Effect of Extracellular Matrix (ECM) on Clara Cell Differentiation Through P21 Regulation

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Abstract: Cell-matrix interactions or disruption affects the cell cycle regulation in various ways and thus the extracellular Matrix (ECM) can control cell differentiation. In this study, Clara cells obtained form both wildtype (wt) and p21 knockout (p21 ko) mice were isolated and cultured on different ECM combinations. A lower expression of cytokeratins 8, 18 and 19 in unattached Clara cells from p21 knockout (ko) mice compared to wildtype (wt) mice was observed. The expressions of cytokeratin 8 and 19 were significantly higher in primary Clara cells cultures when laminin formed part of the ECM composition. The cell-matrix disruption also played an important part on Clara cells differentiation process.

Key words: ECM, Clara cells, cytokeratins, laminin, wildtype, composition

INTRODUCTION

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; 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 differentiation 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 $\mu g\ mL^{-1}$. The effect of ECM composition on Clara cell differentiation 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 pentabarbitone (Sagatal™). p21 ko mice wee 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, cell were plated onto 16-well glass chamber slides (Gibco) which had been pre-coated with

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

Extracellular Matrix (ECM) combinations	Concentration
Lam/Fn/Coll IV	50 $\mu g\ mL^{-1}$ of each added simultaneously
Coll IV/Fn	50 $\mu g\ mL^{-1}$ of each added simultaneously
Coll IV/Lam	50 $\mu g\ mL^{-1}$ of each added simultaneously
Lam/Fn	50 $\mu g\ mL^{-1}$ of each added simultaneously
Coll IV	50 $\mu g\ mL^{-1}$
Lam	50 $\mu g\ mL^{-1}$
Fn	50 $\mu g\ mL^{-1}$

Table 2: Details of the primary antibody used for immuno-cytochemistry and their relative concentrations

Antibody against	Concentration	Supplier	Catalogue number
cytokeratin 8	1/10	ICN	10526
cytokeratin 18	1/10	ICN	10500
cytokeratin 19	1/10	ICN	11417

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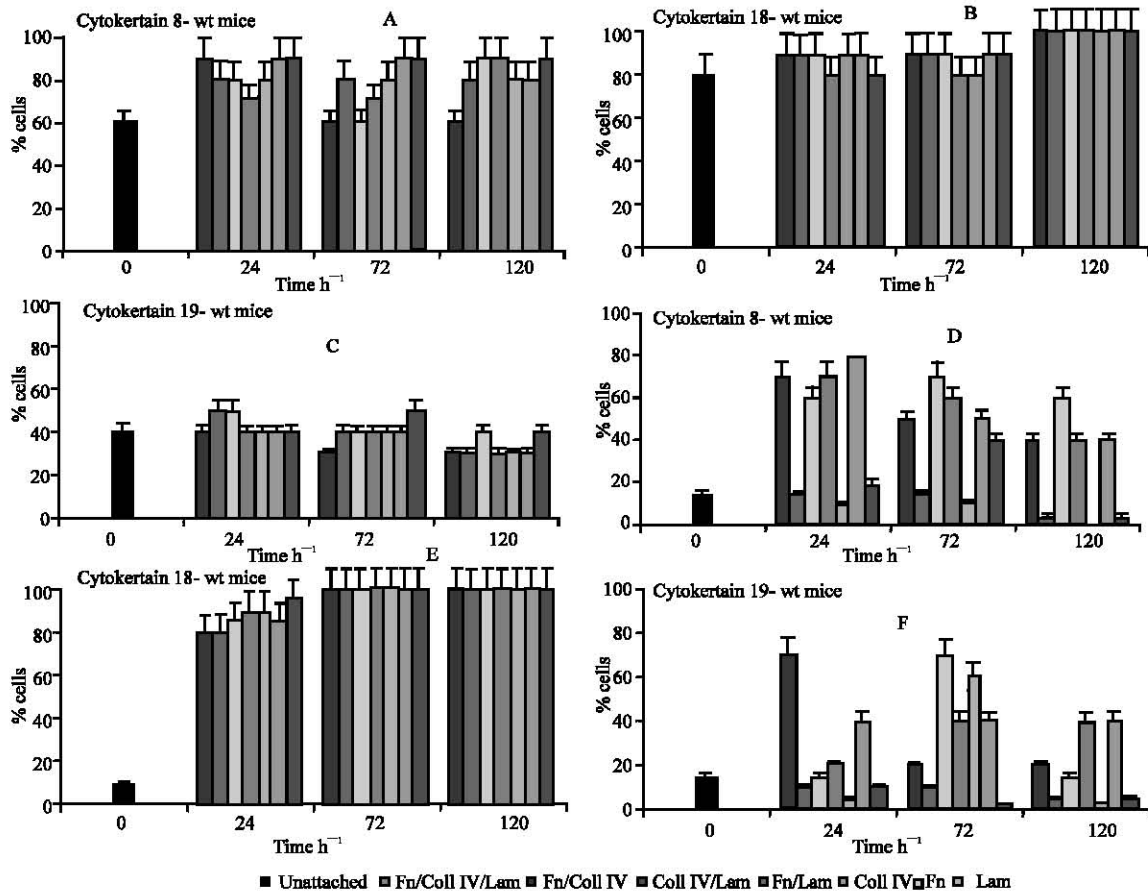
Fig.1: Variation in the expression of cytokeratins 8, 18 and 19 in Clara cells at time 0, 24, 72 and 120 h in both wt and p21 ko mice in different matrix conditions. At time 0 h, the expression of cytokeratin 8, 18 and 19 is lower in Clara cells from p21 ko mice compared to cells from wt mice (A – F). The expressions of cytokeratin 8 and 19 in Clara cells from p21 ko mice were higher when laminin part of the ECM composition (D and F)

appropriate ECM and incubated at 37°C, 5% CO_2/air. 16-well chamber slides (wells having 6mm diameter) (Gibco) were coated with 50μg of ECM overnight at 4°C. The next morning the chamber slides were washed with sterile PBS and stored and -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 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. Primary antibody, details and concentrations as described

in Table 2 was applied for 2 h, followed by three 5 min washes with TBST. Secondary antibody was put on for 30 min then washed three 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.

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.
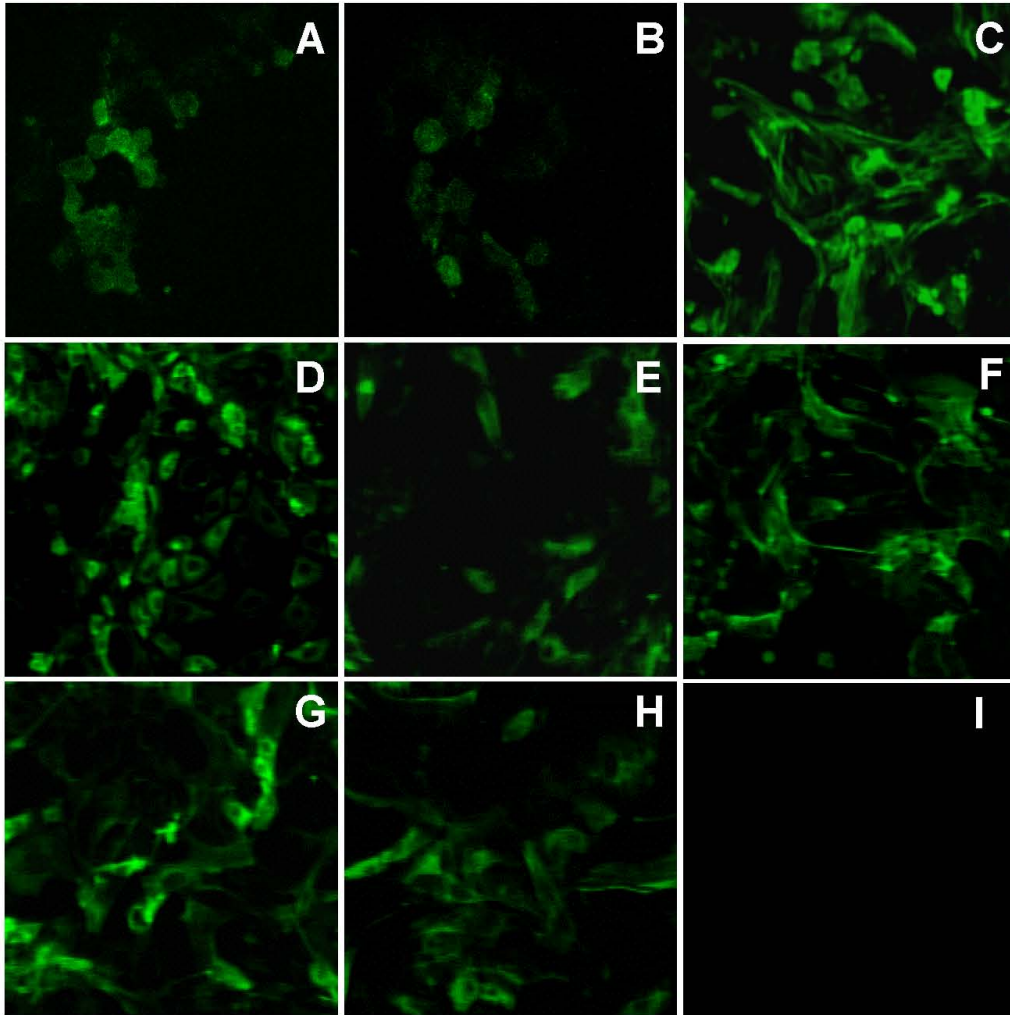
Fig. 2: Cytokeratins 8, 18 and 19 expression in different matrix conditions. Cytokeratin 19 expression were found to be lower in Clara cells from p 2 1 ko mice (B) compared to cell from wt mice (A) at time 0 h. No differences in the expression of cytokeratin 18 was found when cells were cultured on collagen IV © and laminin (D). The expression of cytokeratin 8 was found to be higher when cells from p21 ko mice were cultured on ECM containing laminin eg collagen IV/laminin (F) compared to an ECM without laminin e g fibronectin/collagen IV (E), while no difference in cytokeratin 19 in wt mice eg collagen IV/laminin (G) and fibronectin/collagen IV (H). C, D, E, F, G and H are at time point 72 h. I is a typical negative control, whereby the primary antibody was omitted. Magnification×200

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 ap value less than 0.05 was considered significant.

RESULTS

Cytokeratins are a good marker of epithelial cells differentiation (Gunning *et al.*, 1992; McBride *et al.*, 2000). In this study three cytokeratins 8, 18 and 19 were studied all of which are present in lung epithelial cells including Clara cells. The variation in the degree of cytokeratin expression gives a good indication on the degree of differentiation from one cell type to another to an intermediate cell.

When cells were freshly isolated, thus unattached at day 0, cytokeratins 8, 18 and 19 expressions were found to be lower (p < 0.05) in p21 ko mice when compared to wt mice. (Fig. 1 and 2).

In p21 ko mice, the cytokeratins 8, 18 and 19 expressions at time 0 when cell are freshly isolated and not attached to ECM, were significantly lower (p < 0.05) when compared to attached cells at time 24, 72 and 120 h.

In wt mice, the ECM composition did not influence significantly the cytokeratins 8, 18 and 19 expression and the expression of cytokeratins 8, 18 and 19 did not change significantly over the time in culture.

When Laminin was part of the ECM composition in cultured Clara cells from p21 ko mice, cytokeratins 8 and 19 expressions were significantly higher (p < 0.05) when compared with ECM without laminin.

DISCUSSION

Lower expression of cytokeratins 8, 18 and 19 in unattached Clara cells from p21 ko mice compared to wt mice. Differences in cytokeratin expression in lung cells have been established and have been used as differentiation markers in lung epithelial cells. (Gunning *et al.*, 1992; Moll *et al.*, 1982; Moll, 1987; Blobel *et al.*, 1984). The expression of cytokeratin 8, 18 and 19 in freshly isolated Clara cells from p21 ko mice was significantly lower (p < 0.05) than cultured cells. By disrupting the cell-matrix interactions, in this case during the isolation procedure, the unattached Clara cells from p21 ko mice could be undergoing differentiation and thus cytokeratins 8, 18 and 19 expression was lower.

No significant changes in the expression of cytokeratin 8, 18 or 19 was observed in Clara cells from wt mice upon the cell-matrix disruption and when cultured on different ECM. Thus changes in cell-matrix interactions could be an important factor for cells to undergo differentiation through a p21-dependent pathway. In p21 ko mice, normal differentiation has been observed, thus p21 is not a mutually exclusive agent that promotes differentiation (Paramio *et al.*, 2001; Cox, 1997; McDonald *et al.*, 1996; Deng *et al.*, 1995).

When Clara cells from p21 ko mice were attached and cultured to an ECM which contained laminin, the expression of cytokeratin 8 and 19 was significantly higher (p < 0.05) than cells cultured in the absence of laminin. Thus in the absence of p21, the presence of laminin was found to be quite important for cells to undergo normal differentiation. The actual pathway by which the presence of laminin and p21 are involved in differentiation in Clara cells is still unclear and further studies need to be carried out.

CONCLUSION

Although the presence of laminin was found to be important in cytokeratin 8 and 19 expression in Clara cells from p21 ko mice, the cell-matrix disruption rather than ECM combinations seems to have greater influence on cell cycle progression in the absence of p21. Thus, further studies have to be carried out in order to understand the role of specific integrins such as laminin on Clara cells regulation.

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