Overexpression of connexin 43 reduces melanoma proliferative and metastatic capacity

A Tittarelli1, I Guerrero1, F Tempio1,2, M A Gleisner1, I Avalos1, S Sabanegh1, C Ortíz1, L Michea1,2, M N López1,2,3, A Mendoza-Naranjo4 and F Salazar-Onfray*,1,2

1Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Santiago 8380453, Chile; 2Millennium Institute on Immunology and Immunotherapy, Institute of Biomedical Sciences, University of Chile, Santiago 8380453, Chile; 3Research Support Office, University of Chile Clinical Hospital, Santiago 8380453, Chile and 4UCL Cancer Institute, University College London, 72 Huntley Street, London WC1E 6DD, UK

Background: Alterations in connexin 43 (Cx43) expression and/or gap junction (GJ)-mediated intercellular communication are implicated in cancer pathogenesis. Herein, we have investigated the role of Cx43 in melanoma cell proliferation and apoptosis sensitivity in vitro, as well as metastatic capability and tumour growth in vivo.

Methods: Connexin 43 expression levels, GJ coupling and proliferation rates were analysed in four different human melanoma cell lines. Furthermore, tumour growth and lung metastasis of high compared with low Cx43-expressing FMS cells were evaluated in vivo using a melanoma xenograft model.

Results: Specific inhibition of Cx43 channel activity accelerated melanoma cell proliferation, whereas overexpression of Cx43 increased GJ coupling and reduced cell growth. Moreover, Cx43 overexpression in FMS cells increased basal and tumour necrosis factor-α-induced apoptosis and resulted in decreased melanoma tumour growth and lower number and size of metastatic foci in vivo.

Conclusions: Our findings reveal an important role for Cx43 in intrinsically controlling melanoma growth, death and metastasis, and emphasise the potential use of compounds that selectively enhance Cx43 expression on melanoma in the future chemotherapy and/or immunotherapy protocols.
been observed in most tumour cell lines and in solid tissue tumours, including melanoma (Huang et al, 1999; Zhang et al, 2003; Haass et al, 2004; Naus and Laird, 2010). The role of GJs in tumour progression has been studied mainly through the ectopic reintroduction of Cx genes into tumour cell lines. Ectopic expression of Cx43 has been shown to reduce cell proliferation in many distinct cancer cells, including in mouse melanoma cell lines (Huang et al, 1998; Fukushima et al, 2007; Absler et al, 2014). The mechanisms that may be involved in the inhibition of tumour cell growth by Cx43 re-expression include enhanced sensitivity to cell death, increased propagation of death signals and inhibition of proliferation via the regulation of specific kinases (Huang et al, 2002; Krutovskikh et al, 2002; Zhang et al, 2003). However, there is limited information about the role of Cx43 expression in cell proliferation/cell death and metastatic capacity in human melanoma. In this regard, Su et al (2000) observed that overexpression of Cx43 in a malignant melanoma cell line resulted in the suppression of the anchorage-independent growth, suggesting that Cx43 may also be a tumour suppressor gene in human melanoma. Recently, Zucker et al (2013) demonstrate that the overexpression of a dominant-negative Cx43 mutant decreases the anchorage-independent growth and increases the invasive potential of human melanoma cells.

In contrast with the established role of Cxs regulating growth of tumour cells in culture, the role of Cxs in tumour cell invasion and metastasis is controversial. Several reports suggest that Cxs might reduce tumour metastasis (Li et al, 2008; Sato et al, 2008; Bodenstein et al, 2010; Plante et al, 2011; Wang et al, 2013), whereas others argue the opposite effect (Naus and Laird, 2010; Stoletov et al, 2013), highlighting cell- and context-specific components of the process. In the present study, we aimed to evaluate the role of Cx43 expression in cell proliferation, apoptosis and metastatic capacity in human melanoma. Using a combination of in vitro and in vivo assays, we provide new insights into the biology of Cx43 in melanoma, and validate Cx43 as an important factor regulating growth, survival and metastasis formation.

**MATERIALS AND METHODS**

**Cell culture.** DFB, DFW, HF and FMS human melanoma cell lines were established and characterised at the Microbiology and Tumor Biology Center (MTC), Karolinska Institute, Sweden (Salazar-Onfray et al, 2002). The lines were derived from metastatic lesions of patients treated at Radiumhemmet, Karolinska Hospital. DFW is a depigmented melanoma subtype obtained from DFB by limiting dilution. Melanoma cells were grown at 37 °C in an atmosphere with 5% CO₂ in RPMI 1640 culture medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10% foetal bovine serum (FBS), penicillin (100 U ml⁻¹), streptomycin (100 μg ml⁻¹) and 1 μM L-glutamine. The pRES-Cx43-transfected cells were grown in the same medium in the presence of geneticin G418 (250 μg ml⁻¹; Invitrogen). Cells were incubated with 300 μM of GJ-inhibitory Cx43-specific mimetic peptide 1848 (GNTQPGPCCNVYC, extracellular loop 1; 95% purity), Cx43-specific gap20 control peptide (EIKKFKGYIEIEHC, cytoplasmic loop; 95% purity) (Mendoza-Naranjo et al, 2011) or 5 μM of the Cx43-specific peptide 5 (P5), which prevent hemichannel opening but do not disrupt GJ communication (VDCFLSRIPTEKT, extracellular loop 2; 95% purity) (O’Carroll et al, 2008). Peptides were purchased from GenScript (Piscataway, NJ, USA).

**Cell immune fluorescence staining and confocal microscopy.** Melanoma cells were grown on poly-l-lysine-coated slides (Sigma-Aldrich, Steinheim, Germany). Cells were washed twice with PBS and fixed with 4% paraformaldehyde (PFA) for 30 min. After gentle washing with PBS, the cells were incubated in ammonium chloride (50 μM) for 10 min, then permeabilised with 0.5% Triton X-100 for 10 min, and blocked with 0.5% bovine serum albumin (BSA). Cells were then incubated with the anti-Cx43 polyclonal antibody (C6219; Sigma-Aldrich) overnight at 4 °C. Samples were stained with a secondary donkey anti-rabbit FITC-conjugated Ab (Poly4064; BioLegend, San Diego, CA, USA) and 5 μg ml⁻¹ Hoechst 33342 (Invitrogen) and mounted using DAKO fluorescence mounting medium. The samples were analysed using a Nikon Eclipse C2si confocal microscope (Plan Apo VC60X OIL DIC N2, NA: 1.4). Images were acquired using the NIS element AR V3.2 software (Nikon, Melville, NY, USA).

**Flow cytometry.** Flow cytometry experiments were performed as described previously (Tittarelli et al, 2014). Cells were fixed and permeabilised with Intracellular Fixation and Permeabilisation Buffer Set (BD Pharmingen, San Jose, CA, USA). Subsequently, melanoma cells were incubated with an F(ab)2 rabbit polyclonal anti-Cx43 directed to the C-terminal domain (C6219; Sigma-Aldrich) followed by a secondary donkey anti-rabbit FITC-conjugated Ab (Poly4064; BioLegend). Samples were acquired on a FACScalibur (BD Biosciences, Franklin Lakes, NJ, USA) and analysed using the FCS Express 4 plus software (DeNovo, Glendale, CA, USA) or FlowJo (version 8.6.6; Tree Star, Ashland, OR, USA).

**Cx43 stable transfection.** FMS cells were transfected using FuGeneHD (Roche Diagnostic, Indianapolis, IN, USA), according to the manufacturer’s instructions, with a empty pRES vector (Clontech Laboratories, Palo Alto, CA, USA) or containing the CMV promoter controlling the expression of the wild-type Cx43 cDNA, as described previously (Becker et al, 2001). Cells were selected in 250 μg ml⁻¹ geneticin G418 (Promega, Madison, WI, USA) 48 h after transfection. Clonal cell populations were obtained by limiting dilution method.

**Calcine-AM dye transfer assay.** The calcine-AM dye transfer assay was performed according to a previously described method (Czyz et al, 2000). Briefly, donor melanoma cells were loaded with 1 μM calcine-AM (C-1359; Sigma-Aldrich) and acceptor melanoma cells were loaded with Dil (15 μg ml⁻¹; D-282; Molecular Probes, Eugene, OR, USA) for 30 min at room temperature. After thoroughly washing with PBS, both cell types were cocultured at a 1:1 ratio (calcine⁺ -donor cells: Dil⁺ -acceptor cells) for 60 min at 37 °C. Calcine transfer from the donor to the acceptor cells was evaluated by flow cytometry, determining the percentage of calcine⁺ cells among the Dil⁺ cells. Calcine transfer assays were carried out in the presence of the Cx43-specific mimetic peptide 1848, control peptide gap20 or P5.

**CFSE cell proliferation assay.** Melanoma cells were synchronised by serum deprivation for 48 h. Thereafter, the cells were labelled with CFSE at a final concentration of 5 μM (37 °C for 10 min), and cultured in serum-supplemented medium for additional 24 h. Flow cytometric analysis of cell proliferation by CFSE dilution was performed as described previously (Rödel et al, 2005).

**[³H]thymidine incorporation assay.** Melanoma cells were synchronised by serum deprivation for 48 h. Thereafter, the cells were cultured in the presence of [³H]thymidine (Topcount NXT; Perkin-Elmer, Waltham, MA, USA) at 24 h according to standard methods. Proliferation was evaluated in cells untreated or pretreated for 4 h (before [³H]thymidine addition) and treated every 4 h with 1848 or gap20 peptides.

**Western blot.** Equal amounts of protein (30 μg) extracted from FMS-EV- or FMS-Cx43-transfected cells were separated by 12% SDS-PAGE followed by electrotransfer to nitrocellulose membranes (Hybond; Amersham Biosciences, Piscataway, NJ, USA). Connexin 43 expression was examined as described (Mendoza-Naranjo et al, 2007), using an anti-human Cx43 polyclonal antibody (Sigma-Aldrich). Total lysates were probed with
anti-β-actin antibody (Sigma-Aldrich) as a loading control. Signal was visualised using a chemiluminescence substrate system (Biological Industries, Amersham Biosciences).

**Apoptotic cell analysis.** FMS-EV- or Cx43-transfected cells were incubated or not with 200 ng ml⁻¹ of tumour necrosis factor-α (TNF-α) for 24 h. Annexin-V and propidium iodide (PI) staining was used to analyse apoptosis according to the manufacturer's instructions (Apopalert; Clontech Laboratories Inc.).

**Mice.** Nonobese diabetic/scid mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and kept in filtered cages under pathogen-free conditions in the high security animal facilities of the Institute of Biomedical Science, University of Chile. All experiments requiring animals were approved by the Faculty of Medicine Bioethical Committee and performed according to Home Office Animal Welfare Legislation.

**Melanoma cell xenotransplantation and metastasis experiments.** Eight-week-old NOD/SCID mice (three per group) were subcutaneously injected into the right flank with 5 × 10⁶ FMS, DFB, FMS-EV or FMS-Cx43 cells. Tumour growth was monitored every 2–3 days by measuring the tumour volume with a calliper. Tumour volume was calculated according to the equation: (longer diameter × shorter diameter)²/2. For lung metastasis assay, 8-week-old NOD/SCID mice (six per group) were injected with 2.5 × 10⁵ FMS-EV cells, FMS-Cx43 cells or saline solution (100 μl) intravenously into the tail vein. After 18 days, the mice were killed and autopsied to analyse melanoma lung metastasis.

**Tumour immunohistochemical and immunofluorescence staining.** Lungs from animals injected with FMS-EV and FMS-Cx43 cells were fixed with 2% PFA for 12 h at 4 °C, dehydrated with 75% ethanol, rinsed in Xilol and then paraffin-embedded. Next, 3-μm sections were obtained, which subsequently underwent treatment with 0.01 mM EDTA for antigen retrieval, followed by a 10 min treatment with 3% hydrogen peroxide to block endogenous peroxidase activity. The samples were thereafter washed two times with 0.05% PBS/Tween. Finally, the sections were incubated with 4% BSA/PBS for 30 min and then labelled with anti-cleaved caspase-3 (asp175) antibody (Cell Signalling Technology, Beverly, MA, USA; 1:50 dilution), or anti-Melan-A (Mart-1) mouse monoclonal antibody (Dako, Tokyo, Japan; 10 μg ml⁻¹), for 1 h at room temperature. The samples were then washed two times with 0.05% Tween/PBS before incubation with the respective secondary peroxidase antibody (Vector Laboratories, Burlingame, CA, USA) for 30 min. Samples were incubated with ABC solution (Vectastain ABC Kit; Vector Laboratories) for 20 min at 37 °C, and revealed with the chromogenic substrate DAB (Vector Laboratories).

Human gonadal tissues were used as a positive control and lung tissues from non-injected animals were used as a negative control.

**For immunostaining assay, an anti-Cx43 polyclonal antibody (Sigma-Aldrich) was used overnight at 4 °C, followed by incubation with a secondary donkey anti-rabbit Alexa Fluor 488-conjugated antibody (Life Technologies, Carlsbad, CA, USA). Samples were additionally stained with 5 μg ml⁻¹ Hoechst 33342 (Invitrogen) and visualised by confocal microscopy (LSM 510; 363 numerical aperture 1.4 oil immersion objective; Carl Zeiss, Jena, Germany).

**Statistics.** Statistical analyses were performed using the GraphPad Prism 3.0 program (GraphPad Software Inc., San Diego, CA, USA). Differences between experimental and control were tested by applying ANOVA test. Results are expressed as the mean ± s.e.m. In all experiments a P < 0.05 was considered statistically significant.

**RESULTS**

**Cx43 expression correlates with GJIC in human melanoma cells.** Connexin 43 expression was analysed in four different human metastatic melanoma cell lines, named FMS, HF, DFW and DFB. Total Cx43 expression was assessed by flow cytometry on permeabilised melanoma cells. As depicted in Figure 1A, DFW and HF cells showed higher total Cx43 levels compared with FMS and DFB cells. To examine the potential role of Cx43 expression in GJ formation between these cells, we analysed its cellular localisation by immunofluorescence and confocal microscopy. Weak positive Cx43 cytosolic and nuclear staining was observed in all melanoma cell lines analysed (Figure 1B). However, Cx43 clustering in cell–cell contact areas (white arrows) was only detected in HF cells, indicating the low frequency of occurrence of Cx43-GJ plaques in melanoma cells.

The presence of functional GJIC among melanoma cells was then monitored by calcein-AM transfer assays in the four cell lines (Figure 2A). Melanoma cells with the lowest levels of Cx43 expression (FMS) had the lowest incidence of dye coupling (FMS: 4.7 ± 0.4% compared with DFW: 6.9 ± 0.8%; DFB: 8.3 ± 1.1%; HF: 8.6 ± 1%) (Figure 2A, lower panel). The transfer of calcein-AM was significantly reduced when the cells were cultured in the presence of an inhibitory Cx43-specific mimetic peptide (1848) compared with cells treated with the control gap20 peptide (Figure 2A, lower panel), indicating that Cx43-GJ channels are required for functional GJIC between these melanoma cells.

Additionally, CFSE dilution assays indicated that FMS melanoma cells with the lowest levels of both Cx43 expression and GJIC displayed the highest rate of in vitro cell proliferation, compared with DFW and DFB cells (Figure 2B).

We then assessed tumour growth rate in NOD/SCID mice xenotransplanted with FMS or DFB, the melanoma cell lines with the lowest and highest Cx43-mediated cell coupling and highest and lowest in vitro proliferation levels, respectively. In line with the in vitro observations, FMS tumours grew faster than DFB tumours in vivo (Figure 2C).

**Cx43 overexpression reduces proliferation rates and increases susceptibility to apoptosis in FMS melanoma cells.** To address the direct effect of Cx43 in melanoma cell proliferation, Cx43-containing vectors or empty vectors (EV) were stably transfected in FMS cells. Connexin 43 overexpression was confirmed by western blotting and flow cytometric analysis of FMS cells (Figures 3A and B). Additionally, we analysed Cx43 subcellular localisation by confocal microscopy. As depicted in Figure 3C, overexpression of Cx43 was associated with the occurrence of Cx43-GJ plaque structures at the cell–cell interface (white arrows), whereas FMS-EV cells showed weak and mainly nuclear Cx43 staining. In accordance with these results, FMS-Cx43 cells showed increased incidence of dye transfer compared with control FMS-EV cells (21.5 ± 2.6 vs 6.7 ± 1.8) (Figure 3D). Dye transfer was markedly reduced when FMS-Cx43 cells were cultured in the presence of peptide 1848, but not in cells treated with the control peptide gap20 or with a Cx43-hemichannel-specific inhibitory peptide (P5) (Figure 3D).

Connexin 43 overexpression induced a significant reduction in the proliferation rate of FMS cells (Figure 3E), which was completely reverted after incubation with the inhibitory Cx43-specific mimetic peptide 1848 (Figure 3E). These data demonstrate that Cx43 expression in melanoma cells regulates their proliferative potential, likely through a mechanism dependent on GJ-mediated intercellular communication.

It has been shown that Cx43 overexpression in prostate cancer cells increases their susceptibility to TNF-α-induced apoptosis (Wang et al. 2007). To establish whether Cx43 expression is
involved in the control of cell survival in human melanoma cells, we measured the percentage of apoptotic FMS-Cx43 and FMS-EV cells before and after TNF-α incubation. Both the basal percentage of early apoptotic cells (annexin-V⁺ PI⁻) and total dying cells (annexin-V⁺) were higher in FMS-Cx43 compared with that in FMS-EV cells (5.9 ± 1.7% compared with 3.2 ± 0.4%; 11.1 ± 2.3% compared with 6.2 ± 1.3%; Figures 4A–C). Tumour necrosis factor-α treatment did not increase the fraction of early apoptotic or total dying cells in FMS-EV (Figures 4A and B). However, the fraction of early apoptotic cells was increased in FMS-Cx43 treated
with TNF-α compared with untreated cells (10.2 ± 1.2% vs 5.9 ± 1.7%; Figures 4A and B). Taken together, these results show that in addition to a reduction in cell proliferation, Cx43 expression increases basal and TNF-α-induced apoptosis in melanoma cells.

**Cx43 overexpression reduces melanoma tumour growth and lung metastasis in xenotransplanted mice.** To investigate the role of Cx43 expression in melanoma tumour growth and lung metastasis formation in vivo, FMS-Cx43 and FMS-EV control cells were injected subcutaneously or intravenously, respectively, in NOD/SCID mice. In line with our in vitro observations, we found that Cx43 overexpression significantly decelerated tumour growth in xenotransplanted mice (Figure 5A). In contrast, tumours arising from control melanoma FMS-EV cells grew significantly faster, and mice had to be killed 16 days after inoculation. Similar data were observed in terms of metastatic spread. Whereas FMS-EV melanoma cells gave rise to multiple metastatic pulmonary foci, the highly metastatic potential of the FMS cells was strongly inhibited by overexpression of Cx43 (Figure 5B). These data illustrate a tumour-intrinsic role of Cx43 in the regulation of melanoma metastatic spread in the absence of an immune response. Moreover, immunohistochemical analysis showed that both the multiple and bigger tumours found in animals inoculated with FMS-EV cells and the less frequent and smaller tumours produced by the FMS-Cx43 injected cells both expressed the melanoma marker Mart-1 (Figure 5C), ruling out a putative role of Cx43 in the dedifferentiation of melanoma cells in vivo. Interestingly, immunostaining showed that FMS-Cx43 metastatic tumours expressed higher levels of both Cx43-based GJs and active caspase-3 in vivo, compared with FMS-EV tumours (Figures 5D and E). Altogether, these results suggest that Cx43 expression in melanoma cells decreases their tumorigenic and metastatic potential influencing both proliferation and survival rates, likely through a GJIC-dependent mechanism.

**DISCUSSION**

The current study provides direct evidence of the impact of Cx43 expression in human melanoma growth and metastatic behaviour in vitro and in vivo, at least in the last steps of metastasis formation, that is, extravasation, survival and growth in a distant organ. The tumour suppressor properties of Cx43 may be complex, cancer type-specific and may involve both channel (GJ and hemichannels)-dependent and -independent mechanisms. A recent study in B16-BL6 cells, a mouse melanoma cell line with low levels of Cx43 expression, reported a decreased proportion of cells in the S phase of the cell cycle after Cx43 overexpression (Ableser et al, 2014). Inhibition in cell proliferation was further associated with an increase in homocellular GJIC between melanoma cells when...
cocultured in the presence of keratinocytes, although restoration of heterocellular GJIC was not observed (Ableser et al., 2014). These data, along with our current findings, suggest that, through a yet unknown mechanism, Cx43-mediated intercellular communication in melanoma control cell growth and proliferation in both human and murine models.

It has been shown that Cx43 reduces proliferation in glioblastomas, osteosarcoma and ovarian carcinoma by inhibiting cell cycle progression through increased p27 expression and inhibition of the S-phase kinase-associated protein Skp2 (Huang et al., 1998; Zhang et al., 2003). Ectopic expression of Cx43 in breast tumour cells induces tumour-suppressive properties through the downregulation of fibroblast growth factor receptor 3 (Qin et al., 2002). Moreover, Cx43 can interact with over 30 distinct proteins, including proteins with a tumour suppressor role such as caveolin 1 (Langlois et al., 2010) and CCN3 (Gellhaust et al., 2004; Sin et al., 2009).

Key molecules associated with cell proliferation/survival and which are permeable to Cx43 channels include NAD\(^{+}\), ATP, Ca\(^{2+}\), IP\(_3\), glutathione and prostaglandin E2 (Decrock et al., 2009). Accordingly, it has been shown that inhibition of Cx43 channels by 18-β-glycyrrhetinic acid decreases cell proliferation by modulating intracellular ATP and Ca\(^{2+}\) levels (Song et al., 2010). Intracellular Ca\(^{2+}\) signalling and the generation of intercellular Ca\(^{2+}\) waves have been involved in key cancer-related processes including escape from apoptosis, perpetuation of growth and metastasis (Monteith et al., 2012). Particularly, higher intracellular Ca\(^{2+}\) concentrations favour cell proliferation by promoting remodelling of the actin cytoskeleton (Monteith et al., 2012).

Our results also showed that expression of Cx43 in melanoma cells increases both basal and TNF-α-induced apoptosis of FMS melanoma cells. Interestingly, FMS-Cx43 metastatic lung tumours displayed higher Cx43-GJ plaques accompanied by active caspase-3. These data are consistent with previous studies showing that restoration of Cx43 expression in tumour cells correlates with the inhibition of tumour growth and expression of genes involved in the regulation of the cell cycle and apoptosis including Bcl-2 and caspase-3/7 (Hattori et al., 2007). Additional studies have shown that Cx43 overexpression stimulates apoptotic cell death via GJ-mediated transfer of proapoptotic signals such as Ca\(^{2+}\) and IP\(_3\) between cells (Decrock et al., 2009; Kameritsch et al., 2013; Carette et al., 2014). Furthermore, the absence of Cx43 and GJIC correlates with cancer stem cell features and expression of epithelial-to-mesenchymal transition markers in pancreatic cancer cells (Forster et al., 2014), both associated with a higher basal viability, resistance to gemcitabine and enhanced clonogenic potential. Restoration of Cx43 expression and GJIC in melanoma cells could have an effect on proliferation, metastasis and induction of apoptosis through inhibition of its cancer stem cell characteristics (Troso, 2003).

Although over 21 different isoforms of Cxs have been characterised in humans, only Cx26 and Cx43 are expressed in melanocytes (Hsu et al., 2000; Ito et al., 2000). During melanoma transformation and progression, the expression of Cxs is down-regulated, thus promoting loss of communication with cells of the local microenvironment (Hsu et al., 2000; Masuda et al., 2001). Our results along with recent evidences (Zucker et al., 2013; Ableser et al., 2014) suggest that Cx43, and not Cx26, can act as a tumour suppressor during melanoma tumorigenesis. Following ectopic expression of GFP-tagged Cx26 and Cx43 in the Cx-deficient B16-Bl6 mouse melanoma cell line, expression of Cx43, but not Cx26, significantly reduced cellular proliferation and anchorage-independent growth in these cells (Ableser et al., 2014).

The role of Cxs in invasion and metastasis is controversial, and whereas several reports indicate that Cxs may reduce tumour...
metastasis (Li et al., 2008; Sato et al., 2008; Bodenstine et al., 2010; Plante et al., 2011; Wang et al., 2013), other studies argue the opposite effect (Naus and Laird, 2010; Stoletov et al., 2013). Li et al. (2008) observed that expressing Cx43 in breast cancer cells decreases their metastatic potential through a mechanism independent of GJIC but, rather, related to N-cadherin expression and apoptosis. N-cadherin expression is involved in epithelial-to-mesenchymal transition in cancer cells by impairing cell polarity and cell–cell adhesion, and increasing a more migratory and invasive phenotype (Wheelock et al., 2008). However, our data point to a role for Cx43 expression regulating the last steps of the metastatic process, when tumour cells have to colonise distant organs such as the lung, and proliferate and survive in this new environment, which requires the reversion of EMT. Furthermore, recent data strongly suggest that Cx43 acts as a tumour suppressor protein, which may predict clinical outcomes in chemotherapy-treated patients of different kind of cancers (Sirnes et al., 2012; Du et al., 2013; Wang et al., 2013). Moreover, we recently showed that Cx43 accumulates at the interface between natural killer (NK) cells and melanoma cells, facilitating the NK cell cytotoxic activity against melanoma (Tittarelli et al., 2014), suggesting a putative impact of tumour-Cx43 expression in immunotherapy outcomes from melanoma patients.

In summary, our findings propose an important role for Cx43 controlling melanoma growth and metastasis, likely through a mechanism dependent of GJ communication and induction of apoptosis. Therefore, it is tempting to speculate that the use of drugs that selectively enhance Cx43 expression on tumours and/or melanoma cells (Yi et al., 2006; Conklin et al., 2007) could enhance the efficacy of cancer therapies.

**ACKNOWLEDGEMENTS**

We thank Professor David Becker (Lee Kong Chian School of Medicine, Nanyang Technological University) for the kind gift of control and Cx43-containing pIRES plasmids.

**REFERENCES**

Ableser MJ, Penuela S, Lee J, Shao Q, Laird DW (2014) Connexin43 reduces melanoma growth within a keratinocyte microenvironment and during tumourigenesis in vivo. J Biol Chem 289: 1592–1603.

Becker D, Ciantar D, Catsicas M, Pearson R, Mobbs P (2001) Use of pIRES vectors to express EGFP and connexin constructs in studies of the role of
gap junctional communication in the early development of the chick retina and brain. *Cell Commun Adhes* **8**: 355–359.

Bodenstine TM, Vaidya KA, Ismail A, Beck BH, Cook LM, Diers AR, Landar A, Welch DR (2010) Homotypic gap junctional communication associated with metastasis suppression increases with PKA activity and is unaffected by PKJ inhibition. *Cancer Res* **70**: 10002–10007.

Carete D, Gilleron J, Chevallier D, Segretain D, Pointis G (2014) Connexin a check-point component of cell apoptosis in normal and physiological conditions. *Biochimie* **101**: 1–9.

Conklin CM, Bechberger JF, MacFabe D, Guthrie N, Kurowska E, Naus CC (2007) Genistein and quercetin increase connexin43 and suppress growth of breast cancer cells. *Carcinogenesis* **28**: 93–100.

Cayz J, Irmer U, Schulz G, Mindermann A, Hülser DF (2000) Gap-junctional coupling measured by flow cytometry. *Exp Cell Res* **255**: 40–46.

Decrock E, Vinken D, De Vuyst E, Krysko DV, D’Herde K, Vanhaecke T, Forster T, Rausch V, Zhang Y, Isayev O, Heilmann K, Schoensiegel F, Liu L, Du G, Yang Y, Zhang Y, Sun T, Liu W, Wang Y, Li J, Zhang H (2013) Functional gap junctions accumulate at the immunological synapse and regulate calcium signaling in T cells. *J Immunol* **187**: 3121–3132.

Du G, Yang Y, Zhang Y, Sun T, Liu W, Yang L, Li J, Zhang H (2013) Thrombocytopoiesis and immunohistochemical expression of connexin 43 at diagnosis predict survival in advanced non-small-cell lung cancer treated with cisplatin-based chemotherapy. *Cancer Chemother Pharmacol* **71**: 893–904.

Forster T, Rausch V, Zhang Y, Sayo I, Hellmann K, Schoenjes F, Liu L, Nessling M, Richter K, Labsch S, Nwaeburu CC, Mattern J, Gladkich J, Giese N, Werner J, Schemmer P, Gross W, Gehrad MM, Gerharder C, Schaefer M, Herr I (2014) Sulforaphane counteracts aggressiveness of pancreatic cancer driven by dysregulated Cx43-mediated gap junctional intercellular communication. *Oncotarget* **5**: 1621–1634.

Fukushima M, Hattori Y, Yoshizawa T, Maitani Y (2007) Combination of non-viral connexin 43 therapy and docetaxel inhibits the growth of human prostate cancer in mice. *Int J Oncol* **30**: 225–231.

Gellhaust A, Dong X, Propson S, Maass K, Klein-Hitpass L, Kibschull M, Fukushima M, Hattori Y, Yoshizawa T, Maitani Y (2007) Combination of non-viral connexin 43 therapy and docetaxel inhibits the growth of human prostate cancer in mice. *Int J Oncol* **30**: 1427–1439.

Huang RP (2002) Connexin 43 suppresses human glioblastoma cell growth of breast cancer cells. *Carcinogenesis* **23**: 969–975.

Kunihiro T, Kanzaki J (2008) The inhibitory effect of connexin 32 gene on metastasis of cell lines UACC903, UACC903(−), UACC904, UACC905, UACC906. *J Invest Dermatol* **132**: 1326–1334.

Langlois S, Cowan KN, Shao Q, Cowan BJ, Laird DW (2010) The tumour-suppressive function of Connexin43 in keratinocytes is mediated in part via interaction with caveolin-1. *Cancer Res* **70**: 4222–4232.

Li Z, Zhou Z, Welch DR, Donahue HJ (2008) Expressing connexin 43 in breast cancer cells reduces their metastasis to lungs. *Clin Exp Metastas* **25**: 893–901.

Masuda M, Usami S, Yamazaki K, Takami Y, Shinkawa H, Kurisaka H, Kunihira T, Kanazaki J (2001) Connexin 26 distribution in gap junctions between melanocytes in the human vestibular dark cell area. *Anat Rec* **262**: 137–146.

Mendoza-Naranjo A, Saéz PJ, Johansson CS, Ramírez M, Mandakovic D, Pereda C, López MN, Kiesling R, Sáez JC, Salazar-Onfray F (2007) Functional gap junctions facilitate melanoma antigen transfer and cross-presentation between human dendritic cells. *J Immunol* **178**: 6949–6957.

Mendoza-Naranjo A, Bouma G, Pereda C, Ramírez M, Webb KF, Tittarelli A, López MN, Kalgears AM, Thrasher AJ, Becker DL, Salazar-Onfray F (2011) Functional gap junctions accumulate at the immunological synapse and regulate calcium signaling in T cells. *J Immunol* **187**: 3121–3132.

Mege G, Richard G, White T (2007) Gap junction: basic structure and function. *J Invest Dermatol* **127**: 2516–2524.

Monteith GR, Davis FM, Roberts-Thomson SJ (2012) Calcium channels and pumps in cancer: changes and consequences. *J Biol Chem* **287**: 31666–31673.

Naus CC, Laird DW (2010) Implications and challenges of connexin connections to cancer. *Nat Rev Cancer* **10**: 435–441.

Neijssen J, Pang B, Neefjes J (2007) Gap junction-mediated intercellular communication in the immune system. *Prog Biophys Mol Biol* **94**: 207–218.

O’Carroll SJ, Alkadhi M, Nicholson LFB, Green CR (2008) Connexin43 mimetic peptides reduce swelling, astrogliosis, and neuronal cell death after spinal cord injury. *Cell Commun Adhes* **15**: 27–42.

Pfenniger A, Wohlewnd A, Kwak BR (2011) Mutations in connexin genes and disease. *Eur J Clin Invest* **41**: 103–116.

Plante I, Stewart MKG, Barr K, Allan A, Laird DW (2011) Connexin 43 suppresses mammary tumour metastasis to the lung in a Cx43 mutant mouse model of human disease. *Oncogene* **30**: 1681–1692.

Qin H, Shao Q, Curtis H, Galipeau J, Belliveau DJ, Wang T, Aloua-Jamali MA, Laird DW (2002) Retroviral delivery of connexin genes to human breast tumour cells inhibits in vivo tumour growth by a mechanism that is independent of significant gap junctional intercellular communication. *J Biol Chem* **277**: 29132–29138.

Rödel F, Franz S, Sheriff A, Gailp U, Heyder P, Hildebrandt G, Schulz-Frnt-Mosig S, Voll RE, Herrmann M (2005) The CFSE distribution assay is a powerful technique for the analysis of radiation-induced cell death and survival on a single-cell level. *Strahlenther Onkol* **181**: 456–462.

Salazar-Onfray F, López M, Lundqvist A, Aguirre A, Escobar A, Serrano A, Alonso JM, Perbell B, Lampe PD, Naus CC (2009) Connexin43 suppresses human glioblastoma cell growth by down-regulation of monocyte chemotactic protein 1, as discovered using protein array technology. *Cancer Res* **69**: 5089–5096.

Sato H, Hagiwara H, Senba H, Fukumoto K, Nagashima Y, Yamasaki H, Ueno K, Yano T (2008) The inhibitory effect of connexin 32 gene on metastasis in renal cell carcinoma. *Mol Carcinog* **47**: 403–409.

Sin WC, Tse M, Planque N, Peribel B, Lampe PD, Naus CC (2009) Matricellular protein Ccn3 (Nog) regulates actin cytoskeleton reorganization in human breast cancer cells. *J Biol Chem* **284**: 29395–29404.

Su YA, Bittner ML, Chen Y, Tao L, Jiang Y, Zhang Y, Stephan DA, Trent JM (2000) Identification of tumour-suppressor genes using human melanoma cell lines UACC903, UACC903(+6), and SRS5 by comparison of expression profiles. *Mol Carcinogen* **28**: 119–127.
Cx43 inhibits melanoma growth and metastasis

Tittarelli A, Mendoza-Naranjo A, Fariás M, Guerrero I, Ihara F, Wennerberg E, Riquelme S, Gleisner A, Kalergis A, Lundqvist A, López MN, Chambers BJ, Salazar-Onfray F (2014) Gap junction intercellular communications regulate NK cell activation and modulate NK cytotoxic capacity. J Immunol 192: 1313–1319.

Trosko JE (2003) The role of stem cells and gap junctional intercellular communication in carcinogenesis. J Biochem Mol Biol 36: 43–48.

Wang M, Berthoud V, Beyer E (2007) Connexin43 increases the sensitivity of prostate cancer cells to TNFα-induced apoptosis. J Cell Sci 120: 320–329.

Wang ZS, Wu LQ, Yi X, Geng C, Li YJ, Yao RY (2013) Connexin-43 can delay early recurrence and metastasis in patients with hepatitis B-related hepatocellular carcinoma and low serum alpha-fetoprotein after radical hepatectomy. BMC Cancer 13: 306.

Wheelock MJ, Shintani Y, Maeda M, Fukumoto Y, Johnson KR (2008) Cadherin switching. J Cell Sci 121: 727–735.

Yi ZC, Liu YZ, Li HX, Yin Y, Zhuang FY, Fan YR, Wang Z (2006) Tellimagrandin I enhances gap junctional communication and attenuates the tumour phenotype of human cervical carcinoma HeLa cells in vitro. Cancer Lett 242: 77–87.

Zhang Y, Kaneda M, Morita I (2003) The gap junction-independent tumour-suppressing effect of connexin 43. J Biol Chem 278: 44852–44856.

Zucker SN, Bancroft TA, Place DE, Des Soye B, Bagati A, Berezney R (2013) A dominant negative Cx43 mutant differentially affects tumorigenic and invasive properties in human metastatic melanoma cells. J Cell Physiol 228: 853–859.

This work is published under the standard license to publish agreement. After 12 months the work will become freely available and the license terms will switch to a Creative Commons Attribution-NonCommercial-Share Alike 4.0 Unported License.