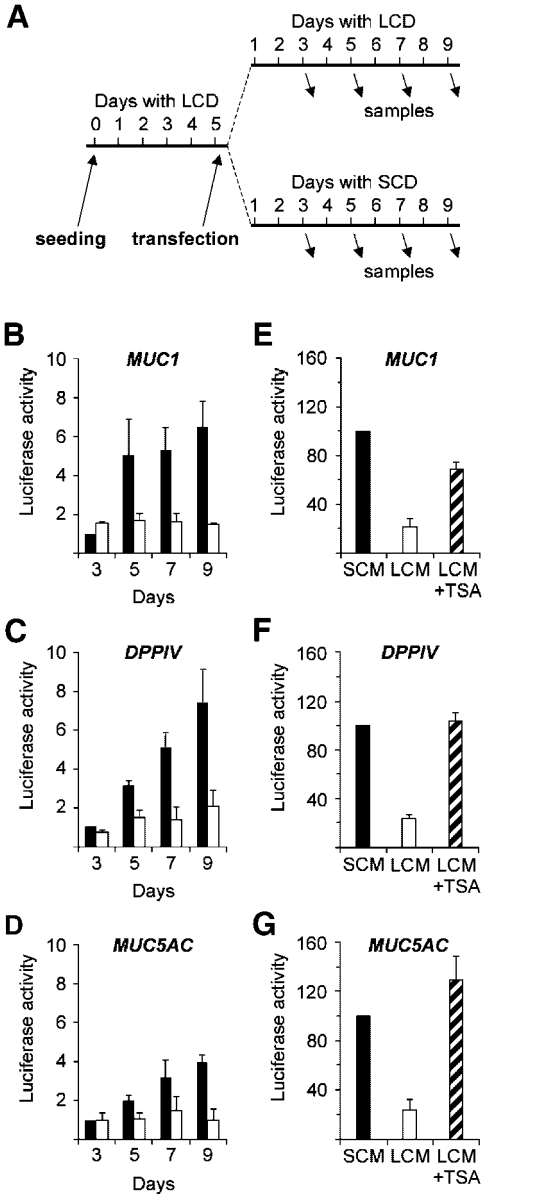

Fig. 4. Differentiation gene expression is transcriptionally regulated at the gene promoter level, and loss of epithelialization represses gene expression by a TSA-sensitive mechanism. Firefly luciferase expression vectors under the control of proximal gene promoters from MUC1 (A), DPPIV (B), and MUC5AC (C) genes were transfected on confluent LCM cultures the 5th day after seeding (Day 0), and were afterwards maintained under LCM conditions (*white bars*) or shifted to an extracellular calcium concentration of 2 mM (*black bars*); samples were taken during the following days (Days 3, 5, 7, and 9) to analyze gene promoter activity, and results are expressed as firefly luciferase activity relative to renilla luciferase activity from a cotransfected normalizer vector (see Materials and Methods section); luciferase activity under differentiating conditions at Day 9 was significantly increased respect to Day 3 and to LCM conditions at any day of the course according to ANOVA analysis and the LSD test ($P < 0.05$). D, E, and F, LCM cell cultures were treated overnight with 5 μM TSA (LCM + TSA, *cross-hatched bars*) or left untreated (LCM, *white bars*) at the 9th day after transfection as in A, B, and C, respectively; results are expressed as the percentage of relative luciferase activity respect to 100% activity in cultures grown under standard culture medium (SCM, *black bars*); the effect of TSA was significant according to the Mann–Whitney test ($P < 0.05$).

not induced during differentiation (see above). Since goblet cell differentiation is partially dependent on the integrity of $p21^{CIP1}$ function in APC-mutated mice (Yang et al., 2001), we hypothesized that $p27^{KIP1}$ induction during differentiation of HT-29 M6 could compensate for the loss of $p21^{CIP1}$ (e.g., triggering differentiation gene expression). To assess this possibility, the MUC1, MUC5AC, and DPPIV gene promoter reporter constructs were transiently co-transfected together with a $p27^{KIP1}$-expression vector. The results showed that $p27^{KIP1}$ increased the transcriptional activity of the reporters in LCM cultures to levels comparable to those reached under standard differentiation conditions (Fig. 5). Therefore, $p27^{KIP1}$ was sufficient to restore, at least in part, the gene transcription program in the absence of calcium-dependent differentiation. These results together point to $p27^{KIP1}$ as a key mediator of the early stage of in vitro differentiation.

Discussion

Our findings revealed that the expression of differentiation genes in the HT-29 M6 cell model takes place by transcriptional activation during a calcium-dependent, early stage of differentiation. Importantly, preventing epithelialization by lowering the extracellular calcium levels abolishes this gene expression program by means of an HDAC-dependent repression mechanism, thus revealing a novel tumor dedifferentiation mechanism. Moreover, the early stage of differentiation also involves the cell cycle exit into G_0, a hallmark of cell differentiation in most adult tissues, and includes the selective induction of the cyclin-dependent kinase inhibitor $p27^{KIP1}$, but not $p21^{CIP1}$. Interestingly, $p27^{KIP1}$ appears as a key transcriptional activator of differentiation gene expression suggesting that retention of its inducibility in tumors contributes to the maintenance of differentiated cancer phenotypes.

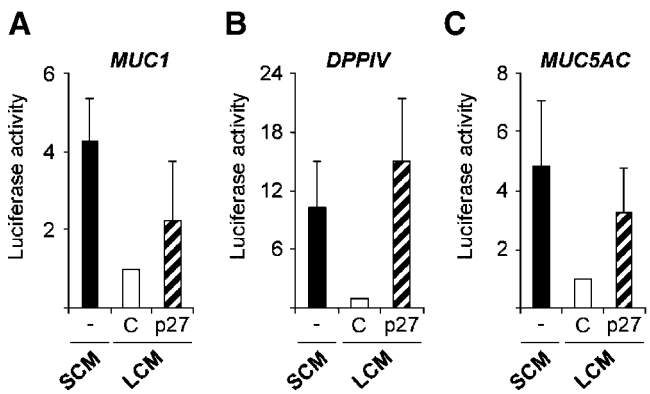

Fig. 5. Ectopic expression of $p27^{KIP1}$ derepresses differentiation gene promoter activities under non-differentiating culture conditions. Cell cultures were transfected as in Figure 4 except for that they were also cotransfected with a pCMV empty vector (C; *white bars*) or the vector driving constitutive expression of a $p27^{KIP1}$ cDNA (*p27*; *cross-hatched bars*); afterwards, cells were allowed to grow under LCM conditions for 9 days and analyzed for normalized luciferase activity. Cultures transfected with the reporter vector alone and grown under standard culture conditions (SCM, *black bars*) are shown as a reference. Results are expressed as the percentage of relative luciferase activity respect to 100% activity in cultures grown under LCM conditions; the effect of $p27^{KIP1}$ was statistically significant ($P < 0.05$, Mann–Whitney test).

The HDAC inhibitor TSA induces the expression of intestinal differentiation markers

There is increasing evidence that HDAC inhibitors are promising for the treatment of a wide variety of tumors (Villar-Garea and Esteller, 2004). The effects of HDAC inhibition are pleiotropic, including cytostasis and the induction of cell differentiation and/or apoptosis, and the molecular events responsible for their antitumor activity have not been completely elucidated (Johnstone and Licht, 2003; Marks et al., 2004). In this context, our results point to the regulation of a differentiation program as one possible mechanism by which TSA impairs tumor progression. Further identification of the molecules that mediate TSA effects in HT-29 M6 cells will reveal novel putative targets for the improvement of HDAC inhibitors as antitumor agents.

$p27^{KIP1}$ role in the control of colon cancer cell differentiation

$p27^{KIP1}$ is a critical regulator of the cell cycle by participating in the cell cycle progression halt induced by inhibitory signals of the G1 phase (reviewed in Sherr and Roberts, 1999). In particular, $p27^{KIP1}$ is typically induced by contact inhibition in a variety of cell types, including epithelial cells (St Croix et al., 1998). Accordingly, we also detected its induction during the early stage of HT-29 M6 in vitro differentiation (Fig. 2). The involvement of $p27^{KIP1}$ in colon tumor progression has been revealed in mice carrying the heterozygous or homozygous ablations of the $p27^{KIP1}$ gene (Moser et al., 1990; Fero et al., 1996; Philipp-Staheli et al., 2002; Yang et al., 2003). Moreover, downregulation of $p27^{KIP1}$ protein levels or its altered subcellular location is becoming a reliable prognostic predictor for advanced disease and poor patient survival in colorectal carcinoma (Liu et al., 1999; Sgambato et al., 1999; Hoos et al., 2002; Li et al., 2002; Hershko and Shapira, 2006, and references therein). Therefore, loss of $p27^{KIP1}$ function appears to play a major role during tumor progression.

$p27^{KIP1}$ alterations have not been found to be associated with increased proliferation of colon tumor cells (Palmqvist et al., 1999), suggesting additional—thus far largely unknown—roles for this protein in carcinogenesis. One possible mechanism has been provided recently by the finding that $p27^{KIP1}$ suppresses Rho A-dependent cell mobility, so that loss of $p27^{KIP1}$ function may favor the acquisition of invasive properties in tumors (Besson et al., 2004). Our results point to regulation of gene expression as an additional function of $p27^{KIP1}$ to promote tumor cell differentiation. The action of $p27^{KIP1}$ on intestinal gene expression in HT-29 M6 cells was revealed in calcium deprived, non-differentiating cells in which overexpression of $p27^{KIP1}$ proved sufficient to induce the activity of the three differentiation gene promoters analyzed. Since $p27^{KIP1}$ is spontaneously induced during differentiation, we propose that $p27^{KIP1}$ plays a role in the control of colon cancer cell differentiation by mediating the calcium-dependent gene expression pathway. Regulation of gene transcription by $p27^{KIP1}$ is not unprecedented as it has been recently reported that antisense oligonucleotides against $p27^{KIP1}$ mRNA downregulate several enterocytic differentiation markers in Caco-2 colon cancer cells without affecting the cell cycle exit by culture confluence (Deschenes et al., 2001). In this respect, we further show that $p27^{KIP1}$ is sufficient to induce the differentiation program under non-differentiated conditions, in addition to its requirement for the maintenance of differentiation reported previously. Our results also extend these data to the regulation of mucin genes (i.e., MUC1 and MUC5AC) that are differentiation genes characteristically expressed by colonic and other gastrointestinal malignancies. On the other hand, whether $p27^{KIP1}$ has also effects on parts of the differentiation program in addition to gene expression cannot be inferred from our results, and it is an issue that merits further investigation.

The observed effects of $p27^{KIP1}$ on differentiation might be the result of its function as a cell cycle protein, that is, by inducing cell cycle exit and by activating pocket proteins it might modulate indirectly the activity of differentiation regulators. Alternatively, it has been reported that $p27^{KIP1}$ regulates several transcription factors at the posttranscriptional level, for instance Sp1 (Wei et al., 2005), so that a similar mechanism may conceivably trigger differentiation gene expression in HT-29 M6 cells thus explaining, at least in part, its cell cycle-independent role in tumor progression. On the other hand, it has been proposed that $p21^{CIP1}$ mediates the cell cycle exit of differentiating enterocytes as part of the differentiation program triggered by the tumor suppressor APC along the intestinal crypt-villus axis (van de Wetering et al., 2002). However, our results showed that $p21^{CIP1}$ was not induced during HT-29 M6 in vitro differentiation suggesting that other cell cycle inhibitors were involved in cell cycle exit in these cells, for example, $p27^{KIP1}$. We also found that overexpression of wild type APC in HT-29 M6 led to $p21^{CIP1}$ transcriptional activation but only in cells that had undergone the in vitro differentiation process and not in proliferating cells (Mayo, Real and Mayol, unpublished results) suggesting that $p21^{CIP1}$ and $p27^{KIP1}$ govern complementary differentiation pathways. These results suggest that the control of differentiation gene expression by $p27^{KIP1}$ becomes critical for the differentiation of colon cancer cells when the APC/$p21^{CIP1}$ pathway cannot be normally induced.

Calcium-dependent and independent differentiation

Our findings suggest a relationship between adhesion contact formation and differentiation gene expression in tumor cells. Since E-cadherin also regulates $p27^{KIP1}$ protein levels (St. Croix et al., 1998), both molecules might link intercellular adhesion and cell cycle exit with differentiation gene expression within the same pathway. This possibility is particularly relevant as loss of E-cadherin function accompanies processes of tumor cell dedifferentiation, including epithelial to mesenchymal transition (Guilford, 1999). In this manner, loss of either E-cadherin or $p27^{KIP1}$ would contribute to cell dedifferentiation during tumor progression. On the other hand, the involvement of other calcium-dependent cellular molecules cannot be ruled out, for instance the recently described calcium sensing receptor which is associated with differentiation phenotypes in colon cancer (Chakrabarty et al., 2003). In this context, it would be of high interest to elucidate in detail the pathways linking the loss of epithelial integrity by LCM with the activation of the transcriptional differentiation program, particularly the pathway mediated by $p27^{KIP1}$.

The finding that part of the in vitro differentiation program occurred in the absence of epithelialization was striking. Particularly, mucus vesicles were still produced in LCM culture conditions with a rate notable low but sufficient to lead to their cytoplasmic accumulation after long-term culture (Fig. 1). Moreover, basal levels of DPPIV could be detected in non-differentiating conditions and even in the HT-29 parental cell line (Fig. 3), which is unable to form cell-to-cell adherent complexes and grows to confluence as a multilayer of mostly undifferentiated cells (Lesuffleur et al., 1990). In this respect, Western blot analysis of mucin protein levels revealed that, while paralleling the induction of mRNA levels during the process of differentiation, still constitutive low protein expression existed in non-differentiating conditions (data not shown). It is thus plausible that calcium acts as an inducer of differentiation—that is, triggering the formation of junctional complexes and inducing differentiation gene expression, while

other mechanisms yet to be ascertained are involved in maintaining the basal expression of some differentiation genes.

New insights into the mechanisms involved in colon cancer cell differentiation based on the HT-29 model

HT-29 cells display alterations typically found in most colorectal cancers, including APC and TP53 mutations. However, the β-catenin/TCF-4 pathway is hardly active in HT-29 despite its APC-altered status (Baulida et al., 1999). Similarly, a fraction of APC-mutated colon tumors do not display accumulation of nuclear β-catenin, a phenotypic marker of β-catenin/TCF-4 activation. In this respect, we also found that $p21^{CIP1}$, a dowstream effector of APC to exert cell cycle inhibition, was not induced during in vitro differentiation. Therefore, HT-29 M6 cells—and possibly, a fraction of colon tumors without β-catenin/TCF-4 activation—might display alterations in their differentiation pathways different from those that lead to stem cell-like phenotypes by β-catenin/TCF-4 activation. Moreover, it has been proposed for a long time that cancer cell phenotypes recapitulate ontogenic aspects of the cell type that undergoes transformation. We found that the process of in vitro differentiation in HT-29 M6 cells led to a multilayer of polarized cells after the early stage of differentiation, and this multilayer reorganized into a monolayer with extended culture (Fig. 1). This situation resembles the organization of the developing epithelium in the intestinal tract, where the formation of the crypt-villi structure and the colon crypts is preceded by a multilayered epithelium still lacking distinct cellular compartments (Arsenault and Menard, 1989). It is thus conceivable that the HT-29 M6 model also recapitulates some mechanisms of the differentiating epithelium at a stage preceding the formation of the β-catenin/TCF-4-regulated crypt-villus structure. Ascertaining this hypothesis, for example, by identifying whether tumors also display developmental patterns of differentiation gene regulation, may help to better define the cell differentiation pathways that operate in colon cancer cells.

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