Loss of Pannexin 1 Attenuates Melanoma Progression by Reversion to a Melanocytic Phenotype*

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Background: Pannexin 1 (Panx1) is a channel-forming glycoprotein that regulates epidermal differentiation and proliferation.

Results: Depletion of Panx1 in melanomas causes cell re-differentiation into a melanocytic-like phenotype and reduced tumorigenesis.

Conclusion: Panx1 is up-regulated during melanoma progression promoting tumor growth and metastasis.

Significance: This is the first report of Panx1 as a proto-oncogene establishing it as a potential target for melanoma treatment.

Pannexin 1 (Panx1) is a channel-forming glycoprotein expressed in different cell types of mammalian skin. We examined the role of Panx1 in melanoma tumorigenesis and metastasis since qPCR and Western blots revealed that mouse melanocytes exhibited low levels of Panx1 while increased Panx1 expression was correlated with tumor cell aggressiveness in the isogenic melanoma cell lines (B16-F0, F10, and BL6). Panx1 shRNA knockdown (Panx1-KD) generated stable BL6 cell lines, with reduced dye uptake, that showed a marked increase in melanocyte-like cell characteristics including higher melanin production, decreased cell migration and enhanced formation of cellular projections. Western blotting and proteomic analyses using 2D-gel/mass spectroscopy identified vimentin and β-catenin as two of the markers of malignant melanoma that were down-regulated in Panx1-KD cells. Xenograft Panx1-KD cells grown within the chorioallantoic membrane of avian embryos developed tumors that were significantly smaller than controls. Mouse-Alu qPCR of the excised avian embryonic organs revealed that tumor metastasis to the liver was significantly reduced upon Panx1 knockdown. These data suggest that while Panx1 is present in skin melanocytes it is up-regulated during melanoma tumor progression, and tumorigenesis can be inhibited by the knockdown of Panx1 raising the possibility that Panx1 may be a viable target for the treatment of melanoma.

Pannexin 1 (Panx1)3 is a glycosylated channel protein (1, 2) that constitutes part of the three-member family of pannexins in mammalian genomes. Pannexins were discovered due to their limited sequence homology to innexins, the invertebrate gap junction proteins (3). However, since their original discovery, the predominant function of pannexins has been documented to rest primarily in their ability to form single membrane channels while their potential role as gap junction proteins capable of mediating direct intercellular communication remains highly contentious and awaits further clarification (4).

Panx1 is ubiquitously expressed in many organs and tissues while Panx2 is abundant in the central nervous system and Panx3 is predominantly expressed in skin, bone, and cartilage (2, 5, 6). Reports on the physiological role of Panx1 channels highlight their importance in paracrine signaling as mechano-sensitive ATP release channels (7). Panx1-mediated ATP release through an interaction with purinergic receptors has been shown to play a role in Ca2+ signal initiation and propagation (8) as well as nucleotide find-me signal release from apoptotic cells to attract phagocytes for cell clearance (9). Panx1 channels contribute to status epilepticus in vivo increasing the severity and duration of seizures (10). In another study Panx1 channel opening under ischemic conditions increased hippocampal pyramidal neuronal cell death (11). All three pannexins have been implicated in keratinocyte (Panx1), neuron (Panx2), chondrocyte, and osteoblast (Panx3) differentiation, and in general pannexins are abundantly expressed in early stages of development (12–16). Furthermore, Panx1 and Panx2 have also been reported to act as tumor suppressors in glioma cells upon overexpression (17, 18).

Skin melanocytes are specialized cells of the basal epidermis that produce melanin pigments (19). Wnt/β-catenin signaling drives differentiation of neural crest cells toward a melanocyte cell fate, and this pathway is also involved in the malignant transformation of melanocytes to melanoma (19, 20). Malignant melanoma is the most deadly of all skin cancers, and although it accounts for only 4% of all skin cancers, it is responsible for 79% of skin cancer-related deaths (21). Various melanoma reference cell models have been used to study this cancer including mouse B16 isogenic melanoma lines described by Fidler et al. (22). The B16-F0 line was used to generate the B16-F10 line by ten successive selections for lung metastasis following intravenous injection in the mouse. B16-BL6 lines
were established from F10 cells that penetrated the mouse bladder (23). Although F10 and BL6 are both metastatic cell lines, BL6 is considered to be more aggressive since it can spontaneously metastasize to the lungs when subcutaneously implanted, while F10 cells can only colonize the lung by direct intravenous inoculation (24).

In the present study, we discovered that Panx1 is significantly up-regulated in B16 melanoma cells compared with the low basal levels observed in normal melanocytes in culture, and Panx1 levels are positively correlated with the aggressiveness of the isogenic melanoma cell lines. Panx1-depleted BL6 cells resemble normal melanocytes in cell morphology, melanin production, reduced migration and growth. Also, in BL6 cells the knockdown of Panx1 down-regulated the expression of vimentin and β-catenin which are markers of malignant melanoma. These proteomic changes indicated a possible effect of Panx1 knockdown in the tumorigenic and metastatic properties of BL6 cells that was confirmed in vivo, as there was a significant reduction in melanoma tumor size in the chorioallantoic membrane (CAM) and in metastasis to the liver of avian embryos.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture Conditions**—Murine melanoma cell lines B16-F0 (ATCC CRL-6322), B16-F10 (ATCC CRL-6475), and B16-BL6 (kindly provided by Dr. Moulay Alaoui-Jamali, McGill University) were cultured in Minimal Essential Medium (MEM) containing 2 mM L-glutamate, 10% FBS, 100 units/ml penicillin, and 0.1 g/liter CaCl₂ and 0.1 g/liter MgCl₂. The isogenic melanoma cell lines. Panx1-depleted BL6 cells resemble normal melanocytes in cell morphology, melanin production, reduced migration and growth. Also, in BL6 cells the knockdown of Panx1 down-regulated the expression of vimentin and β-catenin which are markers of malignant melanoma. These proteomic changes indicated a possible effect of Panx1 knockdown in the tumorigenic and metastatic properties of BL6 cells that was confirmed in vivo, as there was a significant reduction in melanoma tumor size in the chorioallantoic membrane (CAM) and in metastasis to the liver of avian embryos.

**Generation of Stable Panx1 shRNA Cell Lines**—BL6 cells were transfected with four constructs from Origene HuSH 29-mer Panx1 shRNA kit in pGFP-V-RS vector (TR30007) plus a non-effective 29-mer scrambled shRNA cassette as a control. shRNA-expressing cells were selected with puromycin, and two of the four constructs were chosen (sh18 and sh21) for Panx1 knockdown (KD) experiments after verifying at least a 70–85% knockdown of Panx1 protein levels by Western blot. Stable cell lines of sh18 and sh21 were maintained under puromycin selection pressure for 2 weeks and periodically examined for concomitant free-GFP expression and effective Panx1 knockdown by Western blot. For chick-CAM assays, further selection of stable Panx1-KD lines was achieved by isolation of single-cell derived clonal lines (n = 3, for sh21 and scrambled controls) that maintained Panx1-KD levels of up to 85% even in the absence of puromycin selection. Cell morphology was evaluated by light microscopy using a Leica Microsystems fluorescent microscope equipped with a Hamamatsu digital camera and OpenLab software.

**Cell Growth and Migration Assays**—Cultured cells were plated into individual wells at a density of 1 × 10⁴ cells/ml. On day 7, cells were trypsinized with 500 μl of 0.25% trypsin-EDTA (Invitrogen), and counted using a Countess Automated Cell Counter (Invitrogen). At least 4 biological replicates were done for statistical analysis with each of the constructs. Scrape wound-migration assays were performed by culturing cells on a grid Petri dish where cells were grown to confluency as a monolayer. After scraping the cell layer with a pipette tip the growth medium was replaced with serum reduced Opti-MEM to minimize cell proliferation. Images were taken along the grid line using a Leica microscope at the time of wounding and at 48 h after the total area of cells that migrated from the scratch line was measured using ImageJ software and divided by the length of the field of view to obtain the distance migrated. Four biological replicates for each of the two shRNA constructs (sh18 and sh21) and scrambled control were used for statistical analysis.

**Melanin Extraction**—Equal numbers of BL6, scrambled controls, sh18 and sh21 cells as well as L10 melanocytes were plated and grown to confluence over a 72 h period. Then the cells were trypsinized and resuspended in fresh growth media. Cells were counted and 10⁶ cells per sample were transferred to a 10 ml tube for centrifugation for 10 min at 200 × g. The pellets were collected and dissolved in 200 μl of 1 M NaOH with 10% DMSO and incubated at 60 °C for 2 h. After mixing, each sample was transferred to a 96-well plate and absorbance at 450 nm was read using a Perkin Elmer Victor3 plate reader. Five biological replicates were processed for each cell line.

**Dye Uptake Assays**—GFP-positive BL6 cells from scrambled control, sh18 and sh21 stable cell lines were plated onto 35-mm dishes at a density of 2 × 10⁵ cells/ml. The next day, cell cultures were rinsed twice with Dulbecco’s-PBS (D-PBS) (1 × with 0.1 g/liter CaCl₂ and 0.1 g/liter MgCl₂, 6H₂O, pH 7.4, Invitrogen), and mechanically stimulated by drip of sulforhodamine dye (2 mg/ml in D-PBS) for 5 min, followed by 10 washes with D-PBS. Imaging and quantification by ImageJ was done as we described previously (2). The integrity of the cell membranes was tested with parallel experiments using dextran-rhodamine as a negative control. Incidence of dye uptake was calculated...
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based on the ratio of dye uptake area to GFP positive area per field of view and statistical analysis was performed using three biological replicates.

Proteomic Analysis and Validation—Four 10-cm plates of scrambled control or shRNA knockdown (sh21) cells were lysed in lysis buffer (8 M urea, 2% CHAPS, 1 mM benzamidine, 25 μg/ml leupeptin, 20 μg/ml pepstatin A, 20 μg/ml aprotinin, 1 μM okadaic acid, 1 μM microcystin, 1 mM sodium orthovanadate, 0.5% amphotelyte, and 40 mM DTT) on ice according to previously established methods (26). The cell lysates were cleared by centrifugation and the protein concentration was previously established methods (26). The cell lysates were based on the ratio of dye uptake area to GFP positive area per field of view and statistical analysis was performed using three biological replicates.

Four independent protein lysates samples (250 μg) of each treatment were separated by 2D-gel electrophoresis using Immobiline DryStrip pH 3–10 NL (13 cm, GE Healthcare) in the first dimension and SDS-PAGE for the second dimension following the manufacturer’s recommendations and according to established protocols (27). Proteins were visualized by Sypro Ruby protein gel stain (Life technologies) and imaged with the Pro-XPRESS 2D Proteomic Imaging System (PerkinElmer Life Sciences). Three images from each treatment were analyzed by the Phoretix 2D evolution software (TT900 S2S and PG220, Non-linear Dynamics) as described earlier (27). All gels were also stained with Gelcode Blue protein stain (Thermo) to enable protein visualization. The spots that had a significant (p < 0.05) change in expression level between the two treatments were isolated from the gels using an Ettn Spot Picker (GE Healthcare). Samples were processed using the MASSPrep Automated Digestor (Waters/Micromass) in the Functional Proteomics Facility at the University of Western Ontario. Peptides were spotted on MALDI plates and MALDI-TOF analysis was performed using a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA) as described (26). Mass spectra were searched against the NCBIr data base of Mus musculus using the MASCOT search engine with carbamidomethyl (C) fixed modification. Peptide tolerance of 50 ppm was used. Validation of protein expression of the identified proteins was done by immunoblotting using cell lysates of scrambled control, sh18 or sh21 cells. Membranes were immunoblotted for vimentin or β-tubulin to enable protein visualization. The spots that had a significant (p < 0.05) change in expression level between the two treatments were isolated from the gels using an Ettn Spot Picker (GE Healthcare). Samples were processed using the MASSPrep Automated Digestor (Waters/Micromass) in the Functional Proteomics Facility at the University of Western Ontario. Peptides were spotted on MALDI plates and MALDI-TOF analysis was performed using a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA) as described (26). Mass spectra were searched against the NCBIr data base of Mus musculus using the MASCOT search engine with carbamidomethyl (C) fixed and oxidation (M) plus phosphorylation (ST) variable modification. Peptide tolerance of 50 ppm was used. Validation of protein expression of the identified proteins was done by immunoblotting using cell lysates of scrambled control, sh18 or sh21 cells. Membranes were immunoblotted for vimentin or Hsc70 in three biological replicates and evaluated by densitometry using β-tubulin as loading controls. In a non-proteomic screen, β-catenin was also used for immunoblotting of scrambled control and shRNA lysates (sh18 and sh21), and quantified by densitometry as described above.

Spontaneous Metastasis Assay in the Avian Embryo—Avian embryo metastasis assays were performed as described previously (28–32). Fertilized chicken eggs (McKinney Hatchery, St. Mary’s Ontario) were incubated in a rotary incubator (Berry Hill) under 70% humidity at 37 °C for 4 days and then were removed from their shell and placed in covered dishes. Avian embryos were incubated for 5 more days in stationary incubators (Berry Hill) with 70% humidity at 37 °C. On day 11 of development, 20 μl of media containing 5.0 × 10⁵ cells (scrambled control and sh21 cells) were implanted between branching vessels on the CAM. The embryos were returned to a humidified 37 °C stationary hatcher for 7 days to allow tumor growth. On day 18, the melanoma tumors along with the livers were harvestested from the embryos weighed and stored at −20 °C. Genomic DNA was extracted from the organs using an ‘Extract and Amplification’ kit (Sigma). A total of 50 mg of liver from each animal was used in the extraction. Quantitative PCR was performed on the extracted organs by diluting the extractions 50 fold in sterile water and adding 4 μM of primers specific for the mouse Alu sequence and avian GAPDH. This was combined with an equal volume of 2X SYBR green (Invitrogen). Reactions were done in duplicate for each primer set in the myIQ cycler (Bio-Rad). A standard curve was generated by quantitative amplification of genomic DNA extracted from serial dilutions of BL6 cells mixed with individual chick liver extracts from untreated embryos as described previously (31). The actual number of tumor cells/50 mg of liver could be determined by interpolation of the Alu qPCR values from experimental samples with the standard curve. Statistical analyses were done using GraphPad Prism (San Diego, CA).

RESULTS

Panx1 Is Highly Expressed in Metastatic Melanoma Cells—qPCR and Western blot analysis indicated that the mouse melanoma isogenic lines B16-F0, B16-F10 and B16-BL6 express substantial levels of endogenous Panx1 which runs as a series of unglycosylated and glycosylated species between 41–48 kDa (Fig. 1A), while there was no detectable expression of Panx2 or Panx3 (Fig. 1, B and C). Interestingly, increasing levels of Panx1 were correlated with the metastatic aggressiveness of these melanoma lines (24), with BL6 cells having the highest level of Panx1 (Fig. 1, D and E). Immunocytochemistry revealed uniform Panx1 cell surface localization in cultured isogenic melanoma cell lines along with some intracellular labeling, reminiscent of the cellular distribution observed upon ectopic expression of Panx1 in mouse neuroblastoma cells (N2A, Fig. 1F).

Panx1 shRNA Knockdown Induces Melanoma Reversion to a Melanocytic-like Cell Phenotype—To assess the role of Panx1 in melanoma progression, we chose the BL6 cell line due to its aggressive phenotype and high expression of Panx1. Upon shRNA knockdown of Panx1 with two distinct constructs (sh18, sh21) together with a scrambled control, stable cell lines were established by puromycin selection. Cell lines with 70–85% Panx1 knockdown were selected as determined by quantification of Panx1 in Western blots (Fig. 2A). The efficacy of the knockdown and specificity of Panx1 targeting was further supported by the observed significant decrease in Panx1 levels in BL6 cells expressing ectopic Panx1 (Fig. 2B). Interestingly, a qualitative comparison of cell morphology by light microscopy showed that Panx1 shRNA cell lines resembled the morphology of normal melanocytes (L10), that express lower levels of Panx1 (Fig. 2B) with longer cell projections and smaller cell bodies than BL6 cells (Fig. 2C). In parallel cultures, more melanoma cells were found to grow and survive in control cultures than under conditions where Panx1 was reduced (Fig. 2D). Also, the amount of melanin extracted from the Panx1 knockdown cells was found to be comparable to the levels extracted from L10 melanocytes, while wild-type BL6 cells and scrambled shRNA control cells had significantly lower melanin content (Fig. 2E). Finally, scrape wounding of confluent cultures in vitro under reduced serum media revealed that cell migration was also...
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Dye Uptake Is Compromised by Depletion of Panx1 in BL6 Cells—Stable BL6 cell lines expressing shRNA constructs sh18, sh21 or scrambled controls were identified by concomitant free-GFP expression and used for dye uptake assays as described earlier (25). Briefly, mechanical stimulation by sulforhodamine B dye drip under conditions of physiological calcium opened the Panx1 channels in control cells. A marked reduction in dye uptake was observed in shRNA stable cells (Fig. 3). Dextran-sulforhodamine controls run in parallel demonstrated that cell membranes were intact (Fig. 5, insets) strongly suggesting that sulforhodamine B dye uptake was mediated via Panx1 channels.

Proteomic Analysis of Melanoma Cells with Reduced Panx1—The entire proteomic profile of control and shRNA (sh21) BL6 cell lines was compared by two-dimensional gel analysis of total cell protein lysates run in parallel, followed by Sypro gel staining and spot analysis (Fig. 4A). SameSpots software found 42 spots with significant changes between treatments. After visual inspection 24 spots were selected for mass spec identification, but only 12 spots had strong enough signal intensity to allow protein identification. Based on three two-dimensional gels for each treatment, we identified eight proteins by mass spectrometry that showed significant (p < 0.05) change in expression upon Panx1 knockdown as compared with the control (Fig. 4B). Western blots confirmed that two of the identified spots corresponding to the malignant melanoma marker, vimentin (Fig. 5), and the heat-shock protein, Hsc70 (Fig. 6), were significantly downregulated upon Panx1 knockdown in sh18 and sh21 treated cells. In separate experiments, β-catenin expression was also significantly reduced upon Panx1 knockdown as shown in Fig. 7A. Immunolabeling revealed lower levels of expression and increased intracellular localization of β-catenin in Panx1 shRNA-treated cells (Fig. 7B).

Knockdown of Panx1 Reduces Melanoma Tumor Growth and Metastasis—An avian embryo model for spontaneous metastasis (29, 30, 32) was used to determine the impact of Panx1 knockdown on the growth and spread of melanoma tumors. Seven days after implantation of BL6 mouse melanoma cells from control (Fig. 8A) and Panx1 sh21 (Fig. 8B) cell lines in the chicken embryo CAM, visible tumors were formed (white arrowheads, Fig. 8). Tumors derived from Panx1 knockdown cells were significantly smaller than those derived from control cells (Fig. 8C). Furthermore, Panx1 knockdown tumors contained less blood than controls which became hemorrhagic at later time points. Spontaneous metastasis to the embryonic liver was assessed by qPCR with primers specific for the mouse Alu sequence (28, 31). As determined by comparison to a standard curve, the estimated number of metastatic mouse BL6 cells in 50 mg of liver was significantly lower for Panx1 knockdown tumors than controls, even at this early time point for assessing metastasis (Fig. 8D). In summary, these results suggest that Panx1 depletion reverts melanomas into a melanocytic-like phenotype, attenuating their tumorigenic and metastatic properties.
DISCUSSION

The physiological role reported for Panx1 channels has been mostly related to ATP release and their paracrine signaling functions (6, 33). However, several cellular responses mediated by Panx1 have also been reported by us and others such as the dysregulation of keratinocyte differentiation in organotypic epidermis of rat epidermal keratinocytes ectopically expressing Panx1 (12). Upon examining other resident cell types of the skin, we discovered low levels of Panx1 expression in a melanocytic cell line while we observed substantial Panx1 protein levels in three isogenic melanoma cell lines. Therefore, we chose an shRNA knockdown approach as opposed to over-expression to study the role of Panx1 in melanoma. The BL6 cell line was selected for further studies since it is the most aggressive melanoma cell line and readily metastasizes (24). Since Panx1 protein levels correlated with increasing aggressiveness of the isogenic cell lines, we hypothesized that Panx1 may play a role in their transformation from normal melanocytes to metastatic melanomas.

Upon Panx1 knockdown a marked change in cellular phenotype occurred that reflected cell morphological changes that mimicked normal melanocytes. Panx1-reduced cells also exhibited higher melanin content and reduced migration and proliferation, which are all characteristics of differentiated
melanocytic cells (34). Phenotypic changes in pannexin-reduced cells were also reported by Swayne et al. (15) where knockdown of Panx2 induced differentiation of neuroblastoma (N2A) cells to have neuritic extensions and express markers of mature neurons. Interestingly, melanoma and neuroblastoma tumor cells are both derived from parental cells of neural crest origin where pannexins appear to be of central importance (20). Since pannexins have been implicated in the differentiation of osteoblasts, chondrocytes and neurons (13–15), it will be interesting to examine their potential role in the malignant transformation of some of these other neural-crest-derived cell lineages.

Distinct from our study, Panx1-overexpressing glioma cells have been reported to be less tumorigenic (18) raising the possibility that, in some tumors of different origin, Panx1 may act as a tumor suppressor rather than promoting an aggressive tumor cell phenotype as we observed in melanomas. While we cannot dismiss this possibility, it is notable that Panx1 overexpressing glioma cells were reported to have increased gap junctional intercellular communication (18) suggesting that the well-documented tumor suppressors, connexins (35), may have been co-regulated by Panx1 overexpression. In the case of the BL6 melanoma cells used in our study, they are clearly gap junctional intercellular communication-deficient, although very low levels of Cx26 and Cx43 are expressed (36). Nevertheless, conflicting studies of molecules acting as tumor suppressors in one context and oncogenes in another have been reported for E-cadherin in glioblastomas where the molecular function of E-cadherin in tumorigenesis is rooted in its subcellular localization (37).

Mechanistically we investigated the changes in the melanoma proteome as a result of Panx1 knockdown in order to determine why there were significant changes in the cellular phenotype. From our proteomic and immunoblotting analyses it was clear that markers of malignancy such as vimentin and β-catenin were significantly down-regulated in Panx1-depleted melanoma cells. Thus, Panx1 knockdown was not only causing a re-differentiation of the melanoma phenotype into a more melanocytic one, but it appeared to have changed the tumorigenic and metastatic properties of these cells. Vimentin has been identified as a useful biomarker for melanoma metastasis, as its overexpression is observed in patients with primary melanoma with hematogenous metastasis but not associated with metastasis to the lymph nodes (38). Vimentin has also been reported to regulate Hsc70, which is one of the heat shock cognate proteins that was also down-regulated in Panx1-reduced cells (39). In a related but distinct study, a relationship between Panx1 and the cytoskeletal network has been established as we...
reported earlier that Panx1 directly binds actin and its trafficking is regulated by the actin cytoskeleton (40). Disrupting the interaction of Panx1 with different cytoskeletal components in BL6 cells may also contribute to changes in migration, cellular phenotype and invasiveness of melanomas, as reported (41).

The role of β-catenin in melanomas has been extensively reviewed (19, 42) and recent reports suggest that the canonical Wnt3A/β-catenin pathway can promote a more differentiated phenotype of melanoma cells with beneficial effects on patient survival (34). Activation of the Wnt/β-catenin pathway can decrease melanoma metastasis and increase pigment production along with melanogenic antigen expression (34). On the other hand, high levels of non-nuclear β-catenin can induce proliferation of melanoma cells (19), decrease the length of melanocyte dendrites and alters cell morphology to a round enlarged shape more typical of melanoma cells (43). In Panx1-shRNA treated cells we observed a moderate decrease in the characteristic cell surface localization of β-catenin, consistent with a less invasive phenotype (44). Upon Panx1 reduction, β-catenin and the canonical Wnt signaling pathway could be

![Image showing Western blots and mass spectrum analysis](image-url)
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Affected resulting in morphological changes that resemble more closely a melanocytic phenotype with lower $\beta$-catenin levels (43).

Since Panx1 knockdown significantly reduced the expression of malignant melanoma markers, we set out to test if the re-differentiated melanoma cells that we observed in vitro would also have altered tumorigenicity and metastatic ability in vivo. The chicken embryo is a robust in vivo xenograft model that allows for the assessment of tumor growth and metastatic phenotypes of human and mouse cell lines (28–30, 32). A significant reduction in tumor size was observed in Panx1-reduced cell lines, while control cells formed large tumors that readily vascularized and bled. These findings clearly suggest that Panx1 is regulating primary tumor growth via altered cell cycle or cell death pathways or through changes in the vascularization of the tumor. In addition, control melanoma cells were more efficient at metastasizing to the liver of the chick embryo. Thus, in keeping with our in vitro studies, our in vivo studies clearly establish that Panx1 not only facilitates growth of the primary tumor but further enhances the ability of tumor cells to metastasize.

FIGURE 6. Heat shock protein 70 cognate is significantly down-regulated upon Panx1 knockdown. A, Western blots normalized to $\beta$-tubulin levels confirmed a significant decrease in Hsc70 expression upon Panx1 depletion (combined separate sh18 or sh21 treatments) as seen in triplicate two-dimensional gels. Student’s $t$ test, $n = 3$, $p < 0.05$. bars: S.E. Molecular markers are in kDa. B, mass spectrum and identification parameters of Hsc70 protein identified by MALDI mass spectrometry. Peaks with red in the mass spectrum were matched to the identified protein. The protein score, number of peptides matched (red) and sequence coverage of the MASCOT peptide mass fingerprint search, are indicated.
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FIGURE 7. β-Catenin expression is reduced in Panx1-depleted cells. A, significant reduction in β-catenin level was observed in cells with ~80% Panx1 knockdown (combined separate sh18 or sh21 treatments) as quantified by densitometry of β-catenin blots normalized to β-tubulin. Student’s t test, n = 3, p < 0.01, bars: S.E. Molecular markers are in kDa. B, immunolabeling of β-catenin (red) in sh21 cells shows an increase in intracellular β-catenin localization compared with the more characteristic cell surface labeling observed in malignant melanoma cells (control). Nuclei: blue, Bar: 20 μm.

Although Panx1-mediated dye uptake in BL6 melanoma cells triggered by mechanical stimulation was significantly reduced by Panx1 knockdown, it is not known how the channel function of Panx1 could directly affect melanoma progression in vivo. However, we hypothesize that ATP release from melanoma cells with high levels of Panx1 may act via purinergic receptors on neighboring cells leading to elevated intracellular calcium and increased proliferation, as has been reported for other cancer lines such as MCF-7 breast cancer cells (45). Furthermore, in human melanoma tumor samples assessed as part of the Human Protein Atlas project, 70% of the malignant melanoma samples assessed exhibited significant Panx1 immunostaining. Out of 20 cancers tested, other tumor types with high levels of Panx1 included lung, colorectal and non-melanoma skin cancer. Therefore, it is possible that Panx1 is significantly up-regulated as melanocytes transform into melanomas and, moreover, a knockdown of Panx1 can reduce the tumorigenic properties.

Collectively, these data may suggest that Panx1 is a potential therapeutic target in the prevention of melanoma disease progression. Importantly, the extracellular domains of Panx1 are exposed (2) and mimetic peptides have been shown to be capable of blocking Panx1 channel function (46) raising the probability that small molecule drugs could be developed for cancer therapies where drug entry into tumor cells would not be a necessity. Also, as Panx1 knockdown can revert mouse melanomas to more melanocytic-like phenotypes we envision potential for other complementary therapies in humans where RNAi, antisense, antibodies or mimetic peptides could be used to inhibit these cell surface channels and reduce the incidence of melanoma tumor progression.

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