PLAKOGLOBIN-DEPENDENT REGULATION OF KERATINOCYTE APOPTOSIS BY RND3

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Summary

The human epidermis is a self-renewing, stratified epithelial tissue that provides the protective function of the skin. The principal cell type within the epidermis is the keratinocyte and normal function of the epidermis requires that keratinocyte proliferation, differentiation and cell death be carefully controlled. There is clear evidence that signalling through adhesion receptors such as integrins and cadherins plays a key role in regulating epidermal function. Previous work has shown that Rho family GTPases regulate cadherin- and integrin-based adhesion structures and hence epidermal function. In this study we show that a member of this family - Rnd3 - regulates desmosomal cell-cell adhesion in that loss of Rnd3 expression leads to an increase in desmosomes at sites of cell-cell adhesion and altered colony morphology. Loss of Rnd3 expression is also associated with resistance to cisplatin-mediated apoptosis in keratinocytes and this resistance is mediated via the desmosomal protein plakoglobin. We propose a novel plakoglobin-dependent role for Rnd3 in the regulation of keratinocyte cell death.
Introduction

The epidermis is a stratified epithelial tissue made up predominantly of specialised epithelial cells known as keratinocytes. The principal role of the epidermis is to act as a mechanical barrier protecting against infection, UV irradiation and dehydration as well as re-epithelialising during wound healing. Keratinocytes are continuously lost from the outermost layers and the epidermis must continually replenish and maintain itself through proliferation, differentiation and apoptosis (Fuchs, 2007). Keratinocyte proliferation occurs in the basal layer of the normal epidermis whilst the suprabasal layers are formed from keratinocytes undergoing terminal differentiation (Fuchs, 2007; Koster and Roop, 2007). Apoptosis is vital for maintaining normal epidermal homeostasis by the removal of unnecessary, harmful and damaged cells (Lippens et al., 2009).

A major determinant in normal epidermal function is cell adhesion, either to neighbouring cells, or to the underlying extracellular matrix (Muller et al., 2008). In addition to maintaining epidermal architecture, cell-matrix and cell-cell adhesion play key roles in regulating signal transduction and hence keratinocyte function (Muller et al., 2008). Keratinocyte cell-cell adhesion is mediated by a number of different types of adhesion structures but one of the principal adhesion complexes in the epidermis is the desmosome. Desmosomes are multi-protein cell-cell adhesion structures, which act to link the internal keratin intermediate filament cytoskeleton to cadherin family adhesion receptors via linker proteins. Desmosomes are comprised of proteins from at least three distinct gene families: cadherins, armadillo proteins, and plakins (Thomason et al., 2010). All desmosomes contain desmoplakin, plakoglobin and at least one isoform of plakophilin as well as the desmosomal cadherins, desmocollin and desmoglein (Thomason et al., 2010). Desmosome function is linked to the tissue integrity and defects in desmosome assembly and function have been implicated in a number of disease processes including pemphigus vulgaris and arrhythmogenic right ventricular cardiomyopathy and cancer (Thomason et al., 2010). In addition to a mechanical role there is evidence that plakoglobin, an armadillo family member with homology to β-catenin, can function to regulate gene transcription (Karnovsky and Klymkowsky, 1995; Zhurinsky et al., 2000; Garcia-Gras et al. 2006).
Previous work in our lab has demonstrated a role for the RhoA downstream effectors ROCK1 and ROCK2 and their role in regulating keratinocyte adhesion and differentiation (McMullan et al. 2003; Lock and Hotchin, 2009). This study focuses on a ROCK1-interacting protein called Rnd3. Rnd3 (also known as RhoE) is a member of the Rnd family of small GTPases (Foster et al., 1996). Most GTPases cycle between an active GTP-bound state and an inactive GDP-bound state, a process that is regulated by GEFs, GAPs and GDIs (Jaffe and Hall, 2005). However, Rnd3 is an atypical GTPase due to its inability to hydrolyse GTP and is unaffected by GAPs, making it a constitutively activate protein (Foster et al., 2006; Nobes et al., 1998). Rnd3 activity is regulated through post-transcriptional modification, localisation and gene regulation (Riento et al., 2005). Rnd3 has been implicated in the regulation of a number of cellular processes including the regulation of the actin cytoskeleton, proliferation, differentiation, and apoptosis (Guasch et al., 1998; Riento et al., 2003; Villalonga et al., 2004; Bektic et al., 2005; Liebig et al., 2009).

In this study we analyse the function of Rnd3 in human keratinocytes and demonstrate that Rnd3 regulates desmosome function and keratinocyte cell death via a plakoglobin-dependent mechanism.
Results

Keratinocytes exposed to prolonged Y-27632 treatment are protected from cisplatin-induced apoptosis and have decreased Rnd3 expression.

The pharmacological inhibitor Y-27632 inhibits ROCK activity and has been widely used to study the function of these kinases (Ishizaki et al., 2000). As part of an analysis of ROCK function in keratinocyte apoptosis, HaCaT cells were incubated in the presence or absence of 5 μM Y-27632 for 48 hours prior to the addition of cisplatin to induce apoptosis. When treated with Y-27632, HaCaT cells showed a loss of actin stress fibres consistent with Y-27632 being an inhibitor of ROCK1 and ROCK2 (Fig. 1A). To monitor apoptosis in these cells we analysed expression of caspase 9, cleavage of which is a characteristic marker of apoptosis (Slee et al., 1999). In the absence of Y-27632 significant cleavage of caspase 9 was observed when cells were treated with 50 μM cisplatin (Fig. 1B). Similar results were observed when cells were treated with Y-27632 for 24 hours. However, prolonged treatment (48 hours) with Y-27632 resulted in no detectable cleavage of caspase 9 indicating protection from cisplatin-induced apoptosis (Fig. 1B). This would be consistent with previously published data in which knockdown of ROCK1 expression or treatment with Y-27632 protected U2OS cells from camptothecin-induced cell death (Ongusaha et al., 2006).

As Rnd3 is a known ROCK1 binding partner (Riento et al., 2003) and has been implicated in having a role in apoptosis (Bektic et al., 2005; Ongusaha et al., 2006; Boswell et al., 2007; Poch et al., 2007) Rnd3 expression was analysed in cells treated with Y-27632 (Fig. 1C). After 24 hours treatment with Y-27632 a decrease in Rnd3 expression was detected compared to untreated cells. The expression of Rnd3 was further decreased after 48 hours treatment with Y-27632 and remained significantly decreased up to 96 hours. These data demonstrate that prolonged treatment with Y-27632 protects keratinocytes from cisplatin-induced apoptosis and this is accompanied by a significant decrease in Rnd3 expression.
Loss of Rnd3 expression protects keratinocytes from cisplatin-induced apoptosis.

Having observed a decrease in Rnd3 expression following prolonged treatment with Y-27632 we analysed whether the loss of Rnd3 played a role in protection from apoptosis. HaCaT cells in which Rnd3 expression was stably knocked-down using shRNA (HaCaT-shRnd3) were treated with cisplatin for 24 hours prior to lysis and caspase 9 expression was analysed. In control cells, expressing a non-silencing shRNA (HaCaT-NSC), a significant loss of caspase 9 was detected in cells treated with 50 μM cisplatin (Fig. 2A). In contrast, no cleavage of full-length caspase 9 was detected in Rnd3-depleted cells (Fig. 2A). As an alternative method for assessing apoptosis we analysed the number of cells with condensed nuclei (Nakagawa et al. 2000). HaCaT-shRnd3 cells were treated with cisplatin for 24 hours prior to fixation and numbers of cells with condensed nuclei analysed. Treatment of control HaCaT-NSC cells with cisplatin resulted in a significant increase in the percentage of cells with condensed nuclei whereas no significant increase in the numbers of condensed nuclei was observed in Rnd3-depleted keratinocytes treated with cisplatin (Fig. 2B). These data indicate that loss Rnd3 expression protects cells from cisplatin-induced apoptosis.

As an alternative strategy we also used transient transfection of HaCaT cells with siRNA oligos to knockdown expression of Rnd3. (Fig. 2C). These cells were treated with cisplatin for 24 hours and caspase 9 cleavage was analysed by immunoblotting revealing that siRNA-mediated depletion of Rnd3 protects HaCaTs from cisplatin-induced caspase 9 cleavage (Fig. 2C). The percentage of cells with condensed nuclei was also analysed. Treatment with cisplatin resulted in a significant increase in nuclear condensation in control cells transfected with non-silencing control siRNA but no significant increase in nuclear condensation was observed in Rnd3-depleted cells (Fig. 2D).

To confirm that the effects we were seeing were not a consequence of off-target effects we ‘rescued’ Rnd3 expression in HaCaT-shRnd3 cells. To do this we transiently transfected HaCaT-shRnd3 cells with a cDNA construct (pCVM5-FLAG-RhoE), which expresses Flag-tagged wild type murine Rnd3 (Riento et al., 2003). The sequence
targeted by the Rnd3 shRNA is human-specific thus the murine Rnd3 should not be silenced. As a control, HaCaT-shRnd3 cells were transiently transfected with pCMV5-FLAG-EV (empty vector). Cells were treated with cisplatin for 24 hours prior to lysis and expression of caspase 9 and Rnd3 was analysed (Fig. 3A). Consistent with data presented in figure 2 a loss of caspase 9 expression was observed in control HaCaT-shNSC cells transfected with empty vector control and HaCaT-shRnd3 cells transfected with the empty vector control were resistant to cisplatin (Fig. 3A). However, in HaCaT cells transiently expressing murine Rnd3 sensitivity to cisplatin was restored (Fig. 3A). Similar results were observed when we used nuclear condensation as a marker for apoptosis (Fig. 3B). These data demonstrate that reconstitution of Rnd3 expression restores sensitivity to cisplatin-induced apoptosis in keratinocytes and that Rnd3 is required for keratinocyte apoptosis.

**Increased desmosomal expression and function in Rnd3-depleted keratinocytes**

In addition to effects on apoptosis, we also observed that loss of Rnd3 expression resulted in altered colony morphology. When cultured, control cells organised into monolayer colonies characteristic of sub-confluent keratinocyte cultures (Fig. 4B, E). In contrast HaCaT-shRnd3 and HaCaTs transiently transfected with Rnd3 siRNA formed colonies with a much more compact organisation (Fig. 4B, E). To quantitate the change in colony morphology inter-nuclear distances were measured for control and Rnd3 knockdown cells. In both HaCaT-shRnd3 and HaCaTs transiently transfected with Rnd3 siRNA a significant decrease in inter-nuclear distance was observed (Fig. 4C, F).

To analyse whether the ‘compaction’ phenotype observed in Rnd3-depleted cells was related to changes in cell-cell adhesion we analysed expression and localisation of adherens junctions and desmosomes. We found no evidence that loss of Rnd3 expression was accompanied by a change in either expression or distribution of the adherens junction receptor E-cadherin (KRR and NAH unpublished data). However, analysis of expression of desmosomal proteins revealed significant changes in Rnd3-depleted cells. Significant increases in expression of desmocollin 2, desmocollin 3, desmoglein 3 and
desmoplakin I/II was observed in Rnd3-depleted cells (Fig. 5A). This increase in expression of desmosomal proteins was reflected in increased staining for desmoplakin I/II at sites of cell-cell contact, indicating an increase in numbers of desmosomes (Fig. 5B) (Kowalczyk et al., 1997; Niessen, 2007). Cytoplasmic desmoplakin I/II staining was also observed in both cell lines but was increased in Rnd3-depleted cells. This cytoplasmic desmoplakin I/II staining is indicative of the formation of non-membrane bound desmoplakin I/II-containing particles that are known to translocate to sites of cell–cell adhesion before being incorporated into maturing desmosomes (Godsel et al., 2005). Consistent with our observation that long-term treatment of keratinocytes with Y-27632 results in decreased Rnd3 expression (Fig. 1C) we also observe increased expression of desmoglein-3 under the same conditions (Fig. 5C).

A number of studies have demonstrated that a depletion of either plakoglobin or desmoplakin I/II leads to a reduction in numbers of desmosomes and decreased cell-cell adhesion, with no apparent effect on adherens junctions (Gallicano et al., 1998; Bierkamp et al., 1999; Vasioukhin et al., 2001; Acehan et al., 2008). We depleted desmoplakin I/II and plakoglobin using siRNA oligos in Rnd3-depleted keratinocytes (Fig. 6A). Knockdown of either plakoglobin or desmoplakin I/II lead to a reversion of the compaction phenotype observed in Rnd3-depleted cells indicating this phenotype is a consequence of increased desmosomal expression and function in Rnd3-depleted keratinocytes (Fig. 6B, C).

**Loss of plakoglobin expression restores sensitivity to cisplatin-induced apoptosis in Rnd3-depleted keratinocytes.**

Having observed that loss of Rnd3 protected cells from cisplatin-induced cell death we asked the question of whether protection from apoptosis in these cells was linked to increased desmosomal expression and function. We used siRNA oligos to deplete plakoglobin and desmoplakin I/II expression in Rnd3-depleted cells (Fig. 7A). These cells were treated with cisplatin for 24 hours and the level of apoptosis analysed (Fig. 7B). Consistent with previous experiments, loss of Rnd3 expression was associated with resistance to cisplatin (Fig. 7B). In cells where both Rnd3 and desmoplakin I/II were
deleted a similar resistance to cisplatin was observed but in cells where both Rnd3 and plakoglobin were depleted sensitivity to cisplatin was restored (Fig. 7B). Knockdown of plakoglobin or desmoplakin I/II expression alone had no effect on the response to cisplatin with comparable levels of cell death to that seen in control cells (Fig. 7B). Thus, these data indicate that Rnd3 regulates keratinocyte apoptosis in a plakoglobin-dependent manner.
Discussion

Loss of Rnd3 expression protects keratinocytes from cisplatin-induced apoptosis.

Apoptosis is a highly regulated process that is critical for normal epidermal homeostasis and we observed that prolonged treatment of keratinocytes with the ROCK1/2 inhibitor Y-27632 protected keratinocytes from cisplatin-induced apoptosis. Caspase-mediated cleavage of ROCK1 is known to induce membrane blebbing, nuclear fragmentation and packaging of nuclear material into blebs at the cell surface and treatment of cells with the ROCKI/II inhibitor, Y-27632, has been shown to prevent ROCK1-induced membrane blebbing (Coleman et al. 2001; Sebbagh et al., 2001). Similarly, Y-27632 has also been shown to protect U2OS cells from camptothecin-induced apoptosis and reduce endotoxin-induced apoptosis in the liver (Ongusha et al. 2006; Thorlacius et al. 2006). Rnd3 is a known substrate for ROCK1 and we observe that, in addition to protecting cells from apoptosis, prolonged treatment with Y-27632 also results in a decrease in Rnd3 expression. Consistent with this, RNAi-mediated depletion of Rnd3 protects keratinocytes from cisplatin-induced apoptosis suggesting that Rnd3 is required for apoptosis. There are a number of reports that implicate Rnd3 in regulation of apoptosis; increased expression of Rnd3 has been reported in cells treated with various apoptosis-inducing stimuli and over-expression of Rnd3 increases the basal rate of apoptosis (Villalonga et al., 2004; Bektic et al., 2005; Poch et al., 2007). However, it should be noted that other reports have described a pro-survival function for Rnd3 whereby use of siRNA to prevent Rnd3 induction by apoptotic agents lead to cell death (Ongusaha et al., 2006; Boswell et al., 2007). We did not observe an increase in Rnd3 expression following treatment with cisplatin and one possible explanation for this might be the p53 status of HaCaT cells. Rnd3 is known to be a transcriptional target of p53 and increased Rnd3 expression has been reported following DNA damage (Villalonga et al., 2004; Ongusaha et al., 2006). HaCaT cells are known to have mutations in both alleles of their p53 gene, resulting in p53 having an extended half-life (Lehman et al., 1993). However, p53 is still induced in HaCaTs following DNA damage suggesting it is still functional (Henseleit et al., 1997; Boswell et al., 2007).
Loss of Rnd3 expression is associated with altered colony morphology

In previous studies we described a role for ROCK1 and ROCK2 in the regulation of keratinoyte differentiation (McMullan et al., 2003; Lock and Hotchin, 2009). Rnd3 is known to interact with ROCK1 and a recent study reported that knockdown of Rnd3 expression was associated with hyperproliferation and that keratinocytes over-expressing Rnd3 showed increased stratification - a key feature of keratinocyte differentiation (Liebig et al., 2009). Whilst we observed that loss of Rnd3 expression resulted in altered colony organisation we find no evidence that this phenotype is associated with changes in the differentiation status of these cells (KRR and NAH unpublished data). These differences in findings may be a consequence of different culture conditions. The data of Liebig et al were obtained using primary keratinocytes, whereas our data were obtained using an immortalised cell line. Alternatively, it may be a consequence of the cells in the Liebig study being cultured in low calcium medium, conditions in which cadherin-mediated cell-cell adhesion is compromised (Chitaev and Troyanovsky, 1997).

Cell-cell adhesion is important for maintaining epidermal architecture and whilst we observed no apparent effect on adherens junction expression and localisation we observed increased expression of most of the components required for desmosomal assembly in Rnd3-depleted cells (Kowalczyk et al., 1997). This increase in expression of desmosomal components was reflected in increased desmosomal staining at sites of cell-cell contact. Disruption of desmosomes using RNAi to knockdown expression of either desmoplakin I/II or plakoglobin resulted in a reversion to control colony phenotype confirming that the ‘compaction’ phenotype was a consequence of increased desmosomal function.

The mechanism by which loss of Rnd3 results in increased expression of desmosomal proteins and an increase in desmosomes at sites of cell adhesion is not known. Whilst desmosomes are well-studied structures, relatively little is known about how their assembly is regulated. There is evidence that the translocation of cytoplasmic non-membrane bound desmoplakin-containing particles to desmosomes is an actin-dependent process (Godsel et al., 2005). One possible mechanism whereby Rnd3 might regulate desmosomes is through RhoA-dependent regulation of the actin cytoskeleton and
activation of RhoA has been shown to accelerate the initial translocation of desmoplakin I/II to desmosomes, although sustained RhoA activity compromised desmosomal maturation (Godsel et al., 2010). Consistent with this, inhibition of RhoA results in loss of desmosomes and keratinocyte dissociation (Waschke et al., 2006). Rnd3 is known to regulate RhoA activity through p190RhoGAP and the RhoA GEF Syx (Wennerberg et al., 2003; Goh and Manser, 2010). RhoA activity during the redistribution of desmoplakin I/II to desmosomes is not affected by p190RhoGAP (Godsel et al., 2010) but this still leaves the possibility that Rnd3 is regulating RhoA through Syx or through some unknown Rnd3 effector.

Desmosomes and apoptosis in Rnd3-depleted cells

Cell-cell adhesion is essential for maintaining epidermal architecture and mechanical strength as well as regulating proliferation, differentiation and apoptosis (Muller et al., 2008). We demonstrated that knockdown of plakoglobin expression disrupted desmosomes, reversed the compaction phenotype and restored sensitivity to cisplatin in Rnd3-depleted keratinocytes. However, despite the fact that knockdown of desmoplakin I/II expression reversed the compaction phenotype in Rnd3-depleted cells, it did not restore sensitivity to cisplatin. This suggests that the resistance to cisplatin-mediated apoptosis in Rnd3-depleted keratinocytes is a function of plakoglobin, rather than desmosomal cell-cell adhesion per se. However, we cannot rule out the possibility that the desmosomes sequester plakoglobin and thus influence plakoglobin signaling in a manner analogous to that described in adherens junctions with β-catenin (Gottardi et al., 2002).

In additional to its desmosomal function, plakoglobin is known to regulate gene expression and one possibility is that plakoglobin regulates expression of pro-survival genes. This would be consistent with data implicating plakoglobin in the development of squamous cell carcinoma through induction of Bcl-2 (Hakimelahi et al., 2000). Nuclear accumulation of plakoglobin and an increase in Bcl-2 expression has also been observed in advanced prostate cancer (Shiina et al., 2005). However, other evidence suggests that
plakoglobin functions as a tumour suppressor with loss of heterozygosity being linked to poor clinical outcomes in breast, ovarian and small cell lung cancers (Aberle et al., 1995; Winn et al., 2002).

In summary, loss of Rnd3 expression protects keratinocytes from apoptosis via a plakoglobin-dependent mechanism. Reduced expression of Rnd3 has been reported in a number of different tumours (Bektic et al., 2005; Chen et al., 2011) and the data presented here suggest that Rnd3 may function as a tumour suppressor gene.
Materials and Methods

Cell Culture

The human keratinocyte cell line HaCaT (Boukamp et al. 1988) was cultured in DMEM supplemented with 50 units/ml penicillin, 50 ng/ml streptomycin (Gibco) and 5% FBS (Sigma). Cells were maintained at 37°C in a humidified 5% CO₂ atmosphere. In some experiments the ROCK inhibitor Y-27632 (Sigma, UK) was added at 12 hour intervals to cell cultures at 5μM.

Generation of Rnd3-depleted keratinocyte cell lines

To generate stable Rnd3-depleted (HaCaT-shRnd3) and control (HaCaT-shNSC) keratinocyte cell lines HaCaT cells were transfected with the shRNAmir human constructs pGIPZ-Rnd3 (Cat. No. RHS4430-98513300) and pGIPZ-NSC (non-silencing control) (Cat. No. RHS4346) (purchased from Open Biosystems) using the Amaxa Nucleofector kit V (Amaxa Biosystems Inc.). For each transfection 3x10⁶ cells/ml and 3 μg of DNA were used and transfections were performed according to the manufacturers instructions. Polyclonal stably-transfected cell lines were established and maintained in puromycin (0.5 μg/ml, Sigma, UK).

Reconstitution of Rnd3 expression

To rescue the expression of Rnd3 HaCaT-shNSC and HaCaT-shRnd3 keratinocytes were transiently transfected with pCVM5-FLAG-RhoE or pCMV5-FLAG using Lipofectamine 2000 (Invitrogen) according to the manufacturers instructions. pCMV5-FLAG-RhoE and pCMV5-FLAG constructs were a gift from A. Ridley (King's College London) (Riento et al., 2003).

Transient transfection of siRNA oligos
HaCaT cells were transiently transfected with RNAi oligos using Lipofectamine RNAiMAX (Invitrogen Cat. No. 13778) as described in the manufacturer’s instructions. Two custom siRNA oligos against Rnd3 were purchased from Dharmacon (oligo A UAGUAGAGCUCUCCAAUCA and oligo B CAAACAGAUUGGAGCAGCU). All Rnd3 knockdown experiments were verified using both oligos. siRNA oligos against plakoglobin and desmoplakin were purchased from Dharmacon (J-011708-10 and J-019800-07 respectively). Non-silencing control (NSC) oligos were purchased from Quiagen (SI03650325). Concentrations of oligo used in each transfection were as follows: Rnd3 A and B oligos were 9 pmol; plakoglobin and desmoplakin were 18 pmol. The concentration of NSC oligo corresponded to the highest concentration used in each experiment.

**Cell lysis and immunoblotting**

Adherent HaCaT cells were scrape-lysed in 3x Laemelli buffer as described previously (Lock and Hotchin, 2009). In experiments where cells were treated with cisplatin, culture media was also collected and centrifuged at 14000 rpm for 1 min to pellet detached cells and subsequently added to the adherent cell lysate prior to boiling. SDS-PAGE and immunoblotting were carried out as described previously (Lock and Hotchin, 2009).

**Antibodies**

Sources of antibodies used in this study are: caspase 9 (Cell Signaling Technology); desmocollin 2, desmocollin 3, desmoglein 3 (Progen); desmoplakin (Santa Cruz); plakoglobin - VB3 (a gift from Vania Braga, Imperial College, London, UK); RhoE (Upstate); tubulin (Sigma); Alexa-594-conjugated Phalloidin (Molecular Probes); Fluorophore-conjugated secondary antibodies were purchased from Jackson Labs (Stratech Scientific, Suffolk, UK).

**Microscopy**
Indirect immunofluorescent staining was performed as described previously (Akhtar et al., 2000). Use of Hoecht 33342 to identify and analyse the percentage of cells with condensed nuclei was carried out as previously described (Nakagawa et al., 2000). Cells were visualised using a Leica DMRB microscope equipped with a Hamamatsu ORCA camera. The images were captured and processed using OpenLab software (Improvision, UK). Phase contrast microscopy was carried out using an Axiovert 135TV microscope (Zeiss) equipped with a QCam FAST1394 camera (Qimaging). Images were captured and processed using QCapture (Qimaging). To measure inter-nuclear distances cells were transiently transfected with RNAi oligos and seeded 48 hours prior to phase microscopy. The distance between neighbouring nuclei was measured using ImageJ 1.42q. Measurements were taken using the line tool set to no scale with a pixel aspect ratio of 1.

**Induction and analysis of apoptosis**

Cisplatin (cis-Diammineplatinum(II)_dichloride) (Sigma, UK), was used to induce apoptosis (Walczak and Krammer, 2000). Both stably and transiently Rnd3-depleted cells were cultured for at least 24 hours before being treated with cisplatin (concentrations specified in text). Apoptosis was assessed by using immunoblotting to analyse caspase 9 cleavage (Slee et al., 1999) and stained with Hoechst 33342 and the percentage of cells with condensed nuclei calculated (Nakagawa et al., 2000).
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References


Figure Legends

Figure 1. Prolonged Y-27632 treatment protects keratinocytes from cisplatin-induced apoptosis and decreased Rnd3 expression.

A. HaCaT cells were treated with 5 μM Y-27632 for 24 hours before cells were fixed and stained with Alexa-594-conjugated phalloidin to visualise F-actin (bar = 30 μm). B. HaCaT cells were treated with 5 μM Y-27632 for 0, 24 and 48 hours. 24 hours prior to lysis cells were treated with cisplatin to induce apoptosis. Whole cell lysates were prepared and immunoblotted with an antibody to full-length caspase 9, loss of which indicates induction of apoptosis. Immunoblotting with an antibody to tubulin was used as a loading control. C. Keratinocytes were treated with 5 μM Y-27632 for up to 96 hours before lysis and immunoblotting with antibodies to Rnd3 and tubulin (loading control). All images and blots are representative of a minimum of 3 independent experiments.

Figure 2. Loss of Rnd3 expression protects keratinocytes from cisplatin-induced apoptosis.

HaCaT cells stably expressing Rnd3 (shRnd3) and control (shNSC) shRNA (A, B) or transiently transfected with siRNA oligos against Rnd3 (siRnd3) and a non-silencing control (siCtrl) (C, D) were treated with cisplatin for 24 hours. Whole cell lysates prepared and immunoblotted with antibodies to Rnd3 to confirm knockdown, full-length caspase 9 to assess apoptosis and tubulin as a loading control (A, C). Cells were also cultured on glass coverslips prior to fixation and staining with Hoechst 33342 to visualise nuclei (B, D). The percentage of cells with condensed nuclei (indicated by arrows in E) was calculated for each treatment (minimum of 400 cells per sample). Data presented in B and D are the mean and standard error from three separate experiments (* p<0.05, *** p<0.001). Representative blots from a minimum of 3 independent experiments are shown in A and C. The data shown in C and D are from experiments using oligo A; similar results were obtained using oligo B.
Figure 3. Reconstitution of Rnd3 expression restores sensitivity to cisplatin-induced apoptosis in keratinocytes.

To reconstitute Rnd3 expression, stable Rnd3-depleted (shRnd3) and control (shNSC) HaCaT cells were transiently transfected with a cDNA vector expressing Flag-tagged wild type murine Rnd3 (pCMV5-FLAG-RhoE) or an empty vector control (pCMV5-FLAG). A. Cells were treated with cisplatin for 24 hours prior to lysis and immunoblotting for full-length caspase 9, Rnd3 and tubulin. Expression of exogenous murine Rnd3 is indicated by the arrow. A representative blot from 3 independent experiments is shown. B. Transfected cells were plated onto glass cover slips, treated with cisplatin and stained with Hoechst 33342 to visualise nuclei. A minimum of 400 cells were counted per sample and the data presented are the mean and standard error of three separate experiments (**p<0.01).

Figure 4. Loss of Rnd3 expression in keratinocytes results in altered colony morphology.

Stably (A-C) and transiently (Oligo A) (D-F) Rnd3-depleted keratinocytes were immunoblotted using antibodies against Rnd3 to confirm knockdown of Rnd3 expression (A, D) and imaged using phase contrast microscopy (B, E) (bar = 30 μm). All images and blots are representative of a minimum of 3 independent experiments. To assess colony structure the mean inter-nuclear distance was analysed using Image J (C, F). A minimum of 45 inter-nuclear distances were calculated for each condition and data are the mean and standard error of 3 independent experiments (** p < 0.01; *** p<0.001). Similar results were obtained using oligo B to transiently knockdown expression of Rnd3.

Figure 5. Loss of Rnd3 expression increases desmosomal protein expression and localisation to sites of cell-cell contact.

Keratinocytes transiently transfected with siRNA oligos against Rnd3 (siRnd3) or a non-silencing control (siCtrl) and cultured in normal growth medium. A. Cells were stained with antibodies against desmoplakin I/II to visualise desmosomes (bar = 30 μm). B.
Whole cell lysates were prepared and immunoblotting using antibodies against desmocollin 2, desmocollin 3, desmoglein 3, desmoplakin I/II and plakoglobin. Expression of Rnd3 was analysed to confirm knockdown and tubulin was used as a loading control. C. Keratinocytes were treated with 5 μM Y-27632 for up to 72 hours before lysis and immunoblotting with antibodies to desmoglein 3 and tubulin (loading control). All images and blots are representative of a minimum of 3 independent experiments. The data shown in A and B are from experiments using oligo A; similar results were obtained using oligo B.

Figure 6. RNAi-mediated disruption of desmosomes restores normal colony morphology in Rnd3-depleted cells.

HaCaT cells were transiently transfected with siRNA oligos against Rnd3 (siRnd3), desmoplakin I/II (siDsp), plakoglobin (siPG), individually or in combination and cultured for 72 hours in normal growth medium. A. Whole cell lysates were prepared and immunoblotted with antibodies to desmoplakin I/II, plakoglobin, Rnd3 and tubulin (loading control). B. Cells were imaged using phase contrast microscopy (bar = 30 μm). All images and blots are representative of a minimum of 3 independent experiments. C. To assess colony structure the mean inter-nuclear distance was analysed using Image J. A minimum of 45 inter-nuclear distances were calculated for each condition and data are the mean and standard error of 3 independent experiments (NS, not significant; ** p < 0.01; *** p<0.001). The data shown are from experiments using oligo A; similar results were obtained using oligo B.

Figure 7. Loss of plakoglobin expression restores sensitivity to cisplatin-induced apoptosis in Rnd3-depleted keratinocytes.

HaCaT cells were transiently transfected with siRNA oligos against Rnd3 (siRnd3), desmoplakin I/II (siDsp), plakoglobin (siPG), individually or in combination and cultured for 72 hours in normal growth medium. A. Whole cell lysates were prepared and immunoblotted with antibodies to desmoplakin I/II, plakoglobin, Rnd3 and tubulin.
A representative blot from 3 independent experiments is shown. **B.** Cells cultured on glass coverslips were treated with 50 μm cisplatin for 24 hours prior to fixation and staining with Hoechst 33342 to visualise nuclei. For each sample a minimum of 400 cells was counted and data presented are the mean and standard error of three separate experiments (**p<0.01, *** p<0.001). The data shown are from experiments using oligo A; similar results were obtained using oligo B.
Figure 1

A

- Y-27632

+ Y-27632

B

Caspase 9
Tubulin
Cisplatin [μM]

Y27632 (hr)

0 25 50 0 25 50 0 25 50

0 24 48

0 24 48 72 96 +Y27632 (hrs)

Tubulin

Rnd3
Figure 5

A

Desmoplakin I
Desmoplakin II
Desmocollin 2
Desmocollin 3
Desmoglein 3
Plakoglobin
Tubulin
Rnd3

siCtrl siRnd3

B

siCtrl

siRnd3

C

0 24 48 72 + Y27632 (hr)

Desmoglein 3

Tubulin
Figure 6

A

Desmoplakin I
Desmoplakin II
Plakoglobin
Tubulin
Rnd3

+  -  -  -  -  -  siCtrl
-  -  +  -  -  +  siDsp
-  -  -  +  -  +  siPG
-  -  -  -  +  +  siRnd3

B

siCtrl
siRnd3
siDsp
siRrd3
siDsp
siRnd3 siDsp
siPG
siRnd3 siPG

C

Mean inter-nuclear distance (AU)

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