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Effect of ECM on Clara Cells' Expression of P21, P27 and P53

[1,2]Renald Blundell and [2]David J. Harrison
[1]Department of Physiology and Biochemistry, University of Malta, Msida MSD06 Malta
[2]Department of Pathology, University of Edinburgh, Medical School, Edinburgh EH8 9AG, Scotland

Abstract: The disruption of cell-matrix interactions influences the expression of a number of cell cycle regulatory proteins such as p21, p27 and p53. In this study, Clara cells obtained form both wildtype (wt) and p21 knockout (p21 ko) mice were isolated and cultured on different ECM combinations and the expression of three cyclin kinase inhibitors p21, p27 and p53 were studied. Distruption of the cell-matrix resulted in a decrease in the expression of cytoplasmic p21 and cytoplasmic p27. Nuclear p53 expression was found to increase in Clara cells lacking p21 and although the variation of ECM combinations did not affect the expression of p53. Although the p27 expression did not significantly changes upon culturing the cells in different compositions of ECM, disruption of the cell-matrix interactions seems to be influential.

Key words: Cell-matrix, wildtype, ECM, cytoplasmic, disruption, interaction

INTRODUCTION

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).

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).

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 $mL.^{-1}$ The effect of ECM composition on Clara expression of Cyclin Kinase Inhibitors (CKI) mainly p21, p27 and p53 were 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 0.5ml pentabarbitone (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, cell were plated onto 16-well glass chamber slides (Gibco) which had been pre-coated with appropriate ECM and incubated and 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

Corresponding Author: Renald Blundell, Department of Physiology and Biochemistry, University of Malta, Msida MSD06 Malta

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

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

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

Antibody against	Concentration	Supplier	Catalogue number
p21	1/10	DAKO	M7207
p53	1/100	Vector Lab	NCL-p53-CM5p
p27	1/200	SIGMA	P2092

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.

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

Three cyclin kinase inhibitors p21, p27 and p53 were studied by immunocytochemistry (Fig. 1-4).

Nuclear p21 expression increased ($p<0.05$) from time 0 to 120 h in culture. Cytoplasmic p21 expression was higher ($p<0.05$) at time 0 when compared to time 24 h.

The expression of cytoplasmic p53 was lower ($p<0.05$) whereas the expression of nuclear p53 was higher ($p<0.05$) in Clara cells from p21 ko mice compared to cells from wt mice. The expression of cytoplasmic p53 is increasing ($p<0.05$) from culture times 24 to 120 h in cells from wtmice. The expression of nuclear p53 is increasing ($p<0.05$) from time 0 to time 120 h in culture in cells from p21 ko mice.

The expression of cytoplasmic p27 was found to be higher ($p<0.05$) in cells from p21 ko mice compared to cells from wt mice at 24 h in culture. No significant differences in the expression of nuclear p27 were observed between cells from wt and p21 ko mice.

No significant differences in the expression of p21, p27 and p53 were observed when Clara cells were cultured on different ECM compositions.

DISCUSSION

Decrease in cytoplasmic p21 upon adherence: In this study an increase ($p<0.05$) in the nuclear p21 expression was observed from time 0 to 120 h in culture. Cytoplasmic p21 expression was higher in unattached Clara cells at time 0 compared to cells at 24 h in culture thus cytoplasmic p21 expression could be increased upon cell-matrix disruption. The functional role of cytoplasmic p21 is still unclear (Asada *et al*., 1999; Tchou *et al*., 1996; Poon and Hunter, 1998; Donato and Perez, 1998; Zhang *et al*., 1999; Jin *et al*., 2000; Gervais *et al*., 2000), thus further studies need to be carried out to understand its functional role. Culturing different Clara cells on a variation of ECM combinations did not significantly affect the expression of p21 thus cell-matrix disruption rather than ECM composition seems to be more important.

Nuclear p53 expression increases in Clara cells lacking p21: The expression of cytoplasmic p53 was lower whereas the expression of nuclear p53 was higher ($p<0.05$) in Clara cells from p21 ko mice as compared to cells from wt mice. Thus it seems that in the absence of p21, there is an increase in p53 stabilisation. The p53 protein is a potent inhibitor of cell growth, arresting the cell cycle at

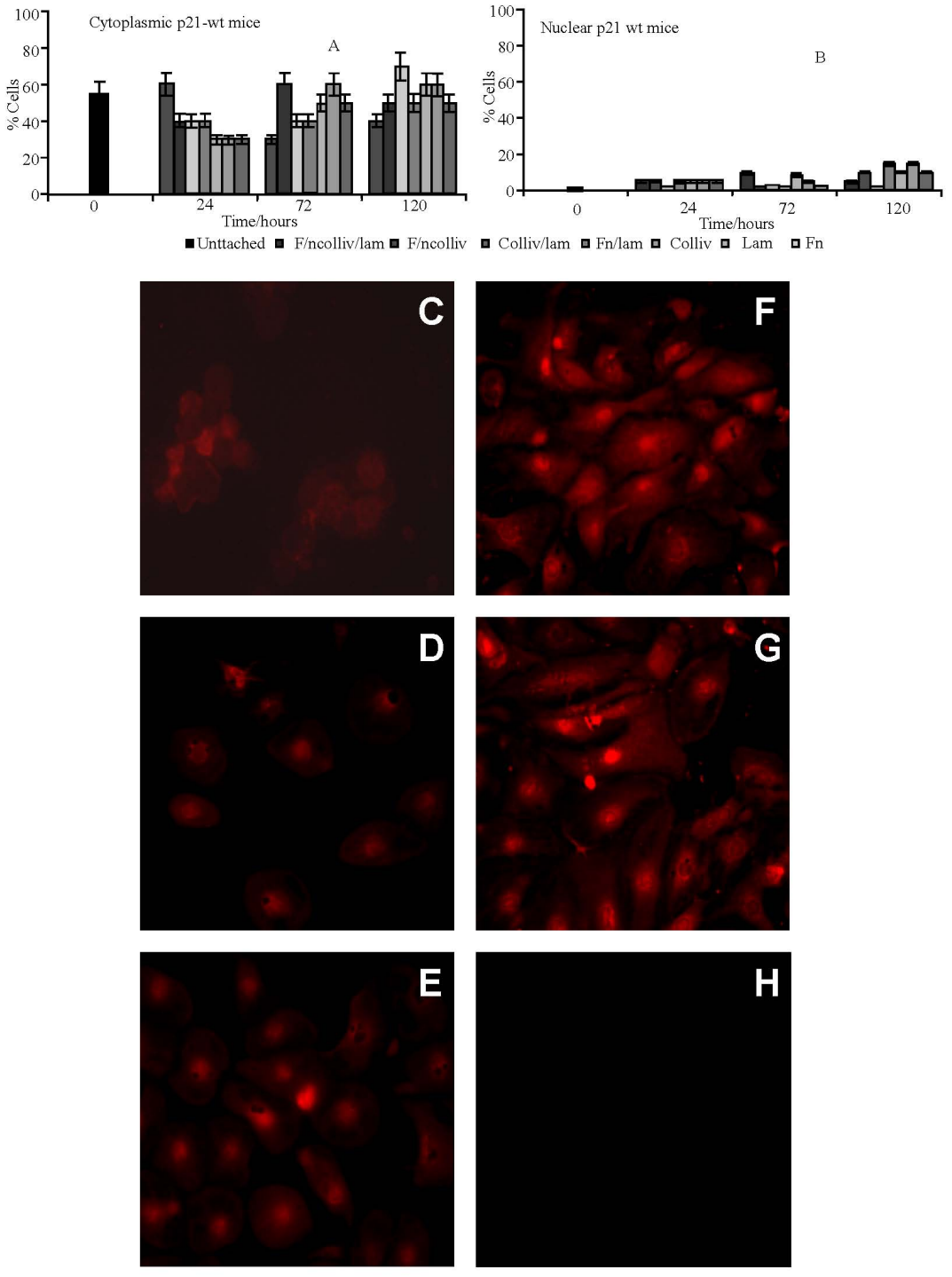

Fig. 1: Nuclear and cytoplasmic p21 expression in Clara cell cultures in different matrix conditions. uclear p21 expression increased ($p<0.05$) from time 0 to 120 h in culture [Graph B, cells at time 0 (C), 24 h (D), 72 h (E), 120 h (F and G). Cytoplasmic p21 expression was higher ($p<0.05$) at time 0 (C) when ompared to time 24 h (A and D). No significant difference in p21 expression was observed when cells were cultured on different ECMs eg laminin (F) and fibronectin/laminin (G). Cells in D and E were ultured on laminin. H is the negative control whereby the primary antibody was omitted. Magnification x 200

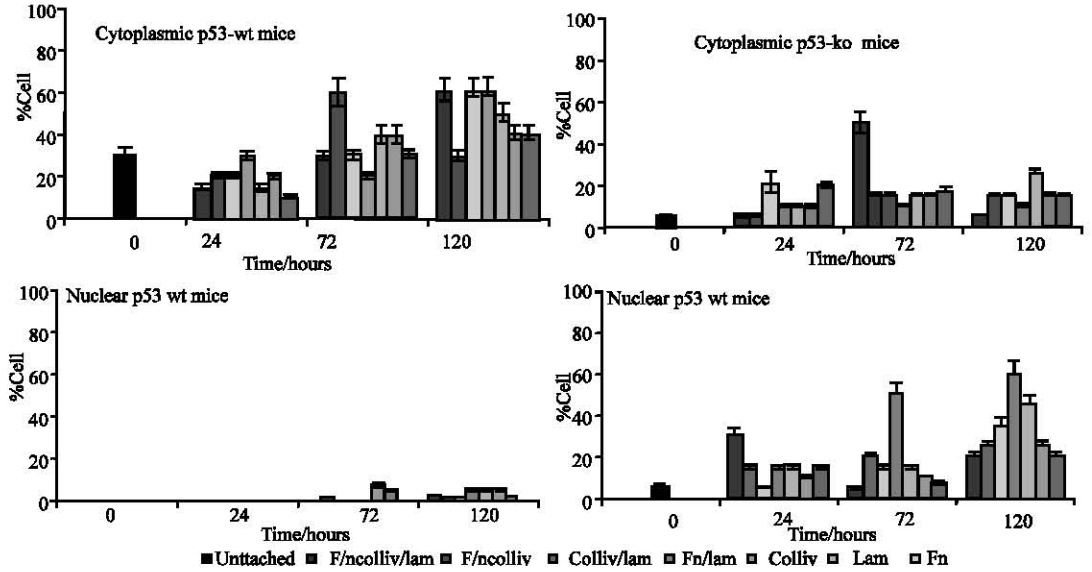

Fig. 2: Cytoplasmic and nuclear p53 expression in Clara cells from wt and p21 ko mice in different matrix conditions. The expression of cytoplasmic p53 was lower ($p<0.05$) (A and B) whereas the expression of nuclear p53 was higher ($p<0.05$) (C and D) in Clara cells from p21 ko mice compared to cells from wt mice. The expression of cytoplasmic p53 is increasing ($p<0.05$) from culture times 24 to 120 h in cells from wt mice (A). The expression of nuclear p53 is increasing ($p<0.05$) from time 0 to time 120 h in culture in cells from p21 ko mice (D). The expression of nuclear and cytoplasmic p53 expression did not significantly when cells were cultured on different ECMs (A-D)

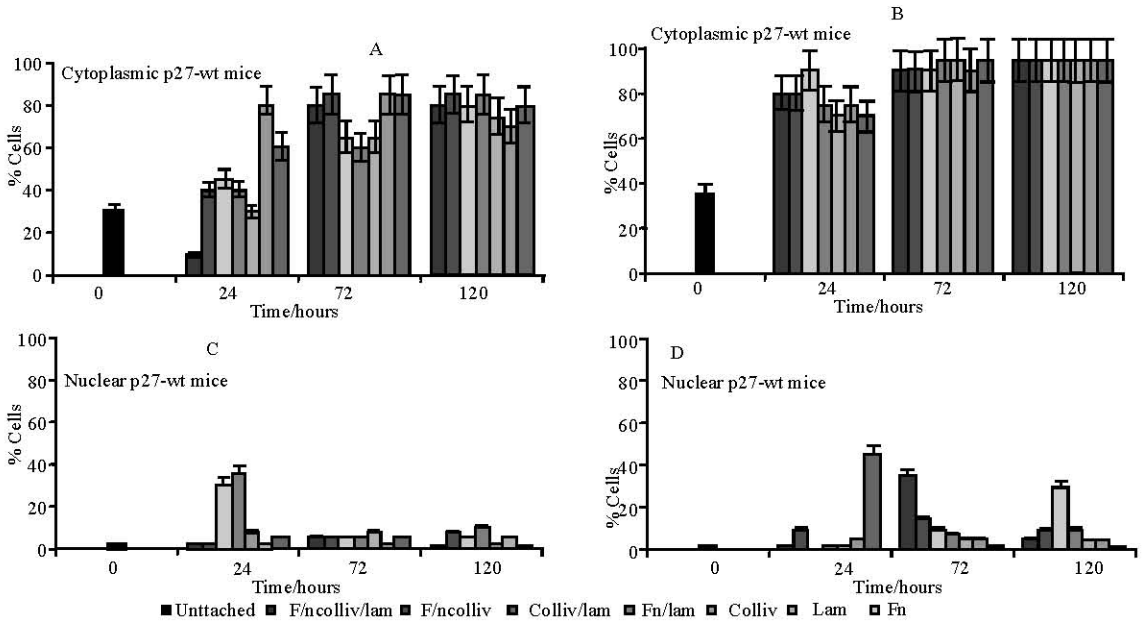

Fig. 3: Cytoplasmic and nuclear p27 expression in Clara cells from wt and p21 ko mice in different matrix conditions. The expression of cytoplasmic p27 was found to be higher ($p<0.05$) in cells from p21 ko mice (A) compared to cells from wt mice (B) at 24 h in culture. No significant differences in the expression of nuclear p27 were observed between cells from wt and p21 ko mice (C and D). No significant differences in the expression of nuclear and cytoplasmic p27 were observed when Clara cells were cultured on different ECMs (A-D)

several points and under some circumstances, activating the apoptotic machinery leading to cell death (Brambilla and Brambilla, 1997; Hupp *et al*., 2000; Ji *et al*., 1997; Jimenez *et al*., 1999; Kaelin, 1999; Kamijo *et al*., 1998;

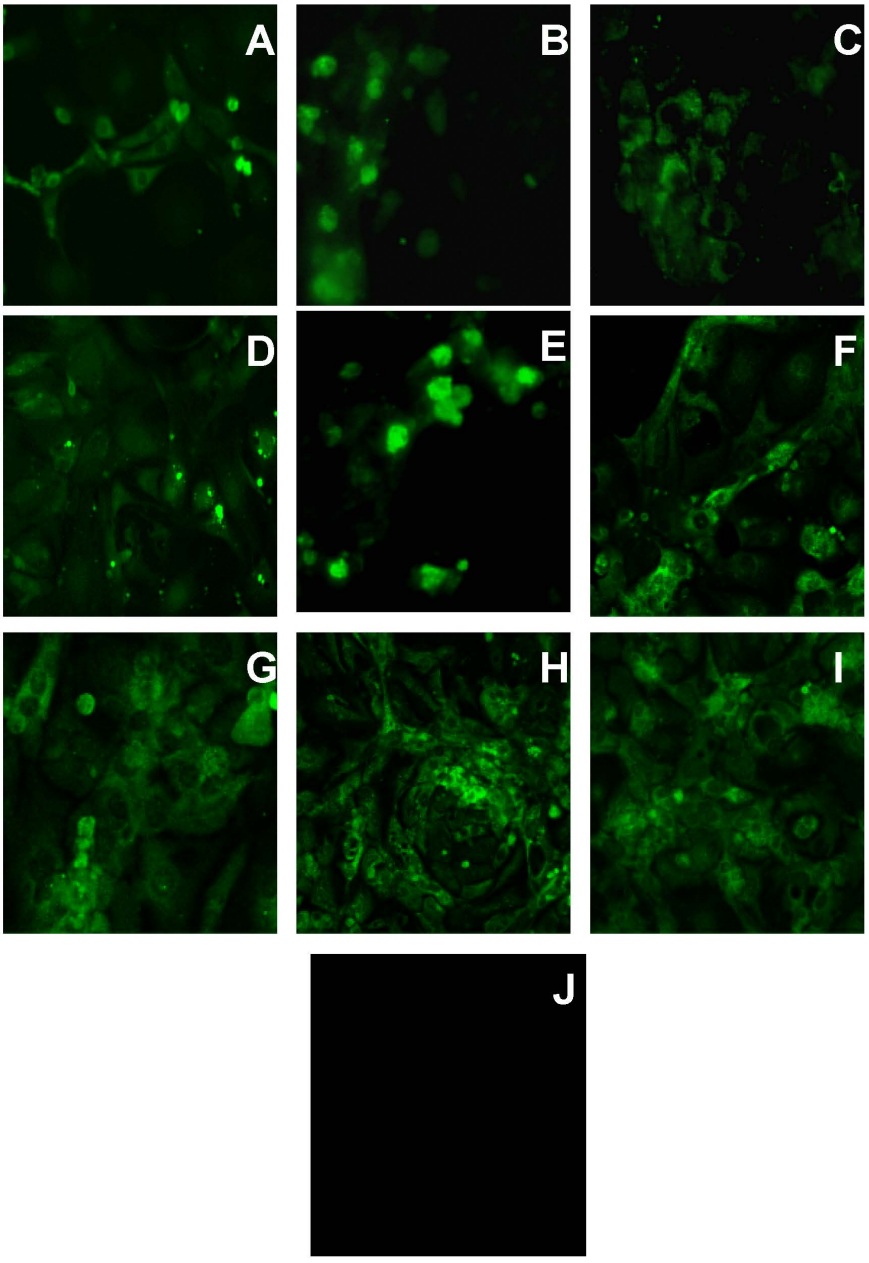

Fig. 4: p27 and p53 expression in Clara cells from wt and p21 ko mice in different matrix conditions. The expression of cytoplasmic p53 was lower ($p<0.05$) whereas the expression of nuclear p53 was higher ($p<0.05$) in Clara cells from p21 ko mice (B) compared to cells from wt mice (A). The expression of cytoplasmic p53 is increasing ($p<0.05$) from culture times 24 hrs (A) to 72 hrs (C) to 120 hrs (D) in cells from wt mice. The expression of nuclear p53 is increasing ($p<0.05$) from 24 hrs (B) to 120 hrs (E) in culture in cells from p21 ko mice. The expression of cytoplasmic p27 was found to be higher ($p<0.05$) in cells from p21 ko mice (G) compared to cells from wt mice (F) at 24 h in culture. No significant differences in the expression of nuclear p27 were observed between cells from wt (H) and p21 ko mice (I) at 120 h in culture. J is a negative control whereby the primary antibody was omitted. Cells in A – E were cultured on Fibronectin/Collagen IV/Laminin while cells in F – J were cultured on Collagen IV. Magnification x 200

Lakin and Jackson, 1999; Lane, 1992; May and May, 1999; Morgenbesser *et al*., 1994; Sigal and Rotter, 2000; Stewart *et al*., 2001; Vaziri and Benchimol, 1999; Vitale *et al*., 1999; Wyllie *et al*., 1994; Yap *et al*., 1999). The role of cytoplasmic p53 is still unclear although cytoplasmic localisation of wildtype p53 has been reported in inflammatory breast carcinoma, colorectal adenocarcinoma, undifferentiated neuroblastoma (Ostermeyer *et al*., 1996; Moll *et al*., 1995), hepatocellular carcinoma and retinoblastoma (Morgenbesser *et al*., 1994). Culturing different Clara cells on a variation of ECM combinations did not affect the expression of p53.

Increase in the cytoplasmic p27 in Clara cells lacking p21 at 24 h in culture: The expression of cytoplasmic p27 was found to be higher ($p<0.05$) in Clara cells from p21 ko mice compared to cells from wt mice at 24 h in culture, but no significant differences in the expression of nuclear p27 were observed in cells from wt and p21 ko mice at any time point in culture. Nuclear p27 negatively regulates G1 progression by binding to cyclin D-cdk 4/6 complexes, cyclin A/cdk2 and cyclin E-cdk 2 preventing their activity (Lloyd *et al*., 1999; Harvat *et al*., 1998; Cheng *et al*., 1999; Rodier *et al*., 2001; Reed *et al*., 1994), but the functional role of cytoplasmic p27 is still unknown although it is thought to be involved in the activation of Cdk 2 complexes (Orend *et al*., 1998; Rodier *et al*., 2001; Levkau *et al*., 1998). Thus the absence of p21 could lead to an increase in cytoplasmic p27 upon cell attachment to ECM and therefore inhibiting G1 progression and maybe lead to cell differentiation. Cytoplasmic p27 was shown previously to be able to inhibit G1 progression (Orend *et al*., 1998; Rodier *et al*., 2001; Tomoda *et al*., 1999; Reynisdottir and Massague, 1997; Soucek *et al*., 1998; Lloyd *et al*., 1999; Nakayama *et al*., 1996; Ishida *et al*., 2000; Resnitzky *et al*., 1995). The p27 expression did not significantly change upon culturing different Clara cells on a variation of ECM combinations, but rather seems to be influenced by the disruption of cell-matrix interactions.

CONCLUSION

In this study a decrease in the cytoplasmic p21 was observed upon Clara cells adherence. Nuclear p53 expression was found to increase in Clara cells lacking p21 and although the variation of ECM combinations did not affect the expression of p53. The expression of cytoplasmic p27 was found to increase in Clara cells lacing p21 at the initial period of the cells culture. Although the p27 expression did not significantly changes upon culturing the cells in different compositions of ECM, disruption of the cell-matrix interactions seems to be influential. 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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