

Published in final edited form as:

J Membr Biol. 2007 August ; 218(1-3): 29–37. doi:10.1007/s00232-007-9025-0.

A Deafness Associated Mutant Human Connexin 26 Improves The Epithelial Barrier *In Vitro*

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Abstract

A large proportion of recessive non-syndromic hearing loss is due to mutations in the *GJB2* gene encoding connexin 26 (Cx26), a component of a gap junction. Within different ethnic groups there are specific common recessive mutations each with a relatively high carrier frequency suggesting a possibility of heterozygous advantage. Carriers of the R143W *GJB2* allele, the most prevalent in the African population, present with a thicker epidermis than non-carriers. In this study, we show (R143W)Cx26 expressing keratinocytes form a significantly thicker epidermis in an organotypic co-culture skin model. In addition, we show increased migration of cells expressing (R143W)Cx26 compared to (WT)Cx26 overexpressing cells. We also demonstrate that cells expressing (R143W)Cx26 are significantly less susceptible to cellular invasion by the enteric pathogen *Shigella flexneri* than (WT)Cx26 expressing cells. These *in vitro* studies suggest an advantageous effect of (R143W)Cx26 in epithelial cells.

Introduction

It has been suggested that intercellular communication via gap junctions play a role in keratinocyte differentiation and wound healing by regulating the passage of ions, small metabolites and second messenger molecules (< 1 kDa) (Goliger & Paul, 1995). Specific connexins are expressed at different layers (or strata) of the normal human epidermis. One of the predominant connexins is connexin 43 (Cx43) which is expressed throughout the suprabasal layers. Extremely low levels of Cx26 are expressed in the basal layer and similarly Cx30 is sparsely expressed in the basal and granular layer (Di et al., 2001; Lucke et al., 1999). The expression and distribution of these epidermal connexins alter during various stages of wound healing. After wounding rat epidermis *in vivo*, Cx26 expression was upregulated in the differentiated cells proximal to the wound but was downregulated in the cells at the wound edge. Using dye transfer analysis the changes in connexin expression corresponded to changes in junctional communication (Goliger & Paul, 1995). In addition,

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the enteric pathogen *Shigella flexneri* can open Cx26 hemichannels allowing the release of ATP (Tran Van Nhieu et al., 2003) suggesting Cx26 may play a role in bacterial cell entry.

Distinct dominant mutations in *GJB2* encoding Cx26 can produce an array of ectodermal phenotypes including hearing loss, neuropathy, hair growth abnormalities and hyperkeratosis (Richard, 2005). These disease associations support an important function for this gap junction protein in epidermal differentiation. Although dominant *GJB2* mutations can result in syndromic disease, recessive *GJB2* mutations only cause hearing loss (Kelsell et al., 1997). Subsequently numerous studies have shown that *GJB2* mutations account for a significant proportion of recessive genetic deafness (Hutchin et al., 2005; Kenneson, Van Naarden Braun & Boyle, 2002; Snoeckx et al., 2005). Within different ethnic groups there are specific common recessive mutations that account for the majority of *GJB2*-related hearing loss, for example 35delG, 235delC and R143W in the European, Japanese and African populations respectively (Brobbly, Muller-Myhsok & Horstmann, 1998; Denoyelle et al., 1997; Kudo et al., 2000). Each ethnic or geographic population has a carrier frequency of between 1-3 % suggesting a possibility of heterozygous advantage. Although there is no clinically defined dermatological signs, data generated from skin biopsies has shown that heterozygotes for the R143W mutation in *GJB2* have a thicker epidermis than wild-type (WT) *GJB2* homozygotes (Meyer et al., 2002). Enhanced cellular viability of HeLa cells overexpressing (R143W)Cx26 compared to those overexpressing (WT)Cx26 has also been shown (Common et al., 2004).

In this study, we demonstrate *in vitro* an extended keratinocyte differentiation programme and increased migration of (R143W)Cx26 expressing cells compared to the wild-type counterpart. We also demonstrate that HeLa cells expressing this Cx26 mutant do not increase cellular invasion by the enteric pathogen *S. flexneri* unlike that of (WT)Cx26 expressing cells.

Materials and Methods

All reagents were supplied from Sigma (Poole, Dorset, UK) unless otherwise stated.

Antibodies

Monoclonal keratin 10 (LH2), involucrin (Sy5), keratin 14 (LL002), keratin 6 (LHK6) and keratin 16 (LL025) antibodies were all generated by CRUK, United Kingdom. Transglutaminase 1 (TG1) (Biogenesis), keratin 2e (Abcam), loricrin (Covance Research Products) and Ki67 (DAKO) antibodies were obtained commercially.

Plasmid constructs

Full length cDNA of (WT)Cx26, (R143W)Cx26, (WT)Cx30 and (WT)Cx31 fused with EGFP, together with EGFP alone, was cloned and inserted into pSIN retroviral vector (Deng, Lin & Khavari, 1997). All clones were verified by automated sequencing using ABI 3700 (Genome Centre, Queen Mary University, London).

Culture of nTERT keratinocytes and HeLa cells

The human cell line nTERT was derived from primary keratinocytes immortalised by telomerase (Dickson et al., 2000). The culture medium consisted of E4 Ham's medium containing 10 % (v/v) foetal calf serum, 2mM glutamine, 1 % (v/v) RM + supplement (final concentration of 400 ng/ml hydrocortisone, 5 µg/ml insulin, 10 ng/ml EGF, 8.4 ng/ml cholera toxin, 5 µg/ml transferrin, 13 ng/ml lyothyronine), 50 units/ml penicillin-G and 50 µg/ml streptomycin sulphate. HeLa cells (ATCC) were cultured in DMEM containing 10 %

(v/v) foetal calf serum, 2mM glutamine, 50 units/ml penicillin-G and 50µg/ml streptomycin sulphate.

Retroviral transduction

A retroviral transduction protocol was carried out in order to create nTERT or HeLa cells that overexpress (WT)Cx26-EGFP, (R143W)Cx26-EGFP, (WT)Cx30-EGFP, (WT)Cx31-EGFP or EGFP alone. Phoenix 293 viral packaging cells were cultured in DMEM medium as described above. They were transfected with the plasmid constructs using a 1:3 ratio of DNA:Fugene 6 reagent (Roche). After 24 hours, cells were selected with 1 µg/ml puromycin for 48 hours at 37°C. The media was replaced and the cells incubated at 32°C for 24 hours before the virus-containing supernatant was collected, filter-sterilised and snap frozen in liquid nitrogen. All retrovirus collections were stored at -80°C until ready for use.

For retroviral transduction, the virus was prepared in a glass container whereby 5 µg/ml (final concentration) polybrene reagent was added and incubated at room temperature for 15 minutes. Keratinocytes were also treated with polybrene by adding a final concentration of 5 µg/ml to the normal culture medium. These cells were transferred to 37°C. After 15 minutes incubation, the polybrene-containing media was replaced with polybrene-treated virus. Cells were centrifuged at 350 × g, 32°C for 1 hour. Upon completion of the centrifugation step, the virus was replaced with fresh normal culture media and returned to 37°C/10 % CO₂. After 24-48 hours, cells were viewed under a UV lamp microscope to confirm expression. In all cases, transduction efficiency was comparable between constructs.

Connexin Localisation

Transduced nTERT cells were plated onto coverslips at a suitable density, and after 24-hours fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30-minutes. The cells were washed in PBS, and mounted using Immu-Mount (Thermo Electron Corporation) containing 10 µg/ml DAPI, before being visualised by laser scanning confocal microscopy (LSM510, Carl Zeiss).

Organotypic co-culture

Organotypic co-culture was carried out following the protocol described by Ojeh *et al.* (2001).

Dye injection

The growing epidermis was removed from the dermis raft using fine forceps, and suspended in low melting point agarose. Small blocks of tissue in agarose were mounted on a vibratome and sections cut at 200µm. Lucifer yellow (LY; 4% in 3M LiCl) was microinjected into cells using microelectrodes with resistance ~100MΩ. Following LY injection slices were fixed in 4% paraformaldehyde in PBS for 30 minutes. Slices were then permeabilized (0.1% Triton-X) and blocked (10% normal goat serum), and subsequently incubated in an anti-LY antibody overnight at 4°C. Following several washes in PBS, the anti-LY antibody was detected using an anti-rabbit FITC-conjugated secondary antibody. Slices were mounted in an anti-fade medium containing DAPI (Vectashield, Vector Laboratories). Immunofluorescence was detected by laser scanning confocal microscopy (LSM510, Carl Zeiss). The number of cells containing LY were counted for each injection site. Data from tissue transfected with (WT)Cx26 were compared to that from (R143W)Cx26 transfected tissue using Student's unpaired T-test (Prism v4; Graphpad Software Inc.).

Co-culture staining

Each tissue was cut in half such that one half was snap frozen whilst embedded in Cryo-M-Bed™ (Bright) and was stored at -80°C . The other half was fixed in 4 % (v/v) paraformaldehyde and embedded in paraffin. Sections were cut at 5-6 μm in thickness.

Haematoxylin and eosin staining

Haematoxylin and eosin staining was performed on the paraffin sections following the standard protocol. Briefly, sections were dewaxed in xylene and rehydrated in sequential washes of 100 %, 90 % and 70 % alcohol. Sections were stained in haematoxylin for 3 minutes, followed by eosin for 2 minutes, dehydrated in 70 %, 90 % and 100 % alcohol and mounted with DePeX (VWR).

The thickness of the cornified envelope (green arrows) and the epidermal/dermal junctions (orange arrows) were measured across three representative areas of three independent sections per sample. This was repeated for three independently grown organotypic co-cultures.

Immunofluorescent staining

Frozen sections were air-dried for 30 minutes and cells were permeabilised in 0.1 % (v/v) Triton X-100 in PBS for 15 minutes. Sections were washed in PBS, blocked in 0.2 % (v/v) gelatin from cold water fish skin for 15 minutes and incubated with primary antibody for 1 hour. All antibodies mentioned in this paper were used at a 1/200 dilution. After three PBS washes, fluorescent secondary antibody (donkey anti-rabbit or donkey anti-mouse Alexa Fluor 568, Molecular Probes) was added at a 1/1000 dilution and incubated for 1 hour protected from light exposure. Sections were washed three times in PBS, incubated with DAPI (0.125 $\mu\text{g}/\text{ml}$) for five minutes and washed three more times. Sections were mounted with immunomount reagent (ThermoShandon, Pittsburg, CA) and viewed under a Nikon Eclipse TE2000-S microscope.

Scratch migration assay

Cells were seeded at 5×10^5 cells per well in 6-well plate culture dishes. Each cell sample was seeded in duplicate and incubated at 37°C for 24 hours. After culturing in serum-free medium (Ham's DMEM) for 24 hours, cells were treated with mitomycin C at 10 mg/ml for 1 hour at 37°C to inhibit proliferation. The media was removed and a "cross" was scratched across the dish using a P1000 pipette tip leaving "scratched" areas with no cells (Boyer et al., 1989). Cells were washed three times in PBS. An area of the "cross" was imaged at 5 \times magnification at 0 hours and 48 hours after the initial scratch to ensure the same part of the scratch was imaged each time. Cells were incubated at $37^{\circ}\text{C}/5\% \text{CO}_2$ in between time points. Images were analysed using ImageJ software program (downloaded from website <http://rsb.info.nih.gov/ij/>) whereby the cell-free area was recorded at each time point. The data was presented as average percentage area migrated.

Proliferation assay

Cellular proliferation was analysed using the alamarBlue assay (Biosource). Cells (1×10^4) were seeded in triplicate in 12-well plates and incubated for 20 hours at 37°C to allow the cells to attach onto the culture dish. Cells were washed with PBS and 10 % (v/v) alamarBlue dye in normal culture media was added. Cells were incubated for 6 hours at 37°C . 150 μl from each sample was removed from each well for fluorescence detection and the remaining dye was washed from the cells before normal culture media was replaced. The assay was performed over three consecutive days, with fresh alamarBlue added 6 hours prior to assay.

Each sample was measured in duplicate by recording the optical density at 570 nm (OD₅₇₀). The fold change (i.e. average OD₅₇₀ each day / average OD₅₇₀ on day 1) per sample was calculated and plotted against time (days).

Bacterial invasion assay

Shigella flexneri serotype 2a (2457T) (ATCC) was grown in LB broth overnight at 37°C in an orbital shaker. The optical density of the culture was measured at 600 nm. Retrovirally transduced HeLa cells were seeded into 24-well plate dishes at 2×10^5 cells per well. The medium was removed and replaced with antibiotic-free DMEM containing 10 % (v/v) foetal calf serum and 2mM glutamine. *S. flexneri* was added to each well of transduced HeLa cells (0.93 OD₆₀₀ units per well, corresponding to 3×10^7 CFUs). The plate was centrifuged at $15 \times g$ for two minutes and incubated at 37°C/10 % CO₂ for 4 hours. Cells were washed three times with PBS and media was added containing 50 µg/ml of gentamycin and incubated at 37°C for 1 hour. Following three washes in PBS, cells were lysed with 0.1 % (v/v) Triton X-100 in PBS. Lysates were serially diluted in PBS and were plated onto LB agar. Bacteria were propagated overnight at 37°C. The number of colonies was counted and statistical analysis was carried out using two-tailed, unpaired t-test.

Statistical analysis

All statistical analysis was carried out either as a one or two-tailed, unpaired t-test as stated depending on the type of experiment performed. p-values equal to or less than 0.05 were considered as statistically significant.

Results

To confirm expression of the EGFP-tagged connexin proteins, cells transduced with the constructs were first visualised by confocal microscopy (fig 1). Aggregations were observed in cells transduced with (WT)Cx26 at the cell periphery which are indicative of gap junction plaques. No such aggregations were observed in the (R143W)Cx26-expressing cells. Organotypic co-cultures expressing (WT)Cx26 or (R143W)Cx26 were subjected to LY dye transfer assays. Whilst (WT)Cx26 allowed dye transfer to adjoining cells, in the (R143W)Cx26 co-cultures the amount of dye transfer was significantly reduced by eight-fold.

Morphological characterisation of organotypic co-cultures overexpressing connexins

The morphological characteristics of keratinocytes on a three-dimensional basis were examined by organotypic co-cultures (Ojeh, Frame & Navsaria, 2001). Haematoxylin and eosin staining showed little difference between the co-cultures using cells transduced with (WT)Cx26-EGFP, (WT)Cx30-EGFP and pSIN-EGFP (vector-only), (fig 2a-c). These co-cultures all exhibited typical histological features of stratified squamous epidermis i.e. nucleated basal cells eventually differentiating into flatter, anucleated cells towards the top of the epidermal layer. (R143W)Cx26 co-cultures showed evidence of a significantly thicker epidermis compared to the other samples despite there being equal numbers of keratinocytes seeded at the start of each organotypic co-culture (fig 2d-e). Observationally, the (R143W)Cx26 co-culture had more densely packed basal keratinocytes compared to the control suggesting an increased number of proliferative cells. Some suprabasal keratinocytes in the spinous layer appeared bigger in size and an eosin-positive layer (stained pink) was also visible. In the latter, some cells appeared to lose their nuclei, whilst others retained very small nuclei. The (R143W) co-culture lacked a clearly defined cornified envelope at day 14, compared to control co-cultures where it had formed by this stage. However, the (R143W) co-cultures have formed a cornified envelope by day 16 (data not shown) indicating delayed terminal differentiation.

The average epidermal thickness measurement of (R143W)Cx26 was 1.6 times higher than that corresponding to the (WT)Cx26 co-cultures ($p=0.003$) (fig 2f). The average measurements of the eosin-positive (pink) layer remained fairly constant in all the co-cultures. Similarly, no differences in the epidermal thickness measurements were found between (WT)Cx26 and (WT)Cx30 co-cultures compared to the vector-only control. This data is comparable to an *in vivo* study (Meyer et al., 2002).

Immunohistochemistry was performed to assess any molecular changes in the differentiation programme of the transduced keratinocytes on organotypic co-cultures using differentiation markers. Normal expression patterns were found for keratin 10, transglutaminase 1, involucrin, keratin 2e and loricrin between all co-cultures (data not shown). This suggests that the onset of late differentiation is similar between all the organotypic models. In contrast a larger number of keratin 14 positive cells were observed in (R143W)Cx26 co-cultures compared to the other co-cultures (fig 3a-d). Keratin 14, a basal cell marker of the epidermis, was not only expressed in the basal layer but was also evident in (R143W)Cx26 expressing cells in the higher layers of the three-dimensional culture.

Proliferative features of organotypic co-cultures and nTERT keratinocytes

No evidence of hyperproliferation was observed, with only weak staining of keratin 6 and keratin 16 observed in all co-cultures (data not shown). The proliferation status was further assessed by the cellular pattern of Ki67 expression in the organotypic co-cultures (fig 3e-h). Ki67 is expressed in the nuclei of actively proliferating cells normally exclusive to those in the basal layer of the epidermis. This expression pattern was observed in the (WT)Cx30 culture (fig 3f) and in the vector-only control (fig 3e). More Ki67 positive keratinocytes were present along the basal layer of the (R143W)Cx26 co-culture (fig 3h) compared to the vector-only control. Similarly, in (WT)Cx26 co-cultures, Ki67 was present at an increased level in the basal layer relative to control co-cultures. These Ki67-positive cells also appeared in the early spinous layer (fig 3g). The increase in number of proliferating (Ki67-expressing) cells in the (WT)Cx26 and (R143W)Cx26 organotypic co-cultures was confirmed by a blind scoring analysis (data not shown). The proliferative status of the cells was also analysed using the alamarBlue assay (fig 3i). The data revealed that keratinocytes overexpressing (R143W)Cx26, (WT)Cx26 and (WT)Cx30 proliferated faster than the vector only control. There was no difference in proliferation between (R143W)Cx26 and (WT)Cx26 transduced cells.

Migratory characteristics of keratinocytes

A scratch assay was performed to assess cell migration (fig 4). Keratinocytes overexpressing (R143W)Cx26 showed an increase in migration by 1.5-fold compared to the (WT)Cx26 equivalent ($p = 0.025$). (WT)Cx26 and (WT)Cx30 cells exhibited an increased migration compared to vector-only control by 3.5-fold ($p = 0.0013$) and 2.7-fold ($p = 0.013$), respectively.

The increase in bacterial invasion seen in (WT)Cx26 expressing HeLa cells is not seen in the (R143W)Cx26 counterpart

Previously, it has been shown that cellular invasion of the gut pathogen *S. flexneri* is associated with opening Cx26 hemichannels in an actin-phospholipase-C-dependent manner (Tran Van Nhieu et al., 2003). We have confirmed and extended these findings (Figure 5). The total number of *S. flexneri* bacteria which invaded into (WT)Cx26 overexpressing HeLa cells was higher than in cells expressing vector-only control. Interestingly two other epidermally expressed connexins, (WT)Cx30 and (WT)Cx31, showed no evidence for enhancing the potential for *S. flexneri* to invade. HeLa cells expressing (R143W)Cx26 do not have the increased levels of bacterial invasion observed in (WT)Cx26 transduced cells

(4670 ± 324.8 bacteria invaded in (WT)Cx26, and 3069 ± 238.3 bacteria invaded in (R143W)Cx26. A significant difference ($p = 0.044$) in bacterial invasion was illustrated between (R143W)Cx26 and (WT)Cx26.

Discussion

Multiple differences were observed between keratinocytes overexpressing human (WT)Cx26 compared to those overexpressing (R143W)Cx26. (WT)Cx26 formed aggregates indicative of gap junction plaques, whereas cells expressing (R143W)Cx26 were devoid of plaque formation. Palmada *et al* (2006) observed the (R143W)Cx26 mutant in the $10,000\times g$ pellet which is consistent with an internal membranous structure. However other studies (Wang *et al.*, 2003) report a plasma membrane localisation. Differences in cell type and expression conditions could explain these contrasting results. However, regardless of the mutant protein's cellular localisation, our dye conductance experiments herein confirm that the (R143W)Cx26 is unable to form functional channels (Mese *et al.*, 2004; Palmada *et al.*, 2006). The dye transfer observed was most likely due to endogenous nTERT connexin expression.

The human (R143W)Cx26 model presented with thickening of the epidermal equivalent, increased number of cells expressing basal marker K14 and increased cellular mobility. Coincidentally, the overall increase in thickness was similar in both our model and in an *in vivo* study (Meyer *et al.*, 2002). This might suggest that there is a limit to the overall thickness of the epidermis before it becomes pathological. Furthermore we provide evidence that, unlike (WT)Cx26, (R143W)Cx26 did not increase *S. flexneri* invasion.

From our data in this study, the increase in cell numbers seen in the (R143W)Cx26 organotypic co-culture showed little difference in proliferation compared to (WT)Cx26 co-cultures, a conclusion further confirmed by the proliferation assay. Since increased proliferation cannot explain the thickened epidermis in (R143W)Cx26 co-cultures, the phenotypic effect may be due to an increased number of undifferentiated basal cells as demonstrated by the Ki67 and K14 immunostaining data, respectively. It is possible that the overall proliferation rate is unaltered but because more undifferentiated cells are present more daughter cells are generated, giving rise to epidermal thickening.

Additionally, skin-thickening conditions such as psoriasis and palmoplantar keratoderma (PPK) display an increase in K6 and K16 expression, markers of hyperproliferation (Leigh *et al.*, 1995). Although the organotypic co-culture model of (R143W)Cx26 showed significant epidermal thickening, no significant changes were seen in K6 expression compared to the control, supporting the notion that the phenotype is not associated with any pathological changes. The very weak staining observed in all the co-cultures is most likely due to the keratinocytes adopting a slight hyperproliferative state – a finding consistent with previous descriptions of organotypic co-cultures (Stark *et al.*, 2004). If the observations were not of a pathological nature, (R143W)Cx26 may exert beneficial properties to the skin.

One way R143W(Cx26) may benefit the skin is by decreasing the ability of bacterial pathogens to invade. As a preliminary experimental model we used the gut invading pathogen *S. flexneri* in HeLa cells as HeLa cells express low endogenous levels of connexins and were previously used to show that Cx26 enhances *S. flexneri* invasion (4). Our data shows that two other epidermally expressed connexins, Cx30 and Cx31, do not have a similar effect. The mechanism behind relative specificity of Cx26 to elicit the invasion increase is yet to be explored. (R143W)Cx26 expressing cells showed reduced bacterial invasion compared with (WT)Cx26 expressing cells. This may be due to the functional disruption of the Cx26 hemichannel since the wild-type hemichannel is reported

to allow *S. flexneri* invasion through the actin-phospholipase-C-dependant pathway in HeLa cells (Tran Van Nhieu et al., 2003). Patients who carry the (R143W)Cx26 variant have increased levels of sodium and chloride in their eccrine sweat glands compared to normal individuals. This physiological finding was postulated to create an osmotic environment that is difficult for microbial colonisation (Meyer et al., 2002). Our model shows that (R143W)Cx26 expressing cells are protected against this gut pathogen compared to (WT)Cx26 counterpart. It could be possible that a similar effect is seen in the keratinocytes expressing (R134W)Cx26. It is interesting to note that Cx26 expression is limited to the basal layer in interfollicular human epidermis and to the epidermal eccrine sweat glands. This specific cellular localisation of Cx26 is consistent with protecting the epidermis from bacterial infection. Similarly, in human colon tissue, Cx26 expression levels appeared to be greatest around the goblet cells – cells that interact closely with the contents (bacterial or otherwise) of the gut (data not shown).

In human cutaneous wounding Cx26 is expressed 24–48 hours post-injury. It is detected near the wound margin and eventually as the wound heals, normal low expression of Cx26 resumes (Brandner et al., 2004). The role of this connexin at times of injury remains elusive. It has been suggested that Cx26 (and Cx30) is massively upregulated in psoriasis to control differentiation (Labarthe et al., 1998; Rivas et al., 1997; Wiszniewski et al., 2000). However, we did not observe any obvious differentiation changes in our Cx26 overexpression model. We postulate that Cx26 regulates the wound healing process, not by promoting it but by controlling and maybe even dampening the process. Our data demonstrated that overexpression of (WT)Cx26 did not promote an increase in migration whereas inhibition of Cx26 (by the R143W mutation) appeared to lead to an effect on keratinocyte migration. In support of our hypothesis, an *in vivo* study in human skin has shown an upregulation of Cx26 and Cx30 at wound margins of non-healing wounds, whereas during spontaneous wound repair these connexins are absent at the leading edge of the regenerating epidermis but are overexpressed behind the wound margin (Brandner et al., 2004). This expression pattern may reflect the proliferative capacity of Cx26 expressing cells which correlates well in our study herein. It has been reported that cells at this location relative to the wound undergo a proliferative burst which possibly provide a pool of extra cells to replace those lost during injury (Hertle et al., 1992; Matoltsy & Viziam, 1970).

Whilst this paper was in preparation, Segre and colleagues reported that transgenic mice heterozygous for involucrin-Cx26 had persistent Cx26 expression in the epidermis (Djalilian et al., 2006). The wounded epidermis was infiltrated with immune cells and the wound remained in a hyperproliferative state with the remodelling phase blocked, resulting in wound healing failure. This *in vivo* data is consistent with our findings and supports the hypothesis that Cx26 suppresses wound healing. In summary, these studies suggest that a non-functional Cx26 channel due to a *GJB2* hearing loss associated mutation confers beneficial properties to the epidermis and possibly other Cx26 expressing epithelium resulting in an improved barrier, accelerated wound repair and protection from infection. As also recently suggested (Djalilian et al., 2006), knock-down of Cx26 expression may have a therapeutic application in wound healing and also as an anti-infective.

Acknowledgments

This work has been funded by the Research Advisory Board of Barts and the London, the BBSRC and the Wellcome Trust to Professor David P. Kelsell. With special thanks to Dr. Jash Vyas for advice and help with the migration assays and Professor Tom MacDonald for the normal human colon and help with interpretation of Cx26 localisation

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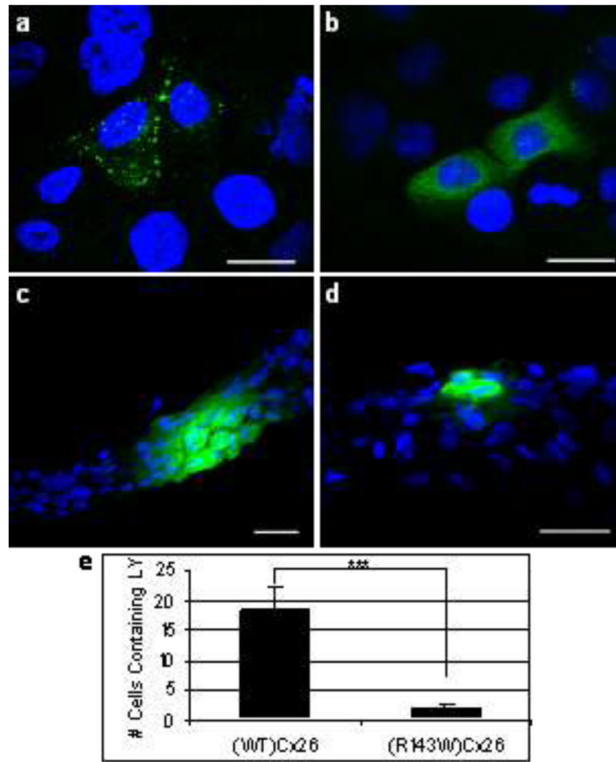


Figure 1. Localisation of (WT)Cx26 (a) and (R143W)Cx26 (b) (green). Note the aggregations of (WT)Cx26 at the cell periphery which are indicative of gap junction plaques are absent in the cells expressing (R143W)Cx26. LY (green) transfer following microinjection of organotypic co-cultures expressing (WT)Cx26 (c), or (R143W)Cx26 (d). Quantification of number of cells containing LY after microinjection of (WT)Cx26 and (R143W)Cx26 expressing co-cultures (e). There is statistical difference between the two (***) represents $p < 0.0001$; unpaired t-test). (a-d): Scale bars represent 20 μm . Nuclei are shown with blue DAPI staining.

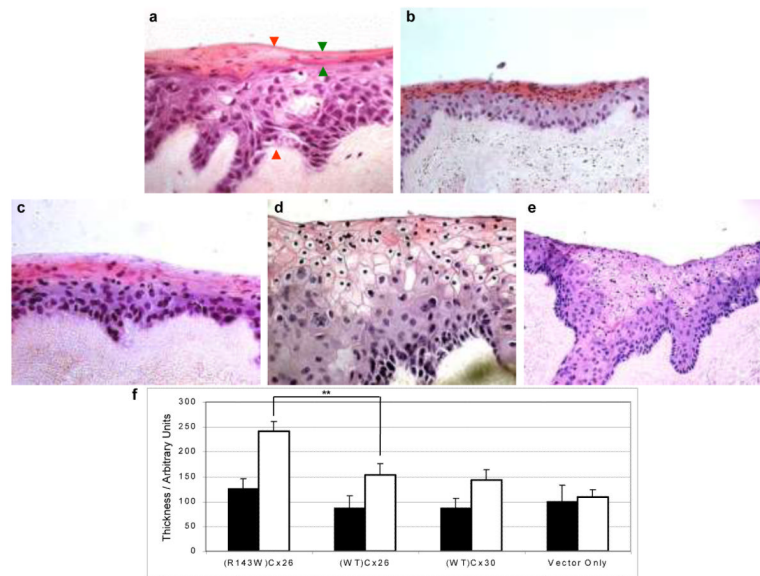


Figure 2.

Histological features of organotypic co-cultures. Haematoxylin and eosin stained sections of a) pSIN-GFP (vector-only); b) (WT)Cx30; c) (WT)Cx26; d) (R143W)Cx26 co-cultures at $400 \times$ magnification and e) (R143W)Cx26 at $200 \times$ magnification. Green and orange arrows shown on a) represent the distance at which the eosin-positive layer and full thickness of the epidermal equivalent were measured, respectively. f) Graph representing the average thickness of the eosin-positive layer (black bars) and average full thickness of the epidermal equivalent (white bars). Unpaired, two-tailed t-test showed that the total thickness of the epidermal equivalent of the (R143W)Cx26 co-culture was statistically different from (WT)Cx26 (** represents $p < 0.01$). Error bars represent the standard error of the mean (SEM), $n = 3$.

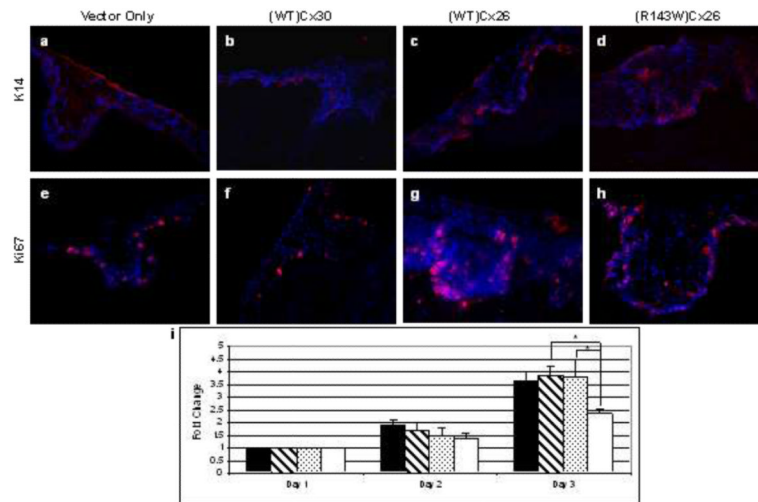


Figure 3. Characterisation of nTERTs overexpressing wild-type (WT)Cx26, (WT) Cx30 and (R143W)Cx26. Keratin 14 immunofluorescent staining (red) of a) vector-only control; b) (WT)Cx30; c) (WT)Cx26 and d) (R143W)Cx26 co-cultures. Ki67 immunofluorescence (red) of; e) vector-only control f) (WT)Cx30 g) (WT)Cx26 and h) (R143W)Cx26 co-cultures. Nuclei are shown with blue DAPI staining. i) Graph showing the average fold change in proliferation over a 3-day time course. The black, striped, speckled and white bars represent nTERTS overexpressing (R143W)Cx26, (WT)Cx26, (WT)Cx30 and vector-only control, respectively. The data represent mean \pm the standard error of the mean (SEM) (n=3). * represents $p < 0.05$.

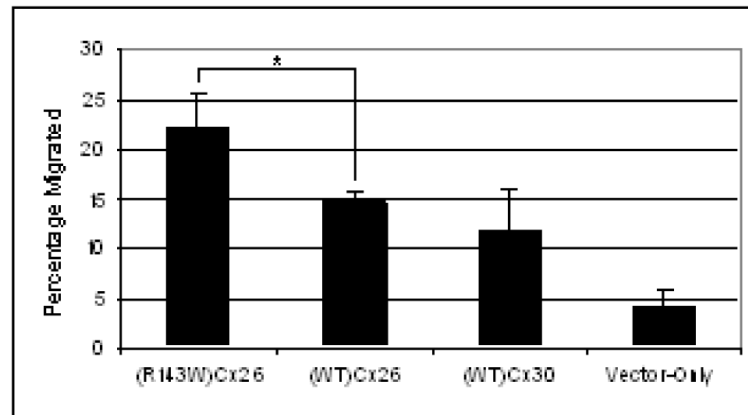


Figure 4. Keratinocyte cellular migration analysis. A monolayer of NTERTS overexpressing (R143W)Cx26, (WT)Cx26, (WT)Cx30, or vector-only control were scratched. Graph shows the percentage of cells migrated after 48 hours. The data represent mean \pm the standard error of the mean (SEM). Comparison was carried out using unpaired, one tailed t-test where a significant difference was found between (WT)Cx26 and (R143W)Cx26. * represents p 0.05.

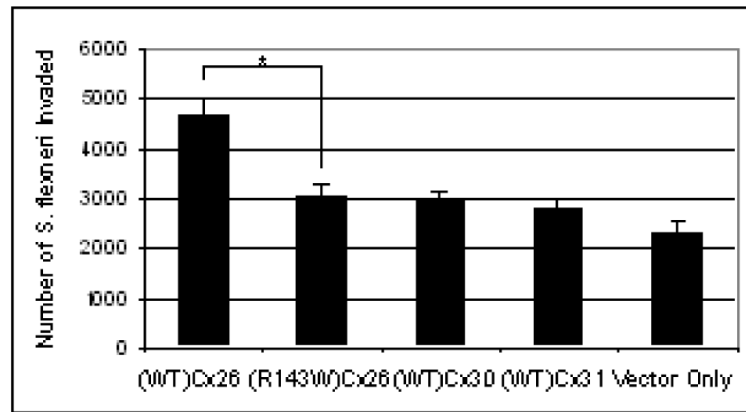


Figure 5. Bacterial invasion via Cx26 hemichannels. Histogram showing invasion of *Shigella flexneri* bacteria in HeLa cells overexpressing (WT)Cx26, (R143W)Cx26, (WT)Cx30, (WT)Cx31 and vector-only control. The data represent mean \pm the standard error of the mean (SEM) $n = 15$. Comparison was carried out using unpaired, two-tailed t-test where a significant difference was found between (WT)Cx26 and (R143W)Cx26. * represents $p < 0.05$.