

## Effect of Changes in the Extracellular Matrix (ECM) Composition on Clara Cell Proliferation and Death Through P21 Regulation

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**Abstract:** Extracellular Matrix (ECM) contains signals that control cell progression, division and death. Cell-matrix interactions or disruption affects the cell cycle regulation in various ways. In this study, Clara cells obtained from both wild type (wt) and p21 knockout (p21 ko) mice were isolated and cultured on different ECM combinations. Different ECM compositions did not influence Clara cells proliferation both in the presence or absence of p21. On the other hand, p21 was found to induce both apoptosis and necrosis in primary Clara cells cultures. The cell-matrix disruption rather than ECM composition seems to have a greater influence on the cell cycle regulation and progression.

**Key words:** ECM, cell-matrix, progression, cell cycle, regulation, necrosis

### INTRODUCTION

Extracellular Matrix (ECM) is a general term that encompasses components of the basement membrane and interstitial connective tissue. The extracellular matrix contains signals that control cell shape, migration, proliferation, differentiation, morphogenesis and survival (Lukashev and Werb, 1998; Boudreau and Jones, 1999; Streuli, 1999).

After an injury to the lung epithelial cells, changes in the ECM composition could be a key regulator in restoring the epithelial barrier otherwise the injury could progress into a disease (Roskelley *et al.*, 1995; Lukashev and Werb, 1998; Dunsmore and Rannels, 1996; Chintala and Rao, 1996; Talpale and Keski-Oja, 1997; Boudreau and Jones, 1999; Streuli, 1999; Ebihara *et al.*, 2000). Cell-matrix interactions or disruptions affect the cell cycle regulation in various ways. A number of studies have shown that by the disruption of cell-matrix interactions, regulated cell cycle progression and influences the expression of a number of cell cycle regulatory proteins such as p21, p27 and p53 (Guadagno *et al.*, 1993; Assoian, 1997; Schwartz and Assoian, 2001; Bao *et al.*, 2002; Ilic *et al.*, 1998; Nagaki *et al.*, 2000; Wu and Schönthal, 1997). Other studies have shown that cell-matrix disruption could lead to apoptosis (Ruoslahti and Reed, 1994; McGill *et al.*, 1997; Frisch and Francis, 1994; Bourdoulous *et al.*, 1998; Kettritz *et al.*, 1999; Sethi *et al.*, 1999; Day *et al.*, 1997).

The main hypothesis of this study is that cell-matrix interaction or disruption regulates cell cycle progression through p21. To study this hypothesis Clara cells from

both wt and p21 ko mice were isolated and cultured on seven different ECM compositions: Fibronectin/Collagen IV/Laminin (Fn/Coll IV/Lam); Fibronectin/Collagen IV (Fn/Coll IV), Collagen IV/Laminin (Collagen IV/Laminin (Coll IV/Lam); Fibronectin/Laminin (Fn/Lam); Collagen IV (Coll IV); Laminin (Lam); Fibronectin (Fn). The final concentration of each ECM composition was 50 µg mL<sup>-1</sup>. The effect of ECM composition on Clara cell behaviour proliferation was studied.

### MATERIALS AND METHODS

**Clara cell isolation and culturing:** Mice (C3H/He strain or p21 ko mice either male or female, between 4 to 8 weeks old) were sacrificed by lethal intraperitoneal injection of 0.5 mL pentobarbitone (Sagatal™). p21 ko mice were kindly provided by Dr. Philip Leder, Harvard Medical School, Boston. Clara cells were isolated and cultured as previously described (Blundell and Harrison, 2005).

Once isolated, cells were plated onto sixteen-well glass chamber slides (Gibco) which had been pre-coated with appropriate ECM and incubated at 37°C, 5% CO<sub>2</sub>/air. Sixteen well chamber slides (wells having 6 mm diameter) (Gibco) were coated with 50 µg of ECM overnight at 4°C. The next morning the chamber slides were washed with sterile PBS and stored at -20°C. Three different types of ECM components were used in this study: Fibronectin (Fn) (Sigma), collagen IV (Coll IV) (Sigma) and laminin (Lam) (Sigma). Fibronectin was used for the basal culture conditions of all cultures. Seven variations of ECM were used for further studies as shown in Table 1. Cells were

Table 1: Different ECMs combinations and the concentrations upon which Clara cells were cultured

Extra Cellular Matrix (ECM) combinations	Concentration
Lam/Fn/Coll IV	50 µg mL <sup>-1</sup> of each added simultaneously
Coll IV/Fn	50 µg mL <sup>-1</sup> of each added simultaneously
Coll IV/Lam	50 µg mL <sup>-1</sup> of each added simultaneously
Lam/Fn	50 µg mL <sup>-1</sup> of each added simultaneously
Coll IV	50 µg mL <sup>-1</sup>
Lam	50 µg mL <sup>-1</sup>
Fn	50 µg mL <sup>-1</sup>

allowed to attach overnight after which the medium was replaced to remove dead and unattached cells. Medium was subsequently replaced every 2 days. Cells were usually fixed at days 1, 3 and 5 by methanol at -20°C.

**Immunohisto-chemistry:** Slides were equilibrated in TBS for 5 min. The slides were block with an appropriate serum in which the secondary antibody was raised. PCNA antibody (P8825, Sigma) (concentration of 1/5000) was applied for 2 h, followed by three 5 min washes with TBST. Secondary antibody was put on for 30 min then washed 3 times for 5 min each with TBST. An Alexa conjugated secondary antibody (diluted 1: 200 in serum) was put on the slide and incubated for 3 min. The slides were then washed three times for 5 min each and mounted using DAKO fluorescent mounting medium. The slides were then visualised either under the fluorescent microscope or confocal microscope.

**Proliferation counts:** Cultured Clara cells were exposed to medium containing 10 µM BrdU for 6 h, after which they were fixed using 80% ethanol at 4°C overnight. After fixation cells were rinsed in PBS for 10 min and incubated in 5M HCl for 45 min at room temperature. Cells were then washed four times in PBS for 5 min each and incubated in 1% H<sub>2</sub>O<sub>2</sub> for 10 min. Cells were washed twice in PBS for 5 min and incubated in blocking solution (PBS+20% rabbit serum+0.05% Tween) for 10 min. The slides was drained and incubated in rat anti-BrdU antibody (Beohringer Manneheim) diluted 1: 10 in blocking solution for 60 min. The slide was then washed three times in PBS for 5 min each and DAB solution (100 µL DAB + 4.8 mL 0.05 M Tris + 100 µL 1% H<sub>2</sub>O<sub>2</sub>) was added for 5 min or until the colour developed. The slides was then rinsed with water and counterstained with haematoxylin, time according to strength of solution. The slide was mounted using DAKO aqueous mounting medium.

**Feulgen staining:** After the culturing cells were fixed in Bouvin fixative overnight at 4°C. The slides were incubated in denaturing solution (5M HCl) for 45 min

at room temperature. The slides were washed for about 15 min using tap water. Slides were incubated in Schiff's reagent for 1 h at room temperature and then washed in tap water until pink colour developed. Slides were counterstained in 0.1% Light Green and mounted in Cedarwood oil and stored at 4°C in the dark.

**Cell counting:** A wide range of variation in the degree of immunohistochemical staining was observed. Thus, strongly stained cells were considered as positive staining, while negatively or weakly stained cells were considered as negative. Experiments and counts were repeated at least three times and standard deviation was calculated using Microsoft Excel. Counts of 500 cells were sufficient to achieve a stable running mean.

**Statistical analysis:** Statistical analysis was carried out using Microsoft Minitab software. The general linear model test (ANOVA) with Bonferoni corrections for multiple tests, was used to find out significant changes in cell behaviour upon cell-matrix disruption and to find out differences in Clara cells from wt and p21 ko mice. Experiments and counts were repeated at least three times. For all tests a p value less than 0.05 was considered significant.

## RESULTS

Clara cell proliferation was evaluated by three method: BrdU incorporation, mitotic counts and PCNA immunocytochemistry and counts (Fig. 1-3).

No significant changes in mitosis and BrdU incorporated positive nuclei of Clara cells isolated from wt mice when compared to p21 ko mice was observed. A decrease (p<0.05) in the cytoplasmic PCNA expression and an increase (p<0.05) in nuclear PCNA expression in Clara cells from p21 ko mice was observed when compared to wt mice.

ECM composition did not significantly affect rates of mitosis, BrdU incorporation, cytoplasmic and nuclear PCNA expression of Clara cells from either wt or p21ko mice.

Mitosis counts were lower (p<0.05) at time 24 h compared to cultured cells at 72 and 120 h from wt and p21 ko mice. Cytoplasmic and nuclear PCNA expressions were lower (p<0.05) from freshly isolated cells at time 0 h compared to cultured cells at 24, 72 and 120 h from wt mice. Nuclear PCNA expression was lower (p<0.05) in freshly isolated cells at time 0 h compared to the cultured cells from p21 ko mice. No change of BrdU incorporation was noted over the time in culture.

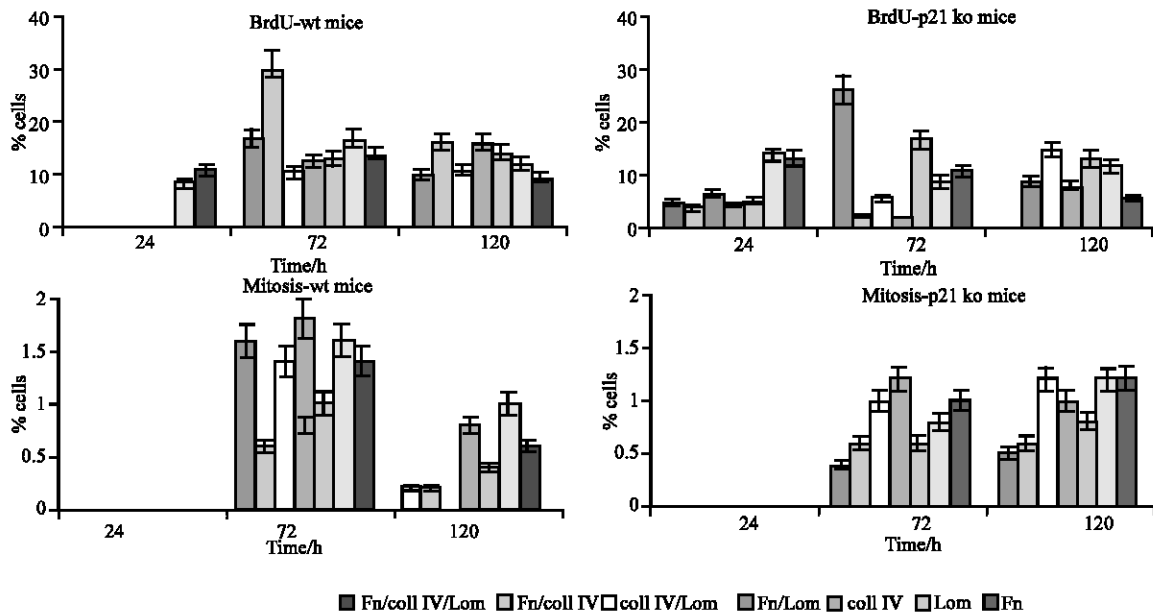


Fig. 1: BrdU incorporation and mitosis counts in Clara cells from wt and p21 ko mice in different matrix conditions. No significant changes in the BrdU incorporation and mitosis counts were observed in cells from wt mice when compared to p21 ko mice. ECM did not change significantly BrdU incorporation and mitosis in cells from both wt and p21 ko mice. Mitosis counts at time 24 h were lower ( $p < 0.05$ ) than at times 72 and 120 h in cells from both wt and p21 ko mice

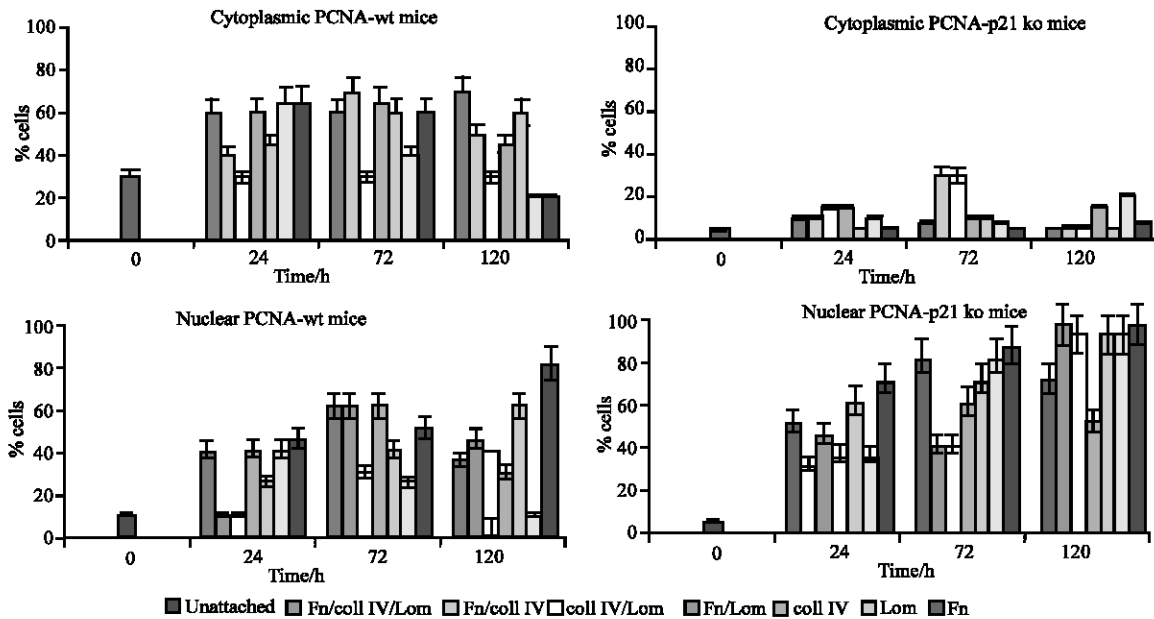


Fig. 2: Cytoplasmic and nuclear PCNA expression in Clara cells from wt and p21 ko mice in different matrix conditions. Cytoplasmic PCNA expression was lower ( $p < 0.05$ ) (A and B) but nuclear PCNA expression higher ( $p < 0.05$ ) (C and D) in cells from p21 ko mice when compared to wt mice. ECM did not change significantly PCNA expression in cells from both wt and p21 ko mice. In cells from wt mice, the nuclear and cytoplasmic PCNA expression of freshly isolated cells at time 0 h was lower ( $p < 0.05$ ) than cultured cells (A and C), while in cells from p21 ko mice, the nuclear PCNA expression was lower ( $p < 0.05$ ) at time 0 h (D) compared to the other time points

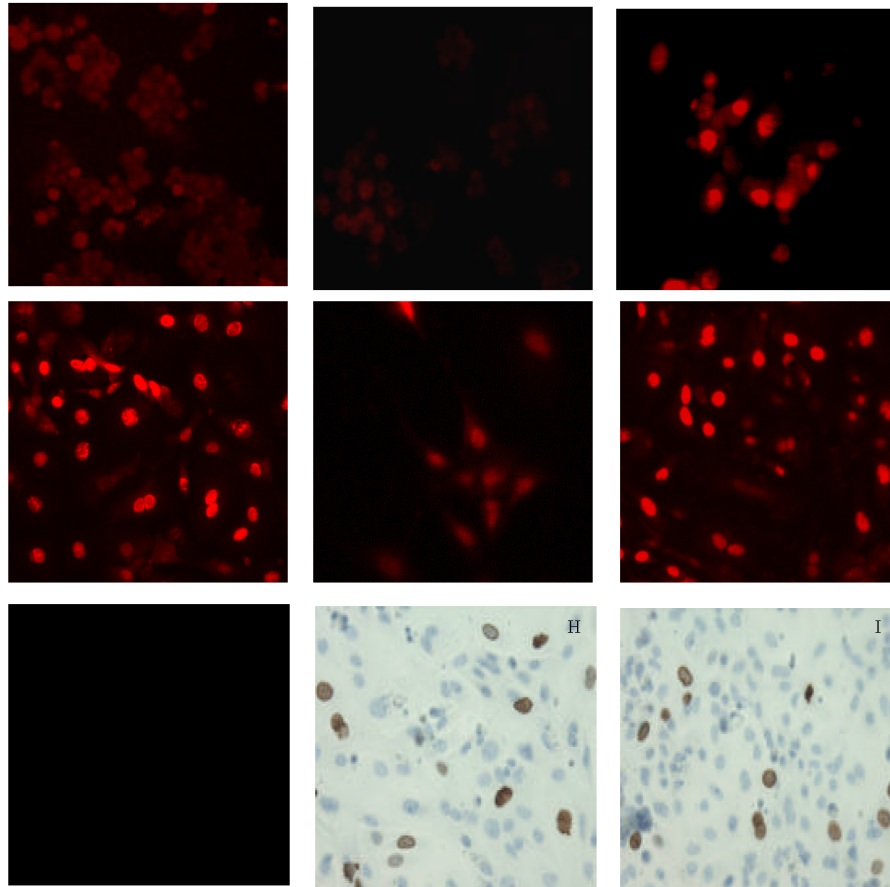


Fig. 3: PCNA expression and BrdU incorporation in Clara cell cultures in different matrix conditions.0 Cytoplasmic PCNA expression was lower ( $p < 0.05$ ) but nuclear PCNA expression higher ( $p < 0.05$ ) in cells from p21 ko mice (D and F) when compared to wt mice (C and E). ECM did not change significantly PCNA expression in cell from both wt (C and E) and p21 ko mice (D and F). In cells from wt mice, the nuclear and cytoplasmic PCNA expression of freshly isolated cells at time 0 h (A) were lower ( $p < 0.05$ ) than cultured cells (C and E). In cells from p21 ko mice at time 0 h (B), the nuclear PCNA expression was lower ( $p < 0.05$ ) compared to cultured cells (D and F). G is a negative control for immunofluorescence whereby the primary antibody was omitted. No changes in the number of BrdU positive nuclei were observed between cells from wt (H) and p21 ko mice (I). Cells in C, D, E, F, G, H and I are at time points 72 h. Cell from C and D were cultured on Fibronectin/Collagen IV/Laminin while cells in E and F were cultured on Fibronectin/Collagen IV. Magnification  $\times 200$

**Effect of ECM variation on Clara cell death in wt and p21 ko mice:** The necrotic and apoptotic dead cells were counted in primary Clara cell cultures at times 24, 72 and 120 h, respectively using Feulgen staining technique and morphological assessment (Fig. 4).

The necrotic rates at time points 72 and 120 h in Clara cells from wt mice were significantly higher ( $p < 0.05$ ) when compared to cells from p21 ko mice. The necrotic rate in Clara cell cultures at 24 h from wt mice were

significantly lower ( $p < 0.05$ ) than cells cultured at time points 72 and 120 h. The apoptotic rate in Clara cells from wt mice at time point 24 h was significantly lower ( $p < 0.05$ ) than cells at 72 h in culture. The apoptotic rate at 72 h was significantly higher ( $p < 0.05$ ) in cells from wt mice compared to cells from p21 ko mice.

No significant differences in apoptosis or necrosis were observed when Clara cells from both wt and p21 ko mice when cultured in different ECM.

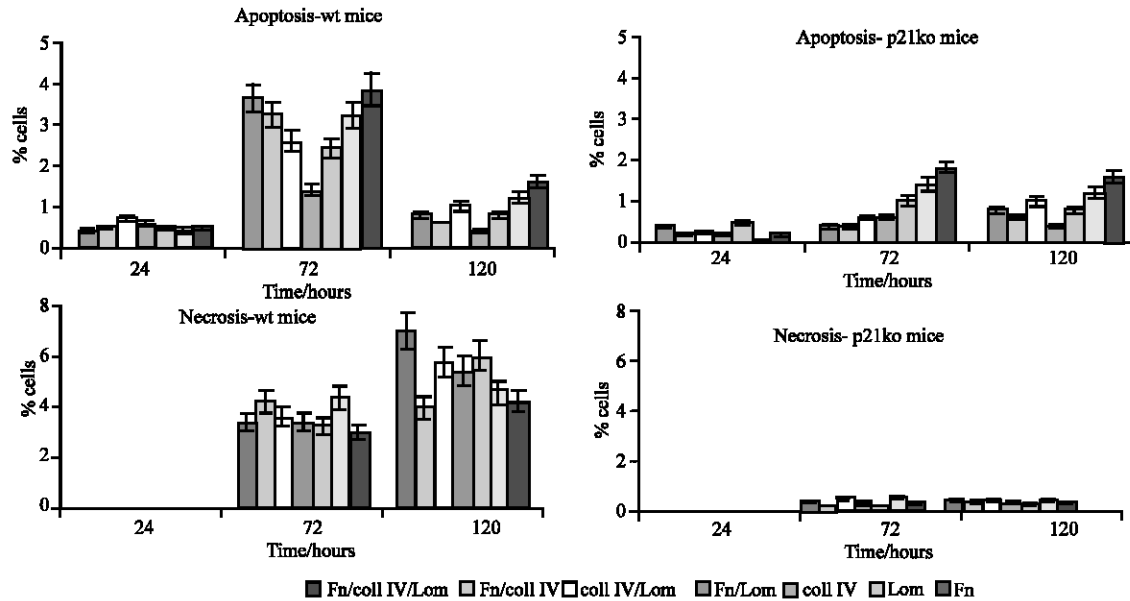


Fig. 4: Apoptotic and necrotic rate in Clara cell cultures from wt and p21 ko mice. The apoptosis rate in cells from wt mice (A) was higher ( $p < 0.05$ ) at time point 72 h compared to cells from p21 ko mice (B). The necrotic rates in cells from wt mice at time points 72 and 120 h (C) were higher ( $p < 0.05$ ) than cells from p21ko mice (D). The necrotic rate at time 24 h was lower ( $p < 0.05$ ) when compared to other time points in Clara cells from wt mice (C). No significant differences in apoptosis and necrosis were observed when cells were cultured on different ECM compositions from both wt and p21 ko mice

## DISCUSSION

### ECM composition does not influence Clara cells proliferation both in the presence and absence of p21:

No significant changes in the proliferation rate were observed in Clara cells from wt mice as compared to cells from p21 ko mice. Thus under these serum-free cell culture conditions, p21 does not affect Clara cell proliferation. Although p21 was shown to inhibit proliferation (Sugibayashi *et al.*, 2002; Harper *et al.*, 1993; El-Deiry *et al.*, 1993; Harper *et al.*, 1995; Niculescu *et al.*, 1998) normal development and proliferation was also described in mice lacking p21 (Brugarolas *et al.*, 1995; Deng *et al.*, 1995; Waldman *et al.*, 1996; McDonald *et al.*, 1996; Weinberg *et al.*, 1997; Alan Wang *et al.*, 1997). The role of p21 regarding proliferation is still unclear and this could be due to different cell types and also due to different culture conditions.

Although culturing Clara cells on different ECM composition did not affect the proliferation rate, the mitotic rate was lower ( $p < 0.05$ ) at culture time 24 h compared to cultured cells at 72 and 120 h from both wt and p21 ko mice. This could be due that cells at 24 h in culture are not as attached to the ECM as cells at 72 and

120 h in culture. Thus proliferation in Clara cells is anchorage dependent although not directly influenced by ECM composition.

A significant ( $p < 0.05$ ) decrease in cytoplasmic PCNA and an increase ( $p < 0.05$ ) in nuclear PCNA expression was observed in Clara cells from p21 ko mice as compared to wt mice. PCNA is required for both DNA replication and DNA repair (Li *et al.*, 1995; Moriuchi, 1990; Oku *et al.*, 1998; Rossi *et al.*, 1999; Rousseau *et al.*, 1999; Yu *et al.*, 2001). Since no significant changes in proliferation were observed using BrdU incorporation and mitotic counts, these changes in the PCNA expression could be attributed to repair.

Nuclear PCNA expression was lower in the freshly isolated cells at time 0 compared to cultured cells from both wt and p21 ko mice, while cytoplasmic PCNA expression was lower only in cells from wt mice. Thus it can be hypothesised that the cell-matrix disruption increases the nuclear PCNA expression in Clara cells from both wt and p21 ko, which is most probably involved in DNA repair. Most probably p21 has a role in controlling transportation of PCNA to the nucleus, since a decrease ( $p < 0.05$ ) in cytoplasmic PCNA expression but an increase ( $p < 0.05$ ) in nuclear PCNA expression was observed in

Clara cells from p21 ko mice when compared to cells from wt mice. The role of p21 in transportation of PCNA to the nucleus has been reported (Li *et al.*, 1994, 1995; Funk *et al.*, 1997; Warbrick *et al.*, 1997; Cayrol *et al.*, 1998; Oku *et al.*, 1998; Rousseau *et al.*, 1999).

#### **p21 can induce apoptosis and necrosis in clara cells:**

Necrotic rate of Clara cells from wt mice at 72 and 120 h in culture were significantly higher ( $p < 0.05$ ) than cell from p21 ko mice. Apoptotic rate was higher ( $p < 0.05$ ) in Clara cells from wt mice at 72 h in culture compared to cells from p21 ko mice. Thus p21 seems to be involved in promoting necrosis and apoptosis death pathways.

There were no significant changes in the apoptosis and necrosis rates by culturing Clara cells on different ECM. A number of studies has shown that when cells are detached from substrate or anchorage is prevented they usually undergo apoptosis (Ruoslahti and Reed, 1994; Frisch and Ruoslahti, 1997; Frisch and Francis, 1994; Schwartz, 1997; Assoian, 1997; Day *et al.*, 1997; Meredith *et al.*, 1993; McGill *et al.*, 1997; Ilic *et al.*, 1998; Vitale *et al.*, 1999; Ketritz *et al.*, 1999; Sethi *et al.*, 1999). The necrotic and apoptotic rates were lower ( $p < 0.05$ ) at culture time 24 h compared to cultured Clara cells at 72 h from wt mice. Cells at 24 h in culture are most probably less attached to the ECM compared to cells at 72 and 120 h in culture, thus some cells could have been detached and not detected in the counts thereby explaining the unexpected low death at 24 h in culture. On the other hand, due to the fact that Clara cells were cultured in a serum free condition, there could be some growth factors missing in the medium and so as culture time in culture increases, the cell death rate also increases. Most probably both statements are correct and the truth somewhere in between. Further studies need to be carried out to find out whether disruption of cell-matrix interaction leads to apoptosis via a p21-dependent pathway.

#### **CONCLUSION**

The cell-matrix disruption rather than ECM combinations seems to have greater influence on cell cycle progression both in the absence and presence of p21. p21 was found to be an important factor for the induction of both apoptosis and necrosis in primary Clara cells cultures. It has been previously shown that disruption of cell-matrix interactions affects the expression of p21 (Assoian, 1997; Wu and Schönthal, 1997; Nagaki *et al.*, 2000; Ilic *et al.*, 1998; Bao *et al.*, 2002) but the exact mechanism is still unclear. Specific integrins

rather than different ECM combinations seem to be more important in the regulation of cell cycle progression in Clara cells. Thus, further studies have to be carried out in order to understand the role of specific integrins adhesion or detachment on p21 expression and on cell cycle progression in Clara cell cultures.

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