Unexpected High Levels of BRN2/POU3F2 Expression in Human Dermal Melanocytic Nevi


TO THE EDITOR

Malignant melanoma is the most aggressive skin cancer. A majority of cancer-associated deaths are due to the invasion of cancer cells to distant tissues. Thus, it is of great importance to understand the mechanisms of metastatic spread. A potential marker for this is BRN2, encoded by the POU3F2 gene, that interacts with SOX transcription factors (Cook and Sturm, 2008; Malik et al., 2018). While some studies consider BRN2 as a positive regulator of microphthalmia-associated transcription factor (MITF), which favors proliferation (Goodall et al., 2004a; Wellbrock et al., 2008), other studies propose a dual function for BRN2 in both tumor proliferation and invasion, which could be controlled via the upregulation or downregulation of MITF, respectively (Simmons et al., 2017; Wellbrock and Arozarena, 2015). BRN2 and MITF are present in two distinct sub-populations of cells in 3-dimensional culture (Thurber et al., 2011) and melanoma patient biopsies (Goodall et al., 2008; Pinner et al., 2009) in which the expression of each transcription factor is mutually exclusive. The relationship between MITF and BRN2 has also been further characterized by showing a role for MITF in the reduction of BRN2 protein levels (Boyle et al., 2011). However, recent single cell gene expression efforts on human primary melanoma cells suggests that the situation may not always be “black and white” with some melanoma cell-expressing genes associated with both high and low levels of BRN2-associated genes in vivo (Ennen et al., 2017).

Here we have assessed the expression levels of BRN2 in various types of melanocytic lesions using extremely sensitive immunohistochemistry with Tyramide signal amplification. We approached this by looking for melanocytic cells positive for both SOX10 and BRN2 (SOX10+/BRN2+). While BRN2 is not detectable in normal melanocytes, strong expression of the nuclear BRN2 protein was seen in dermal nevi and congenital dermal nevi (Figure 1a). In epidermal melanocytic lesions, we observed much less BRN2 positivity (Figure 1a and Supplementary Figure S1). The proportion of SOX10+/BRN2+ cells was quantified and stratified based on their location in skin using Inform Cell Analysis software (Perkin Elmer, Waltham, MA). The results demonstrate that most dermal nevus cells, particularly in dermal congenital nevi, express BRN2. In epidermal lesions, the proportion of BRN2-positive cells is much lower than that in the dermal lesions, although it increases during progression (Figure 1b). Few or no BRN2-positive cells were observed in benign epidermal nevi, with slightly higher levels in superficial spreading melanomas and invasive epidermal malignant melanomas, although not approaching the very high levels seen in
A schematic description of the BRN2 positivity of melanocytic lesions is presented in Figure 1c.

Our findings are in agreement with recent studies in which benign epidermal nevi were found to not express significant BRN2 protein, while the expression increases during disease progression (Zeng et al., 2018). Hence, we set out to further investigate the control of BRN2 expression in the congenital dermal nevi. Previous studies in melanoma have found that activation of the mitogen-activated protein kinase pathway in melanoma, in particular, the mutation of BRAF (Goodall et al., 2004b), nuclear accumulation of β-catenin (Goodall et al., 2004a), PI3K activation (Bonvin et al., 2012), or E2F1 (Zeng et al., 2018) activate the promoter of BRN2. BRN2 has recently been shown to suppress apoptosis and reprogram DNA repair, suggesting that BRN2 contributes to the generation of melanoma with a high somatic mutation burden (Herbert et al., 2019). We therefore examined the expression of BRN2 in relation to the activation of either the mitogen-activated protein kinase (BRAF V600E mutation and extracellular signal-regulated kinase 1/2 phosphorylation) or PI3K (PTEN) pathway in 10 congenital dermal nevi (Figure 2 and Supplementary Table S1). All the congenital dermal nevi expressed high levels of PTEN, therefore suggesting that the PI3K/protein kinase B pathway is not activated in these lesions. In addition, five of the 10 were positive for the BRAF V600E mutation as assessed.

Figure 1. SOX10/BRN2 expression in human normal skin or dermal melanocytic nevi. (a) Images of 4-plex fluorescent immunohistochemistry with Tyramide signal amplification staining of SOX10/BRN2 in normal skin, benign, and congenital dermal nevi. De-identified patient samples were subjected to histopathological diagnosis by an experienced pathologist (DL). Red arrowheads denote melanocytic cells positive for SOX10. Yellow indicates melanocytic cells positive for BRN2. Green indicates SOX10/BRN2+ cells. Dashed yellow line demarcates the epidermal basal layer or different structures. Yellow Bars = 600 μm; Red Bars = 200 μm. (b) Heatmap showing the percentage of SOX10/BRN2-positive cells in the epidermal and dermal compartments in melanocytic lesions. Red = the highest correlation (one); yellow = the lowest correlation (zero). (c) Schematic representation of the proportion of BRN2-positive cells in various melanocytic lesions. HF, hair follicle; MM, malignant melanoma; SG, sebaceous gland.
using a specific antibody for staining. Interestingly, BRAF V600E staining did not correlate with BRN2 expression or phosphorylated extracellular signal–regulated kinase 1/2 staining (Supplementary Table S1). This data may suggest a different form of control of BRN2 expression in congenital dermal nevi compared with cutaneous melanoma cells.

While we observed strong BRN2 staining in congenital dermal nevi, there was also some weaker BRN2 staining in the invasive epidermal malignant melanomas. To investigate whether BRN2 expression is associated with melanoma survival in a large cohort of melanoma patients, we analyzed the testicular germ cell tumor skin melanoma cohort, which restricts us to assessing the POUSF2 RNA but not the protein levels. Across this cohort, POUSF2 levels just reach the level of significance ($P = 0.048$) (Supplementary Figure S3) for correlation with overall survival but not for disease-free survival ($P = 0.18$), suggesting a somewhat complex relationship between the two variables. Notwithstanding, it is possible that the propensity of certain tumor cells to metastasize may not depend on the overall level of gene expression but rather may be dependent on the presence of just a few positive cells that may initiate metastatic spread.

In conclusion, the levels of BRN2 expression in melanocytic cells throughout melanoma progression in vivo is becoming clearer. It does not appear to be involved in the day-to-day function of normal melanocytes. In primary epidermal lesions, the proportion of melanocytic cells positive for BRN2 increases only mildly during progression. Strikingly, the expression of BRN2 protein is overwhelmingly the highest in dermal congenital nevi, and to a lesser extent, in non-congenital benign dermal nevi, suggesting that high BRN2 expression may be in some way associated with the localization of melanocytic cells in the dermal component, away from the familiar cues from associated keratinocytes. However, this begs the question as to why these lesions carry such high levels of BRN2 expression, because only very large giant congenital nevi (which we did not study) are at increased risk for transformation. BRN2 may possibly function in non-metastatic melanocytic cells to some degree regulating the expression of “neural” type genes, as congenital lesions probably emanate from the neural crest-derived melanocytes trapped in the dermis during development. It was previously shown that such lesions harbor a more neural gene expression profile than other epidermal melanocytic lesions (Hamid et al., 1989).

**CONFLICT OF INTEREST**

The authors state no conflict of interest.

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**AUTHOR CONTRIBUTIONS**

Conceptualization: AC, GW, GB; Formal Analysis: AC, GW, DL; Funding Acquisition: GW, GB; Investigation: AC, BF, HYH; Project Administration: GW, GB; Resources: DL, BG; Supervision: GW, GB; Writing: AC, GW, GB.

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**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2019.12.007.

**REFERENCES**

Bonvin E, Falletta P, Shaw H, Delmas V, Goding CR. A phosphatidylinositol 4,5-bisphosphate 3-kinase 

### Data availability statement

Not applicable.

### ETHICS STATEMENT

Experiments were undertaken with the University of Queensland Human Research Ethics Committee A & B approval (approval number 2011001201/HREC/11/QPAH/363). Patients gave written, informed consent to participate.

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High BRN2 Expression in Dermal Nevi

**Figure 2. Analysis of the upstream regulating pathways of BRN2 expression in vivo.** Images of three different congenital dermal nevi stained for BRN2, PTEN, BRAF V600E mutation, or phosphorylated ERK1/2 in consecutive section using immunohistochemistry. Bars = 200 μm. See Supplementary Table S1 for quantitation information. ERK, extracellular signal–regulated kinase.


SUPPLEMENTARY MATERIALS AND METHODS

Clinical diagnosis
De-identified patient samples were subjected to a histopathological diagnosis by an experienced pathologist (DL). Experiments were undertaken with the University of Queensland Human Research Ethics Committee A & B approval (approval number 2011001201/HREC/11/QPAH/363). Patients gave written, informed consent to participate.

Multiplexed Fluorescent Immunohistochemistry with Tyramide Signal Amplification
Sections from paraffin-embedded lesions were dewaxed and treated with Dako low pH antigen retrieval solution at 100 °C for 15 minutes (Carpinteria, CA). Endogenous peroxidase activity was quenched in 3% hydrogen peroxide and sections blocked with 5% bovine serum albumin. Primary BRN2 (D2C1L Rabbit mAb #12137, Cell Signaling Technology, Danvers, MA) and Sox10 (sc-17342, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were applied, followed by the appropriate secondary antibodies. After washing, the signal was amplified using a Perkin Elmer Tyramide amplification kit (FITC and CY5, respectively). The slides were washed, counterstained with DAPI, and coverslipped using Dako mounting medium. Slides were scanned using the Vectra spectral imaging system (PerkinElmer, Waltham, MA). Images were scanned at ×4 magnification. Multispectral images were captured using Phenochart software and scanned from 420 nm to 720 nm using Vectra 3.1 Automated quantitative pathology imaging system. Three multispectral images per sample were analyzed using InForm analysis software. Positive cells were segmented and counted per ×20 magnification field.

Immunohistochemistry
Sections (5 μm) were incubated with primary antibodies (BRAF V600E was detected using the Ventana system; BRN2 - D2C1L Rabbit mAb #12137, Cell Signaling; PTEN - 138G6 Rabbit mAb #9559, Cell Signaling; phosphorylated extracellular signal-regulated kinase 1/2 - 20G11 Rabbit mAb #4376, Cell Signaling) overnight at 4 °C before incubation with the appropriate biotinylated secondary antibodies and before incubation with the streptavidin horseradish peroxidase conjugate for 20 minutes (1:600 dilution in phosphate-buffered saline; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and subsequently probed with the chromagen substrate 3-amino-9-ethylcarbazole for 5 minutes (DakoCytomation, Glostrup, Denmark). All the slides were counterstained with routine hematoxylin.

Melanoma survival analysis
To calculate the melanoma survival measures associated with gene expression, we used the Gene Expression Profiling Interactive Analysis database (http://gepia.cancer-pku.cn).
Supplementary Figure S1. Antibody staining in nevi and melanomas. Images of 4-plex fluorescent immunohistochemistry with Tyramide signal amplification of melanocytic lesions using SOX10 and BRN2 antibodies. Upper panel—epidermal lesions; lower panel—compound lesion. Red arrowheads denote melanocytic cells positive for SOX 10. Yellow indicates melanocytic cells positive for BRN2. A dashed yellow line demarcates the epidermal basal layer. Invasion indicates areas of invasion of the dermis. Yellow Bars = 600 µm; Green Bars = 400 µm. MM, malignant melanoma; SSM, superficial spreading melanoma.
**Supplementary Figure S2.** BRN2 staining in nevi and melanomas. Comparison of three tumors of each subtype. Images of 4-plex fluorescent immunohistochemistry with Tyramide signal amplification staining of BRN2 in (a) benign dermal and congenital nevi, (b) epidermal lesions from benign nevi to invasive MM. Red arrows point to the major location of tumor within either the epidermis or dermis. Yellow Bars = 600 μm; Green Bars = 400 μm; Red Bars = 200 μm. MM, malignant melanoma.

**Supplementary Figure S3.** POU3F2 RNA expression and melanoma survival across the SKCM cohort. (a) Significant association between high or low levels of POU3F2 gene expression and overall survival in SKCM patients across the testicular germ cell tumors cohort ($P = 0.048$). (b) Association between high or low levels of POU3F2 and disease-free survival. Dotted lines indicate the 95% confidence intervals. HR, Cox PH hazard ratio; SKCM, skin cutaneous melanoma; TPM, transcripts per million.
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Abbreviation: ERK, extracellular signal–regulated kinase.