

# Chronic acid exposure leads to activation of the *cdx2* intestinal homeobox gene in a long-term culture of mouse esophageal keratinocytes

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## Summary

To explore mechanisms whereby Malpighian keratinocytes can transdifferentiate into an intestinal-like epithelium, as observed in the early steps of Barrett's esophagus (BE) development, long-standing cultures of esophageal keratinocytes derived from normal mouse esophageal explants were developed. These cells were able to form multilayers and to differentiate on filter support by the formation of differentiated layers of basal cells (cytokeratine 14 positive) on which secondary suprabasal cell layers (cytokeratine 4 positive) spontaneously developed. Thus, these cultured cells, referred to as P3E6, reproduced, at least in part, the proliferation and stratification pattern existing in the normal esophagus. Because chronic exposure to acid pH is known to be a critical factor for BE development, culture medium at pH 3.5 was added into the apical chamber of cell cultures. This led to a decrease in the overall number of cells but it did not affect cell proliferation. Furthermore, external acid

environment triggered expression of the GFP reporter gene fused downstream of the *cdx2* intestinal homeogene regulatory sequences in P3E6 transfected cells. Expression of the endogenous CDX2 protein, detected by western blot and immunocytochemical analysis, correlated with promoter activation. These findings demonstrate that chronic exposure of esophageal keratinocytes to acid pH induces transcription of *cdx2*, an intestinal specific homeobox gene known to play a critical role in the differentiation and maintenance of intestinal epithelial functions. The results suggest that chronic acid exposure can modify the fate of P3E6 esophageal keratinocytes towards an intestinal program. This can be a key step in the development of intestinal metaplasia often observed in esophagus-cardia junction.

Key words: Esophagus, Keratinocytes, Intestinal metaplasia, Barrett's esophagus

## Introduction

The complex processes that direct cell-specific differentiation are not completely understood, but once the differentiation program is activated cells usually maintain their specific phenotype. However, in certain pathological conditions the epithelium undergoes changes that transform cells into different phenotypes (Tosh and Slack, 2002). The more evident example remains the pathological condition leading to metaplasia, in which the epithelium acquires a new phenotype (Slack, 1985; Slack, 1986; Tosh and Slack, 2002). Although metaplasia is frequently observed in a variety of pathological conditions, the transition that occurs in the progression from normal epithelium to metaplastic epithelium is still not well understood (Eguchi and Kodama, 1993; Slack and Tosh, 2001; Tosh and Slack, 2002). Histological analysis of metaplastic tissue and experimental induction of transdifferentiation of normal epithelia have shown that modification of the local environment may play a crucial role (Kerneis and Pringault, 1997; Gordon et al., 1997; Blau et al., 2001). Change in pH is one environmental factor that has been shown to have relevant effects on redirecting the genetic program (Kraus et al., 1994; Martin and Kurtz, 1993; Fitzgerald et al., 1997). In particular, an important role has been attributed to acidity in the

development of the intestinal metaplasia of the esophagus or Barrett's esophagus (BE) where the normal squamous epithelium of the lower esophagus is replaced by columnar epithelium, principally of intestinal type (Spechler and Goyal, 1996; Sharma, 1999; Jankowski et al., 2000). Although other pathological modifications such as chronic inflammation or continuous erosion of the Malpighian epithelium might be considered, acid reflux is widely assumed to be the main promoter of the intestinal development of Barrett metaplasia (Kumble et al., 1996; Ouatu-Lascar et al., 1999; Kumble et al., 1997; Reid, 1991). However, few studies have documented cellular responses to acid and the molecular pathways involved in these responses (Fitzgerald et al., 1996; Souza et al., 2001).

These studies have been partly hampered because of the absence of adequate culture models. Previous attempts have been restricted to explants culture of esophageal mucosa (Fitzgerald et al., 1996; Kaur et al., 2000) or short-term primary cultures (Compton et al., 1998; Banks-Schlegel and Harris, 1983; Banks-Schlegel and Green, 1981) limited by the rapid death of the culture or immortalized cell lines, where the genetic regulation is altered by the immortalization process (Rheinwald and Becket, 1980; Stoner et al., 1982; Stoner et al., 1991; Palanca-Wessels et al., 1998; Mothersill and Seymour,

1989). Recent studies have shown that animals such as mice, rats and rabbits can provide *in vivo* models for intestinal metaplasia of the esophagus (Vaezi et al., 1995; Goldstein et al., 1997; Ouatu-Lascar et al., 1999; Xu et al., 2000). Apart from the anatomic-pathologic observations and the evaluation of the severity of the lesions, the molecular mechanisms underlying the metaplastic process that take place in the esophagus cannot be investigated in these models. Histological studies do not provide a better understanding of the molecular and cellular processes leading to the syndrome and these models require complicated surgical manipulations.

Insight into the mechanisms that direct cellular changes to establish and maintain the metaplastic phenotype may be gained by studying, at the molecular level, the expression in the esophageal cells of key developmental genes such as the intestinal transcription factors that might be activated during the transdifferentiation process. Candidates that might play a function in triggering intestinal metaplasia are the rodent caudal-related *cdx1* and *cdx2* homeobox genes that encode transcription factors involved in intestinal cell differentiation and proliferation (Freund et al., 1992; James and Kazenwadel, 1991; James et al., 1994). *In vitro* and *in vivo* studies of *cdx2* homeobox gene expression have suggested that this transcription factor is important in the early steps of differentiation and maintenance of the intestinal cell phenotype (Suh and Traber, 1996; Troelsen et al., 1997; Mallo et al., 1997; Soubeyran et al., 1999).

In an attempt to investigate the role of acid pH in the intestinal transdifferentiation process of esophageal cells, we have chosen an approach consisting in the establishment of a model of normal, long-term culture of mouse esophageal keratinocytes in a double-chamber system. In this model, acid exposure effect on *cdx2* activation was investigated. Here, we show that low pH exposure of the apical compartment of the double-chamber culture was sufficient to induce expression of the specific intestinal transcription factor Cdx2 in differentiated mouse esophageal cells.

## Materials and Methods

### Tissue and mouse esophagus cultures

The esophageal epithelium was obtained from Balb/c mice. The esophagus of each single mouse was treated separately. Esophagus was removed and placed in DMEM/Ham's F12 medium (Gibco BRL) supplemented with 1% penicillin-streptomycin 250 µg/ml amphotericin. The esophagus was then opened longitudinally. The mucosa was separated from the muscularis externa by clamping forceps on the muscular edge and peeling off the mucosal layer. The recovered mucosal tissues were washed three times in supplemented DMEM/Ham's F12 medium and cut into sections of approximately 1.5×1.5 mm<sup>2</sup>. Tissue sections were transferred in 35-mm plastic petri dishes and incubated at 37°C. They attached to the plastic support after 4 to 7 days. Growing epithelioid cells surrounding the explants appeared after 2-3 days. Confluent cultures were reached 20 days after seeding. Subsequent subcultures were obtained after trypsinization and removal of remaining necrotic explants. A cell growth curve of cultures was then performed in triplicate and used to calculate the cell doubling time (see Results section). These cells deriving from one single mouse were referred to as P3E6. Growth curves were established on plastic supports or on filters of Transwell devices. Dead cells were estimated by the Trypan blue exclusion dye and the number of live cells was counted in a Malassez chamber. Epithelioid cells for long-term cultures could be obtained, thus, providing a permanent source of material.

### Cell culture

The mouse esophageal cells (P3E6) were routinely grown in DMEM/Ham's F12 medium, supplemented with 10% fetal bovine serum, 5 µg/ml transferrin, 5 µg/ml insulin, 1 nM triiodothyronine, 30 nM sodium selenite, 10 ng/ml epidermal growth factor (EGF), 0.5 mg/ml cholera toxin, 5 mg/ml hydrocortisone, 1% non-essential amino acids, 1% penicillin-streptomycin 250 µg/ml amphotericin and 7.5% bicarbonate, at 37°C in a 10% CO<sub>2</sub> atmosphere. Experiments were performed between passages 11 and 13 on cells grown on either plastic support, glass coverslips or permeable Transwell filters (6.5 mm or 24 mm insert diameter, 0.4 µm pore size; Costar, Cambridge, MA, USA). Caco2 TC7 cells (kindly provided by M. Rousset, INSERM, Paris) were grown in DMEM, 25 mM glucose medium (Gibco BRL) supplemented with 20% fetal bovine serum, 1% non-essential amino acids and 1% penicillin-streptomycin at 37°C in a 10% CO<sub>2</sub> atmosphere. Cells were passaged (1/10 dilution) just before they reached confluency (4-5 days) and medium was changed every two days.

### pH assay

For the pH assay, cells were seeded onto the porous filters of Transwell devices (Costar, Cambridge, MA, USA) at a concentration of 3×10<sup>4</sup> cells/mm<sup>2</sup>. Cells were cultured at pH 7.4 until day 16 (D 16) (when they reached confluency and differentiation) and then the medium equilibrated at pH 3.5 was introduced into the upper (apical) chamber of Transwell devices. D 16 was therefore considered as D+0 of the pH assay. For convenience, subsequent days during the pH assay were designed as D+'n'. DMEM/Ham's F12 complete medium at pH 3.5 was prepared by the addition of 0.1 N HCl in the proportion necessary to achieve the desired pH. In order to assure that any observed cellular change was not attributable to a change in medium osmolarity, control cells were grown in DMEM/Ham's F12 complete medium with an added volume of distilled water equivalent to the acid-treated medium. Sets of cultured cells from the same passage were grown either at pH 3.5 or at pH 7.4. After 2 days, three filters for each pH assay were fixed for immunocytological analysis (6.5 mm diameter) or used for western blot analysis (24 mm diameter).

### Immunocytochemistry

Epithelial cells grown on glass-slides or on porous filters were fixed in ice-cold methanol for 10 minutes, and labeled with mouse monoclonal anti-cytokeratin 4 (dilution 1/10; ICN Pharmaceuticals) and anti-cytokeratin 14 antibodies (dilution 1/10; ICN Pharmaceuticals). Antibodies were revealed by either FITC-conjugated anti-mouse Ig antibody (1/100 dilution; Amersham Pharmacia Biotech) or Cy3-conjugated anti-mouse Ig antibodies (1/100 dilution; Jackson ImmunoResearch). An Alexa 488 conjugated anti-fluorescein goat IgG fraction (Molecular Probes) was also used (30 minutes, dilution 1/50). Nuclear staining was performed by incubation with 0.2 mg/ml propidium iodide for 30 seconds. To analyze the expression of CDX2 protein, cells were fixed in PFA (3.7%) and permeabilized with 2% Triton X-100. Staining was performed using a rabbit polyclonal anti-CDX2 antibody (kindly provided by M. German, Dept. of Medicine Hormone Research Institute, UCSF, San Francisco, CA). A FITC-conjugated anti-rabbit Ig antibody (dilution 1/100; Amersham Pharmacia Biotech) was then used to reveal CDX2 expression and localization. All specimens were examined under a fluorescence microscope equipped for fluorescence and inter-differential contrast (Leica DMR, Cambridge, UK).

### Protein extraction and immunoblot analysis

Cells were grown on 24 mm diameter filters for immunoblot analysis. Cells were washed three times with ice-cold sterile PBS, then harvested using a cell scraper and centrifuged at 6000 g (10 minutes, 4°C). Cell pellet was resuspended in lysis buffer (SDS Laemmli buffer, BioRad)

supplemented with a complete cocktail of protease inhibitors (Sigma). Protein concentration was measured by the BCA protein assay (Pierce), as recommended by the manufacturer. Proteins were separated using 10% SDS-polyacrylamide gel electrophoresis and then transferred to nitro-cellulose membrane (0.45 mm). Following transfer, membranes were incubated for 1 hour in blocking solution (5% dry non-fat milk in PBS containing 0.05% Triton X-100), and then incubated overnight at 4°C with the anti-CDX2 (kindly provided by M. German, Dept. of Medicine Hormone Research Institute, UCSF, San Francisco, CA) or the polyclonal anti-actin (dilution 1:100; Sigma), or the polyclonal anti-GFP antibodies (dilution 1:100; Clontech Laboratories, Palo Alto, CA). Membranes were washed in 3% dry non-fat milk in PBS containing 0.05% Triton X-100 and incubated with anti-rabbit or anti-mouse peroxidase-conjugated secondary antibody (1:10,000 dilution; Amersham Pharmacia Biotech) for 30 minutes. Immunoblots were revealed using an enhanced chemiluminescence system (ECL™, Amersham Pharmacia Biotech).

**Confocal and electron microscopy analysis**

P3E6 cells grown on porous filters were fixed and stained as described above and then examined by confocal laser scanning microscopy (CLSM, Leica, Wetzlar, Germany). A series of filters cultured in parallel were embedded according to a standard procedures and ultrathin sections (Leica ultracut UCT) were examined by transmission electron microscopy (JEOL JEM-1200 EX II microscope).

**Construction of the recombinant plasmid containing the *cdx2* promoter region**

A 659 base pair *BglIII/KpnI* fragment containing the DNA sequence of the mouse *cdx2* gene promoter was produced by PCR using the following primers 5' – GAAGATCTCCTTCTGCCTGAGAATGTAC – 3' and 5' – TCTGTCTGACTCCAGACGAAGCCATGG – 3' (GenBank U00454). The PCR reaction product was digested with *BglIII* and *KpnI* restriction enzymes and cloned upstream of the coding sequence of the GFP reporter gene into the multiple cloning site between *BglIII* and *KpnI* of the pEGFP plasmid (Qiagen). The recombinant vector was then transfected into Epicurian coli XL1-Blue Competent cells (Stratagene) and recovered by a maxi plasmid preparation kit (Qiagen). The construct was confirmed by PCR and sequence analysis. The recombinant plasmid named pcdx2EGFP was then used for transfection experiments.

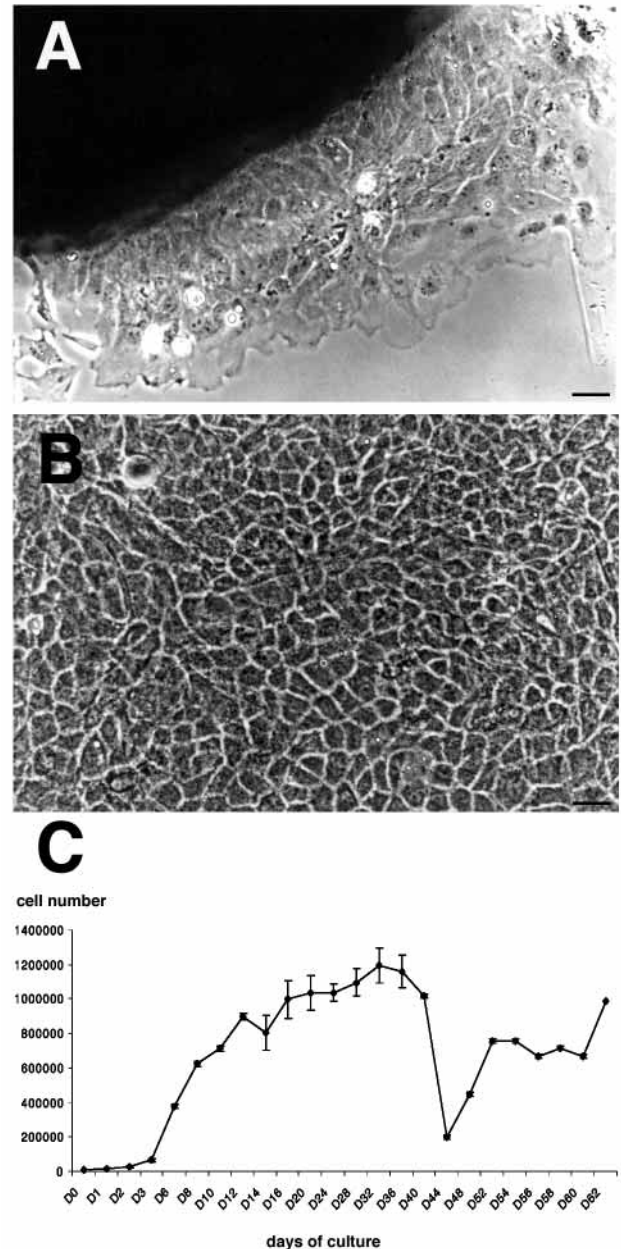
**Transfection of P3E6 cells by pcdx2EGFP plasmid**

Cells were plated at a density of  $1 \times 10^5$  per 35 mm diameter well and transfected 24 hours later with 5 µg of pcdx2EGFP plasmid mixed to 15 µl Lipofectin (Lipofectamine kit, Invitrogen Life Technologies). After a 5 hour incubation, cells were washed and fed with fresh DMEM/Ham's F12 medium. Stable transfectants were selected in medium supplemented with 0.5 µg/ml of geneticin (Gibco BRL) and the resulting P3E6K2 cells were routinely grown in geneticin medium. PCR analysis on stable transfected cells P3E6K2 confirmed the presence of intact inserted construct of pcdx2EGFP (data not shown). Transient transfection of the plasmid DNA in Caco2 cultured cells on glass slides was performed by using the same procedure and the GFP expression was checked at 48 hours post-transfection using epifluorescent microscopy.

**Results**

**Esophageal mouse keratinocytes growth in culture**

Normal mouse malpighian epithelium explants were seeded in Petri dishes and successfully cultured. The explants were able to attach onto plastic supports after day 4 to day 7 with a very



**Fig. 1.** Culture of mouse esophageal keratinocyte cells. (A) Phase contrast microscopy view of epithelial cells in primary culture. The dark area represents the explant tissue. (B) Phase contrast microscopy view of esophageal epithelioid cells at confluency (day 11). Scale bar, 10 µm. (C) Growth curve of long-term culture of normal mouse esophageal cells. Cell number is given as mean ± s.e.m. of three independent cultures.

high efficiency (80%). All attached mouse esophagus specimens rapidly displayed a surrounding “skirt” of rapidly growing cells after few days (Fig. 1A). The attainment of growing cells was reproducible from esophagus explants of different mice. After trypsinization and removal of the remaining tissue fragments, the cells were cultured for 11 days resulting in confluent, homogenous cells showing typical epithelioid morphology characterized by a polyhedral configuration and close apposition (Fig. 1B). In particular a

primary culture of cells referred to as P3E6 was subsequently used in this study. These cells have been frozen and thawed several times without impairing the plating and subculture efficiencies. So far, the cultures have been maintained until passage 16. Thus, long-term culture of nontumoral, nontransformed epithelioid cells was achieved. A typical growth curve (repeated three times) is depicted in Fig. 1C. A 24-48 hour lag phase was followed by a linear exponential growth phase of (D7-12), after that a plateau phase was reached (D22-44). This plateau was maintained up to D40-44 before the occurrence of a cell 'crisis'. This growth curve allowed the definition of the doubling time of cells to about 30 hours, which corresponded to the average doubling time of epithelial cells in culture. The remaining cells that continued to grow again thereafter have not been analyzed in the present study.

### P3E6 cells proliferated and differentiated as 'Malpighian-like' multilayers of esophagus keratinocytes

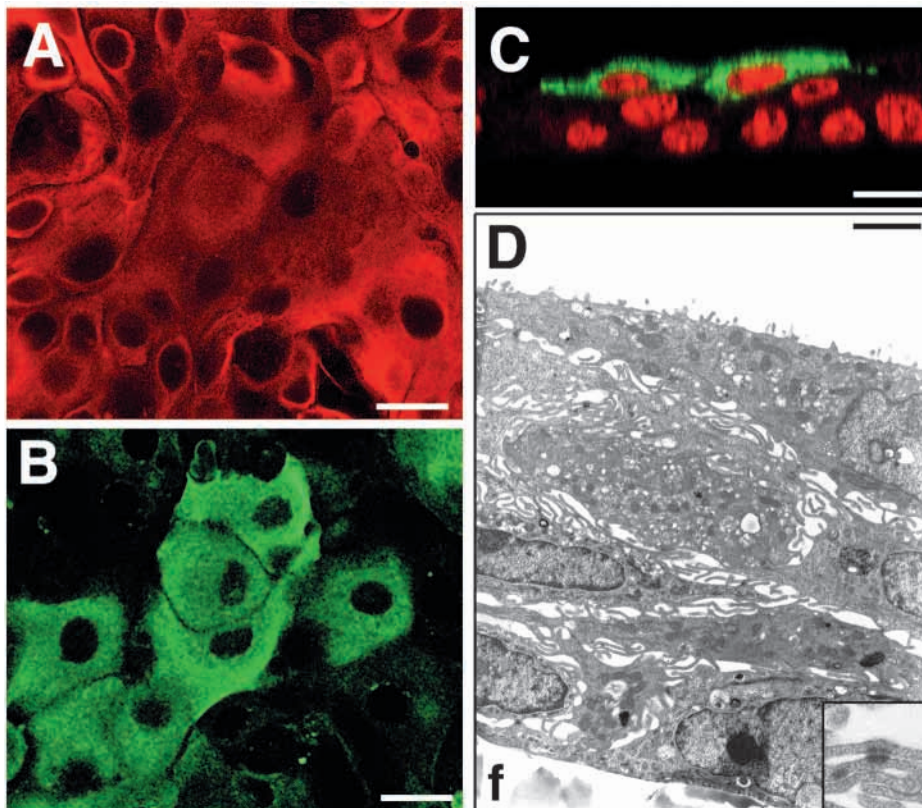
P3E6 cells (passage 6) were seeded onto porous filter support and checked for their ability to proliferate and differentiate. A similar growth curve and cell doubling time to that obtained for P3E6 cells growing on plastic support was obtained for cells growing on filters (data not shown). Confocal analysis of immunocytochemistry of esophageal keratinocytes cultured for 16 days revealed a pattern of expression of cytokeratins typical of the malpighian epithelium of the esophagus. Cytokeratin 14 (CK14) was strongly and uniformly expressed (Fig. 2A), whereas cytokeratin 4 (CK4) was only expressed in the suprabasal cell layer (Fig. 2B). Thus, just as it occurs in vivo, the CK4 staining was restricted to the upper layers, whilst the CK14 staining was observed only in the 2-3 basal layers of

stratified cells. When xz sections were analyzed multiple layers of cells could be observed, as shown by labeling of nuclei with propidium iodide (Fig. 2C). TEM analysis confirmed that epithelial cells formed multilayers when grown on filter supports, forming numerous interdigitations and making contact via desmosomes (Fig. 2D).

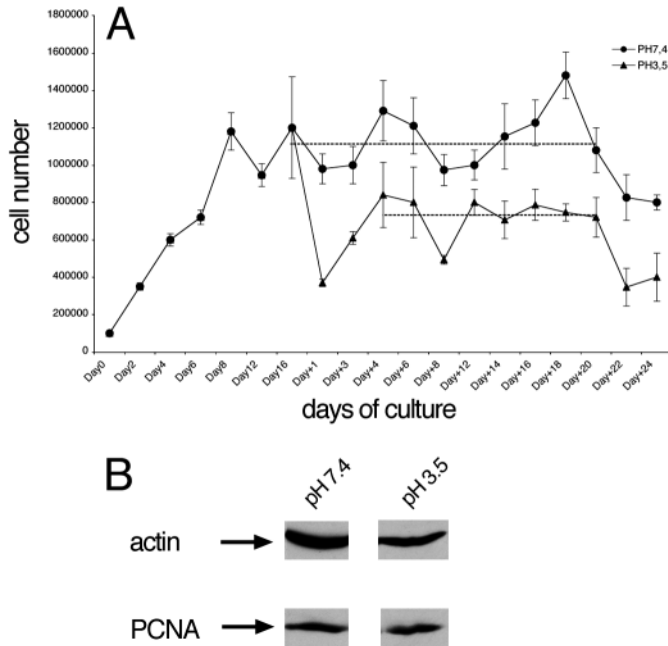
### Effects of acid pH on P3E6 cell growth

In attempts to investigate the role of acid pH on esophagus cells in physiological conditions, exposure to pH 3.5 was started at D16 (D+0 of acidification) when cells became confluent, differentiated and formed multilayers, a condition resembling the in vivo cellular organization. The porous filters allowed us to have independent access to the apical and to the basal chamber of the culture. Acidified medium (pH 3.5) was added only to the apical chamber of the culture, while neutral medium (pH 7.4) was maintained in the basolateral chamber. In these conditions, no significant passage of medium was observed between the upper and the basolateral chamber when checked by analyzing the pH of the medium of the two chambers during the experiment. Interestingly, the acid medium was not buffered by the neutral medium present in the basolateral chamber, despite the absence of tight junctions. This correlates with the results obtained by Orlando et al. (Orlando et al., 1992) showing the presence of barriers to paracellular permeability in rabbit esophageal epithelium.

Fig. 3 shows the effect of acidity on cell growth of P3E6 cultured at pH 7.4 or after continuous exposure to pH 3.5 on their apical side. Exposure of cells to pH 3.5 induced a significant decrease of the total cell number at D+1 of acid exposure. A plateau was then reached and remained constant between D+4 and D+20. However, when a new steady state was reached, the total number of P3E6 cells incubated at pH 3.5 on their apical side was decreased by 42% when compared with P3E6 cells maintained at pH 7.4 (Fig. 3). This suggests that acidification of the medium exerts a direct effect on the efficiency of multilayer formation. However, no significant decrease in CK4 expression was observed in the acidified and control cells (data not shown). In order to assess whether acid



**Fig. 2.** Characterization of long-term cultures of esophageal cells grown on filters. (A-C) Confocal analysis of immunocytochemistry labeling. (A) CK14 was strongly expressed in the basal cell layer, whereas (B) CK4 was expressed in suprabasal cells. (C) xz confocal section after labeling of nuclei (red) and CK4 (green) showing the cell stratification and the suprabasal epithelioid distribution of CK4 positive cells. Scale bar, 20  $\mu\text{m}$ . (D) Transmission electron microscopy view of pluristratified mouse esophageal epithelioid cells grown on filter. Insert: enlarged view of a desmosome (f: filter). Scale bar, 2  $\mu\text{m}$ .



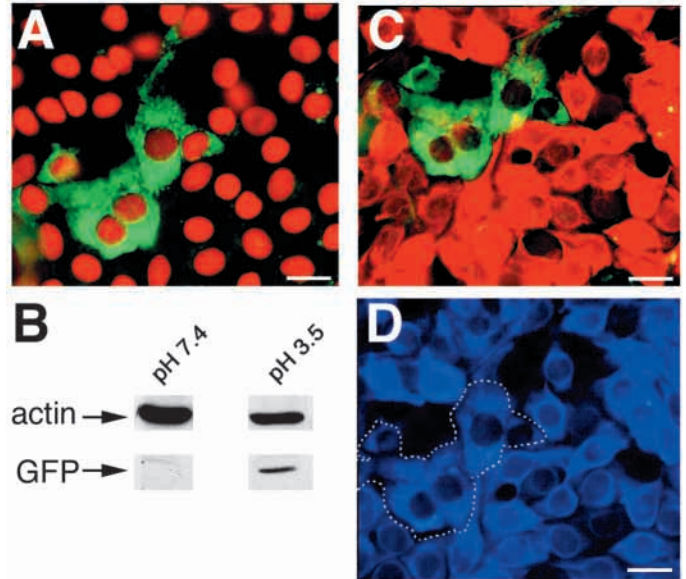
**Fig. 3.** Effect of pH on esophageal epithelial cell growth and proliferation on a transwell filter device. Cells were grown by maintaining neutral pH into the basolateral chamber and adding acid pH or neutral pH into the apical chamber. (A) Cell number is given as the mean  $\pm$  s.e.m. of three independent cultures. (B) Cell lysates of cells at D+1 were used for immunoblotting using anti-PCNA antibody. Equal amount of protein per lane (10  $\mu$ g) were analyzed.

treatment affected cell proliferation, we examined PCNA expression. No difference in cell proliferation was noted after D+1 between cells exposed to acid or neutral medium at their apical pole (Fig. 3B). In contrast, when cells were exposed to acid pH on both sides, cells rapidly died (data not shown).

**Long-term acidification of cultured cells lead to activation of the *cdx2* promoter**

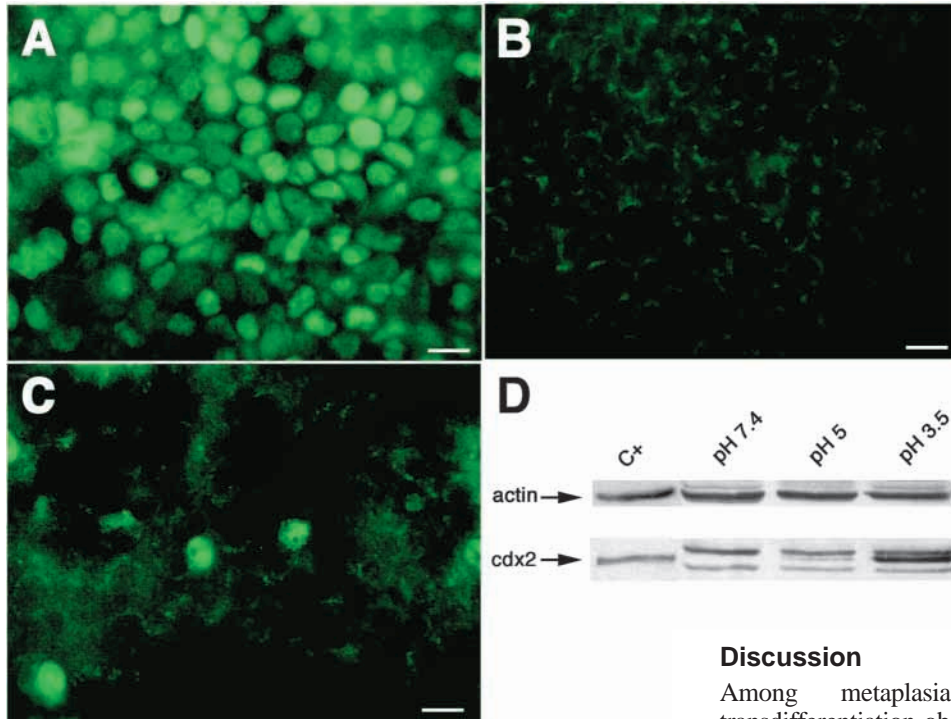
The vector *pcdx2*GFP consisting of the regulatory sequences of the mouse *cdx2* gene inserted immediately upstream of the coding sequence of the green fluorescent protein (GFP) reporter gene was constructed to test whether chronic exposure to acidity could induce activation of the promoter of the *cdx2* intestinal specific homeobox gene. Functionality of the construct *pcdx2*GFP was checked by transfecting the intestinal Caco-2 cell line, known to express high levels of CDX2 endogenous protein. Immunofluorescent detection of GFP after transient transfection (48 hours) was observed as expected (data not shown). Therefore, the *pcdx2*GFP plasmid was considered suitable for the subsequent experiments.

The *pcdx2*GFP construct was stably transfected into P3E6 cells (P3E6K2) after selection with geneticin. These cells were then checked for GFP expression during the pH assay. On the basis of results obtained with the cell growth curve, the pH assay was performed by starting acid exposure at D16 (D+0) and maintained for a further period of 22 days (D+22). At intervals of two days, two filters cultured at each pH condition (pH 7.4 and pH 3.5) were fixed in methanol and checked for GFP expression. Persistent exposure to pH 3.5 of the apical



**Fig. 4.** GFP expression under the control of the *cdx2* promoter in long-term cultured mouse esophageal epithelioid cells maintained at pH 3.5. (A) Pluristratified ‘Malpighien-like’ cells exposed for 18 days at pH 3.5 stained with propidium iodide (nuclear staining) showing GFP-positive cells (B) Western blot analysis of GFP expression on cell lysates of cells exposed for 18 days at pH 3.5, revealed a 27 kDa band corresponding to the GFP protein that is absent in cells grown at pH 7.4. (C) Same field as in (A) stained for CK14 antibody (red). Note that GFP-positive cells remained green indicating that they were CK14 negative. Scale bar, 20  $\mu$ m. (D) The same field as in (A) stained for CK4; the dotted line delineates the GFP-positive cells that also stain for CK4.

side of P3E6K2 cells induced transcription of the GFP reporter gene as demonstrated by fluorescence microscopy analysis and western blot (Fig. 4). GFP-positive cells were observed at D+10 of exposure to pH 3.5. Between D+10 and D+14 the number of positive cells remained stable (8/12  $\pm$  3 cells/filter of 0.33 cm<sup>2</sup>). The number of positive cells significantly increased at D+16 of exposure to pH 3.5. An average of 35 ( $\pm$ 6) cells per filter were found positive at D+16. Fig. 4A shows a representative field of a filter at D+18 of exposure to pH 3.5, on which an islet of GFP-positive cells was observed. No GFP expression was observed during the same time of exposure of P3E6K2 cells to pH 7.4 (data not shown). Consistent with these observations, GFP expression was detected by western blot analysis only in the acid-exposed P3E6K2 cell extracts (Fig. 4B). To better characterize the degree of differentiation of P3E6K2 cells that expressed GFP under the control of *cdx2* promoter, immunocytological analysis of CK14 and CK4 was performed. Fig. 4C,D show the same field of cells as figure 4A after an additional staining with the anti-CK14 and the anti-CK4 antibodies. GFP expressing cells were CK14 negative (Fig. 4C) but CK4 positive (Fig. 4D). Thus, the regulatory sequence of the *cdx2* intestinal homeogene controlling the expression of GFP seemed to be activated only in the suprabasal differentiated P3E6 cells, suggesting that modification of the gene expression program by acid exposure occurred preferentially in the proliferative compartment.



**Fig. 5.** CDX2 expression analysis in esophageal cells grown on filters and in cell lysates. (A) Caco2 cells, used as control, were all CDX2 positive. (B) Esophageal cells at D+18 grown at pH 7.4 exhibited no CDX2 staining, whereas (C) positive nuclear staining was observed in some cells grown at pH 3.5. Scale bar, 20  $\mu$ m. (D) Western blot analysis of CDX2 protein expression performed on lysates of cells at D+18 at pH 7.4 (lane 2), pH 5 (lane 3), and pH 3.5 (lane 4). Lane 1 represents the positive control for Cdx2 protein in Caco2 cell lysate (C+).

#### P3E6 cells expressed the endogenous CDX2 protein when exposed at acid pH

We then checked whether the acid-induced GFP expression under the control of *cdx2* regulatory sequence in P3E6K2 cells was consistent with the induction of expression of the endogenous Cdx2 protein. Nontransfected P3E6 cells were seeded on filters and the pH assay was repeated as described above. At the same time intervals as those chosen for GFP expression analysis in P3E6K2 cells, immunocytochemical analysis was performed using an anti-CDX2 antibody on P3E6 cells. Fig. 5B,C show immunocytochemical analysis on P3E6 cells exposed to acid or neutral pH. Caco2 cells, used as a positive control, displayed homogenous nuclear staining of endogenous CDX2 protein as expected (Fig. 5A). No staining was detected in P3E6 cells cultured (D+18) at pH 7.4 (Fig. 5B). Fig. 5C shows a representative field of P3E6 cells at D+18 cultured at pH 3.5 and as expected expression of Cdx2 protein was confined to P3E6 cell nuclei. Furthermore, the number of endogenous Cdx2 positive cells corresponded to the number of P3E6K2 cells in which the construct Cdx2-promoter fused to GFP. These results were consistent with those obtained by western blot analysis (Fig. 5D), confirming pH-dependent induction of Cdx2 protein expression. Intestinal Caco-2 cells, used as a positive control, displayed a signal at 33 kDa corresponding to the molecular weight of CDX2 protein. Expression of Cdx2 in P3E6, not detected at pH 7.4, was observed as a function of pH (pH 5 and pH 3.5). It has to be noted that in the western blot detection, two additional bands of lower and higher molecular weight were always detected in P3E6 cell extracts but not in Caco-2 cells extracts. These additional bands did not vary with decreasing pH, and did not display the expected molecular weight of Cdx2 (33 kDa), suggesting that they could correspond to non-specific immunoreactions.

#### Discussion

Among metaplasia of epithelial cells, intestinal transdifferentiation observed in the lower part of the esophagus illustrates the potential of cell plasticity, since a Malpighian squamous epithelium can be replaced by a columnar simple epithelium, which displays many characteristics of columnar simple intestinal cells (Barrett, 1950; Spechler and Goyal, 1996; Sharma, 1999; Jankowski et al., 2000). Many intestinal-specific markers have been reported in this metaplastic process, which seems to be multifactorial, since, clinically, it is associated with chronic acid and biliary salt reflux from the stomach, and inflammatory cell infiltration (Baberzat, 1998; Jankowski et al., 2000). The culture model described in this study appears to be appropriate to study the early steps of the mechanisms leading to this metaplasia. Two main issues were addressed, first, the effects of low pH on the transdifferentiation process of normal esophageal cells into an intestinal cell phenotype and, second, the origin of this metaplasia that could occur from esophageal cells themselves. Controversy exists as to the origin of the columnar intestinal epithelial cells during the metaplastic process as these cells may originate from squamous cell transdifferentiation, from migration of the gastric cells localized below the 'Z' line that form the landmark between esophagus and cardia stomach, or from migration from the esophageal glands (Baberzat, 1998; Chaves et al., 1999; Harrison et al., 2000; Jankowski et al., 2000). In this study, we demonstrated that under defined culture condition of mouse esophageal cells, chronic acid exposure can induce expression of the intestinal gene marker *cdx2*. Furthermore, this beginning of intestinal transdifferentiation process was initiated in normal esophageal, squamous cells.

Previous attempts to culture normal esophageal epithelium have been limited mainly to explant cultures of rabbit (Stef et al., 1981), rat (Stoner et al., 1982) or human esophageal mucosa (Resau et al., 1990; Vocci et al., 1983; Mothersill and Seymour, 1989) that are limited in time and do not allow chronic exposure to low pH. To the best of our knowledge, we have for the first time, set up a system of long-term culture of normal mouse esophageal cells. Remarkably, the cultured cells reached the same cellular organization as observed in Malpighian epithelium in vivo,

showing homogeneous expression of cytokeratin 14 and cytokeratin 4 in basal and suprabasal layers, respectively. On average we could obtain four layers of cells. In addition, culture on filter support provided the advantage that we could have independent access to each side of the cell culture. The reproducibility of both the proliferation and specialization patterns of the described primary esophageal cell culture indicated that these cultures represented a useful tool for investigating pathological mechanisms in functioning esophageal cells. These cultures represented a controlled system in which the possible role of acid exposure in the transdifferentiation process of normal esophageal cells into an intestinal cell phenotype could be studied.

Acidification of the apical medium induced a rapid decrease in the number of growing cells but cells rapidly adapted to this acid environment, did not arrest proliferation and persisted in culture. Conversely, cells grown on plastic supports or exposed at both sides at low pH, rapidly arrested proliferation and died (data not shown). These results are in agreement with other findings showing that esophageal cells possess an H<sup>+</sup>-extruding mechanism at their apical surface to adapt to the degree of lowering pH (Grinstein et al., 1998; Tobey et al., 1998).

To assess the effect of low pH on the conversion of normal esophageal cells into intestinal cell type, we focused, in our study, on a very early marker of intestinal differentiation, the intestinal transcription factor CDX2, assumed to be a candidate that is activated during the first steps of the esophageal-intestinal transition. The rationale resides in the fact that CDX2 has been shown to be crucial in the early step of intestinal cell differentiation and in maintenance of intestinal phenotype (Freund et al., 1992; James and Kazenwadel, 1991; James et al., 1994; Suh and Traber, 1996; Soubeyran et al., 1999). Interestingly, we have found expression of CDX2 in human Barrett esophagus explants (data not shown) and this has been confirmed by a recent work (Akashi et al., 2002). It was shown that CDX2 expression was initiated at the stage of esophagitis and was maintained as strong nuclear staining in Barrett epithelium. These data support our hypothesis that implies that CDX2 could be an early event leading to the development of Barrett's esophagus (BE).

The implication of CDX2 in intestinal metaplasia has been recently demonstrated in the intestinal metaplasia of the stomach where CDX2 was ectopically overexpressed, suggesting that it could play a major role during intestinal metaplasia formation in the stomach (Bai et al., 2002). Furthermore, it has been shown that mice in which *cdx2* gene has been inactivated by homologous recombination developed multiple intestinal polyp-like lesions that did not express CDX2 and that contained areas of squamous metaplasia in the form of stratified squamous epithelium, similar to that occurring in the mouse esophagus (Beck et al., 1999). This indirectly suggests a key role of *cdx2* in governing the differentiation process of squamous cells into intestinal cells. In addition, it has been shown that acid exposure significantly increases the activity of p38 MAPK in a Barrett's adenocarcinoma cell line (Saouza et al., 2002); this consolidates our results since other reports have shown that CDX2 plays a role in mediating p38 function in enterocyte differentiation (Houde et al., 2001). Besides the observation of CDX2 expression in BE, the factor(s) responsible for its activation and its expression have not been studied before. In this study, we demonstrated that in a long-term culture model of mouse esophageal cells the activation and the expression of Cdx2 may result from chronic acid

exposure alone. Furthermore, this change in genetic program occurs in well-defined esophageal cultured cells, thus, suggesting that BE can arise from Malpighian cells themselves, at least under some particular physico-chemical conditions. Interestingly an electron microscopy study revealed that P3E6 cells exposed to acid pH showed enlarged extracellular spaces that were absent in cells exposed to neutral pH (data not shown). A recent report by Tobey et al. (Tobey et al., 1996) presented this feature as recurrent in patients having gastroesophageal reflux. This may correlate with an early pathological event that, in the long term, could give rise to the development of intestinal metaplasia. However, in our study, a complete, typical metaplasia with well-differentiated intestinal cells could not be reached by acid exposure alone. Expression of other intestinal markers such as villin could not be observed by western blot analysis. However, villin is expressed at a late stage of the intestinal transdifferentiation process of esophageal cells, when cells have assumed morphologically an intestinal-like phenotype. According to the literature, although acidity is considered as the main factor associated to BE, the pathology is described as consequence of multifactorial and multistep processes. Thus, a complete transdifferentiation program probably needs other inducing environmental factors. This in vitro model could be a potent tool to explore the role of acidity and modifications of the physico-chemical microenvironment on specific gene expression. One can speculate that P3E6 cells exposed to low pH must extrude protons and thus that Na<sup>+</sup>/H<sup>+</sup> exchanger could be involved, even indirectly, in the pathway leading from pH 3.5 exposure to activation of the *cdx2* gene.

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