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Genetic variation in IRF4 expression modulates growth characteristics, tyrosinase expression and interferon-gamma response in melanocytic cells

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Summary

A SNP within intron4 of the Interferon Regulatory Factor4 (*IRF4*) gene, rs12203592*C/T has been independently associated with pigmentation and age-specific effects on nevus count in European-derived populations. We have characterised the cis-regulatory activity of this intronic region and using human foreskin-derived melanoblast strains we have explored the correlation between *IRF4* rs12203592 homozygous C/C and T/T genotypes with TYR enzyme activity, supporting its association with pigmentation traits. Further, higher *IRF4* protein levels directed by the rs12203592*C allele were associated with increased basal proliferation but decreased cell viability following UVR, an etiological factor in melanoma development. Since UVR, and accompanying IFN γ -mediated inflammatory response is associated with melanomagenesis we evaluated its effects in the context of *IRF4* status. Manipulation of *IRF4* levels followed by IFN γ treatment revealed a subset of chemokines and immuno-evasive molecules that are sensitive to *IRF4* expression level and genotype including *CTLA4* and *PD-L1*.

Significance

GWAS have identified associations between an intronic *IRF4* SNP with nevus count and pigmentation, two of the strongest predictors of melanoma. The *IRF4* alleles (rs12203592*C/T) show differential cis-regulatory activities and are targeted by MITF. Analysis of primary melanocytic cells reveals a link between *IRF4* genotype and growth, pigmentation and survival in response to UVR. Furthermore, *IRF4* variance, working through differential expression levels, can influence key IFN γ -induced cytokines and immuno-modulatory signals. These data provide insight into the role of *IRF4* in nevi formation and regression as well as potential interactions between melanocytes and immune cells relevant to melanomagenesis and immunotherapy responses.

Keywords

IRF4, MITF, tyrosinase, interferon-response, UVR-response, melanocyte, melanoma

Running Title

IRF4 effects growth, pigmentation, UV and IFN γ response

Introduction

Comparative genomics, genome-wide and locus-specific allele association studies have been extensively exploited in order to identify genes and alleles associated with pigmentation traits (Sturm, 2009), as well as benign and malignant melanocytic neoplasms (Law et al., 2012). Frequently, GWAS can identify sequence polymorphisms strongly associated with multi-factorial diseases and quantitative traits that reside in non-coding regions of the genome and impact on functions such as gene regulation and locus control (Visser et al., 2015). Melanocytic neoplasms range from benign precursor lesions, both congenital and acquired nevi, to *in situ* and malignant melanomas, an aggressive cancer that originates from neural crest-derived melanocytes (MC) (Shain and Bastian, 2016). Melanoma tumours have a very high rate of metastasis and a large level of heterogeneity, varying greatly in their proliferation, invasion and differentiation characteristics (Fane et al., 2017; Goodall et al., 2008; Quintana et al., 2010). The pathogenesis of melanoma is dependent on environmental, host and genetic factors (Berwick et al., 2016).

Interferon regulatory factor4 (IRF4) belongs to a winged-helix-turn-helix family of DNA-binding proteins that play an important role in immune system development and function (Paun and Pitha, 2007). IRF4 is predominantly expressed in blood cells with key roles in lymphoid, myeloid, and dendritic cell lineages (Busslinger, 2004; Lohoff et al., 2002; Tailor et al., 2006) and adipocytes, where it acts as a potent repressor of differentiation (Eguchi et al., 2008) with lipolytic actions (Eguchi et al., 2011). It is also expressed in MC, nevi and primary melanomas (Alonso et al., 2004; Natkunam et al., 2001; Praetorius et al., 2013; Wang et al., 2014) and was initially implicated as a marker for melanocytic lesions (Sundram et al., 2003).

Numerous epidemiological studies have reported a single nucleotide polymorphism (SNP) rs12203592*C/T in *IRF4* with a strong association with nevus count (Duffy et al., 2010a; Duffy et al., 2010b; Kvaskoff et al., 2011). This polymorphism occurs within the intron4 region of the *IRF4* gene,

and has been shown to reside within a putative enhancer regulatory element of the *IRF4* locus (Praetorius et al., 2013). The rs12203592*C ancestral *IRF4* allele has been reported in Chinese, Japanese and African populations while only European populations harbour the rs12203592*T allele at a frequency of 16.7% (Praetorius et al., 2013). Variants in *IRF4*, notably rs12203592, are also reported to be strongly associated with eye and hair colour and skin tanning in response to sunlight (Han et al., 2008; Nan et al., 2009; Sulem et al., 2007). Notably, a strong genotype-by-age interaction for total body nevus count has also been reported for this SNP, which is also a predictor of melanoma risk (Duffy et al., 2010a).

Involvement of *IRF4* has been described in detail in several types of lymphoma and leukemia (Gualco et al., 2010), including male-specific childhood ALL (Do et al., 2010). In this latter study it was shown to be located within a regulatory element, controlling expression of *IRF4* through binding of the transcription factor AP2 α (TFAP2 α) (Do et al., 2010). Interestingly, in Burkitt Lymphoma B-cells (Raji), cells the rs12203592*C allele was shown to strongly bind TFAP2 α to elicit a trans-repressive effect on the *IRF4* enhancer activity (Do et al., 2010), which is in contrast to its role as a melanocytic enhancer element whereby binding of TFAP2 α to the C-allele trans-activates the region while disrupted binding to the T-allele reduces the cis-activity which correlates with lower levels of *IRF4* expression (Praetorius et al., 2013). Consequently, the lighter pigmentation phenotype associated with homozygosity at the *IRF4* SNP T-allele is attributable largely to reduced expression of tyrosinase expression due to lower *IRF4* levels (Praetorius et al., 2013).

Interferon gamma (IFN γ) is a pro-inflammatory cytokine that orchestrates numerous immunological signalling pathways mainly promoting proliferation, maturation, differentiation and chemotaxis in a range of immune cells (Pestka et al., 2004; Trinchieri, 2010). Through these mechanisms IFN γ has been shown to prevent tumour development (Gollob et al., 2005; Kaplan et al., 1998; Shankaran et al., 2001). However, other studies highlight its paradoxical role associated with tumour proliferation and/or metastasis (Gorbacheva et al., 2002; Lollini et al., 1993; Taniguchi et al., 1987; Zaidi and Merlino, 2011). Studies have reported a pro-tumorigenic effect of sustained low-level IFN γ doses in several tumours including melanoma (He et al., 2005; Le Poole et al., 2002). Zaidi et al, have proposed that an IFN γ signalling response contributes to UV-induced melanomagenesis through increased survival and immuno-evasion of UV-damaged melanocytes (Zaidi et al., 2011; Zaidi and Merlino, 2011). This study highlights that the highest melanoma risk is reminiscent of intermittent burning doses of UV-B (not UV-A) and that contextual IFN γ function may depend on the cellular microenvironment (Zaidi et al., 2011), which can be further dictated by cellular genotype.

Despite numerous reports associating IRF4 with melanocytic cell function through effects on pigmentation, nevus count and melanoma sub-type development the mechanistic role of IRF4 in melanocytic cell biology remains poorly characterised. This study aims to functionally characterise the effects of the rs12203592*C/T non-coding SNP on IRF4 enhancer activity and determine if variation in IRF4 protein levels can influence melanocytic pigmentation, growth, and UVR and IFN γ responses.

Results

***IRF4* intron4 has cis-regulatory activity in melanocytic cells**

To investigate the cis-activity of the *IRF4* intron4 region containing the rs12203592*C/T SNP both alleles were cloned into the pGL2-promoter luciferase reporter vector as either the full intronic region or a 600bp fragment lacking a large repeat region comprising 11 copies of a 52bp sequence (Fig. S1A). The cis-activity of these constructs was determined in melanocytic (A2058 and MM96L) and non-melanocytic (HEK293 and HELA) cell lines with luciferase activity above the empty vector control only observed in the melanocytic cells (Fig. S1A). Moreover, in the melanoma cells the constructs containing the C-allele exhibited greater activity than the T-allele consistent with earlier studies (Praetorius et al., 2013; Visser et al., 2015), while the presence of the repeat region had only a modest effect on reporter activity. Luciferase reporter activity of this region correlated with IRF4 expression in the cell types used as determined by quantitative PCR (qPCR) (Fig. S1B) and immunoblot (Fig. S1C) suggesting that the cis-regulatory activity is restricted to cells expressing *IRF4*.

Identification of 5' and 3' cis-regulatory regions flanking the *IRF4* intron4 SNP

To further extend this analysis we generated a series of 5' and 3' deletion constructs using the 0.6kb region as the 'full length' reference. A number of sequential 5' deletions were generated, designated $\Delta 2$, $\Delta 3$, and $\Delta 4$, and transfected into A2058 melanoma cells. Within the region 5' of the SNP, a number of potential M-Box elements have been identified (Praetorius et al., 2013) (Fig. 1 region highlighted in blue) with deletion of this region resulting in complete loss of luciferase activity above that of the background vector in all lines tested (Fig. 1A upper panel). Sequential deletion of this region from the 3' end to the SNP resulted in a similar loss of activity, despite the presence of the 5' M-box element (Fig. 1A lower panel) indicating the presence of a 3' consensus site that is crucial for enhancer activity. Deletion of region from the 3' end was enough to reduce the regulatory activity by 50% ($\Delta 6$ construct) and was completely abolished when entire region was deleted ($\Delta 1$ construct) as shown in Figure 1A (lower panel). While previous reports identified three potential M-box elements in this region (Praetorius et al., 2013), our analysis suggests one element was most likely to be a true MITF binding site based on the presence of a T residue flanking the core E-box motif (Aksan and Goding, 1998). Accordingly, we generated point mutations in this site, whereby two nucleotides within the core E-box motif and the flanking T residue were altered, to confirm its importance in

regulating the IRF4 intronic enhancer activity. Firstly, electrophoretic mobility shift assay (EMSA) analysis using melanoma cell nuclear extracts was performed using probes for the tyrosinase M-box consensus site (Aksan and Goding, 1998) or putative WT IRF4 M-box that retarded the migration of a DNA-protein complex of similar size while the complex was not detected using the mutated IRF4 M-box probe (Fig. 1B). The introduction of the same mutation into the *IRF4* 0.6C reporter constructs revealed a significant reduction in the cis-activity of the region compared to the WT controls (Fig. 1C). IRF4 candidacy as a MITF target gene was further supported by siRNA-mediated knockdown of MITF and IRF4 in A2058 melanoma cells, where MITF knockdown decreased IRF4 mRNA and protein levels similar to those observed following IRF4 siRNA, while IRF4 knockdown had negligible effect on MITF expression (Fig. 1D-E).

We next sought to characterise the crucial 3' cis-regulatory region that we identified in our initial 3' deletion series that is positioned 51bp downstream of the rs12203592 SNP and extending 120 bases representing the region (Fig. 1 highlighted in yellow) between the 3' end of the $\Delta 1$ deletion and the 3' end of the $\Delta 5$ deletion (Fig. 1A lower panel). This was performed by generating an incremental set of internal deletion mutants, in both the C and T variant background, covering this region by removing 10, 20, 30, 60, 90 or 120 base pairs respectively to generate clones that were accordingly designated $\Delta 10$, $\Delta 20$, $\Delta 30$, $\Delta 60$, $\Delta 90$ and $\Delta 120$, shown schematically in Figure S1D-E. Analysis of the luciferase activity driven by these deletion mutants in both A2058 and MM96L melanoma cells revealed that while removal of the first 10bp of the 120bp region (0.6C/T- $\Delta 10$ mutant) produced a reduction in cis-activity, a further deletion of 10bp (0.6C/T- $\Delta 20$ mutant) completely reduced the activity to that of the empty backbone vector in the context of both variant alleles (Fig. S1D-E). Despite total loss of cis-activity in the 0.6C/T- $\Delta 20$ mutant we cannot discount the possibility that this deletion has dissected a consensus site that extends beyond that point, hence these data suggest that the crucial 3' cis-element is positioned in the first 30bp of the 120bp region, a region henceforth referred to as $\Delta 30$. To clarify the importance of the $\Delta 30$ region we performed a comparative genomic analysis to examine the evolutionary sequence conservation of this region. Multiple sequence alignment revealed that the 20bp deleted in the $\Delta 20$ and $\Delta 30$ mutant clones is highly conserved between mammalian species examined (Fig. 1F and S2A). Moreover, a TGT[C/G]C sequence at the 3' end of the $\Delta 30$ region is completely conserved in all 31 species analysed. Accordingly, we targeted the 100% conserved TGT sequence, which was mutated to CAG in both C and T allele contexts, to examine the effect on the cis-activity of the IRF4 enhancer region. Transfection of the WT and CAG mutant constructs in both A2058 and MM96L melanoma cells revealed a considerable reduction in cis-activity close to that of the backbone (pGL2 promoter) vector (Fig. 1F).

An EMSA was performed to study the protein-DNA interaction within the $\Delta 30$ region of IRF4, and to determine if mutation of the TGT sequence disrupted this binding. Accordingly, MM96L melanoma nuclear extracts were incubated with radiolabelled probes for the IRF4 $\Delta 30$ -TGT (wild type) or the IRF4 $\Delta 30$ -CAG (mutant) sequences (Fig. 1G). In addition a probe for the OA25 high affinity BRN2 binding site was included in this analysis. The Oct-1 and BRN2 (N-Oct-3) proteins have been shown to bind to the OA25 probe previously (Smit et al., 2000). Interestingly, the IRF4- $\Delta 30$ -TGT probe produced a strikingly similar complex profile to the OA25 probe in this analysis suggesting this site might be a target for one of these POU domain proteins because of its similarity to a consensus POU binding site (Fig. 1G). Importantly, the CAG mutation introduced into the $\Delta 30$ sequence almost completely abolished the DNA-protein complex migrating at the BRN2 level while only partially disrupting the complex migrating with Oct-1 (Fig. 1G).

BRN2 regulates IRF4 expression in melanocytic cells

In light of the EMSA data implicating BRN2 as potentially binding to the $\Delta 30$ region of the *IRF4* enhancer we wished to explore the role of BRN2 in regulating IRF4 expression in melanocytic cells. As BRN2 is highly expressed in melanoblast (MB) cells and is downregulated as they differentiate into melanocytes (MC) (Cook et al., 2003; Cook et al., 2005), we first sought to determine if IRF4 expression levels were different between MB and MC cells, and secondly if IRF4 expression correlates with BRN2 expression in this primary culture model. Western blot analysis of four independent strains grown as MB cells or induced to differentiate into MC as described previously (Cook et al., 2005) revealed a striking decrease in IRF4 levels as cells transitioned from MB to MC (Fig. 2A-B). Interestingly, this decrease correlates with the previously reported down-regulation of the BRN2 transcription factor as a consequence of MC differentiation, although while BRN2 expression is generally absent at the end of the differentiation process, IRF4 levels are maintained albeit at lower levels than that detected in MBs (Fig. 2A-B). To further test the role of BRN2 in regulating IRF4 expression two melanoma cell lines, A2058 and MM96L, and a primary MB cell strain QF1566 were subjected to BRN2 siRNA-mediated knock down using three independent siRNAs (designated si1, si2, si3). In all three lines IRF4 protein expression was reduced in response to BRN2 knockdown relative to the negative control siRNA (Fig. 2C). Moreover, exogenous over-expression of BRN2 in the melanoma lines produced the reciprocal effect with a 2.7 and 3.1 fold increase in IRF4 in A2058 and MM96L cells respectively (Fig. 2D).

Effect of *IRF4* rs12203592 genotype and expression level on melanocytic cell growth characteristics

Given the well recognized and intricate relationship between growth, UV exposure and pigmentation with the development of acquired nevi or melanomagenesis (Shain and Bastian, 2016), we sought to assess growth characteristics, pigmentation gene expression and UVR response of our primary melanocytic strains in the context of IRF4 intronic variance.

MTT-based cell viability and DAPI stained nuclei count were employed to investigate the growth characteristics of the primary strains. Five MB strains per genotype were seeded at the same number into 24 well plates after which cells were counted and assessed by MTT assay at days 1, 3 and 7 post-plating. MTT assay data for the MB strains is shown in Figure 3A and S2D, with pooled analysis revealing that the C/C homozygote variant strains were significantly more proliferative than T/T variant strains across all the time points ($P < 0.01$) (Fig. 3A). This observation was supported by DAPI nuclei staining showing significantly higher proliferation at Day 3 and Day 7 post-seeding in C/C variant strains (Fig. 3B and S3A). We further corroborated these findings by performing BrdU uptake assays comparing the C/C and T/T genotyped MB strains at Day 3 post-seeding. A significantly higher percentage of fluorescence was detected in C/C MB cell strains ($P < 0.01$) indicating a greater proportion of cells in S-phase or mitosis, during which BrdU gets incorporated (Fig. 3C).

To determine if higher proliferation is related to increased IRF4 protein expression, A2058 and HT144 melanoma cells, engineered to stably overexpress IRF4, were assayed for cellular proliferation by trypan blue exclusion cell count with increased growth rates evident in both lines over-expressing IRF4 compared to the empty vector control (Fig. 3D). Moreover, a clonogenic assay on these lines further supported the growth-promoting effect of IRF4 (Fig. 3E). Overexpression of IRF4 was confirmed by immunoblot of these melanoma cell lines as shown in Figure S2E.

Effect of *IRF4* rs12203592 genotype and IRF4 protein levels on UVR response in melanocytic cells

Given that UVR exposure is linked to acquired nevi and melanomagenesis it is plausible to propose that IRF4 association with nevus count may in part be attributable to an altered UVR response based on IRF4 genotype. We initially examined the effect of *IRF4* rs12203592 variance on post UVR survival using five MB cell strains homozygous for either C- or T-alleles which were subjected to UV-B irradiation at 50mJ/cm² dosage (under 1 MED). Cell survival was determined by MTT assay and showed that a single dose of UVR was enough to decrease cell viability in C/C strains at all time points tested, 24hr ($P < 0.001$), 48hr ($P < 0.05$) and 72hr ($P < 0.05$) (Fig. 4A). Interestingly, the T/T strains exhibited an initial increase in viability followed by a steady decline over the latter time points, however viability remained significantly higher than the C/C variant strains at all time points (Fig. 4A). Post-UVR viability was also tested using A2058 and HT144 melanoma lines over-expressing IRF4. Reduced cell number was evident in both lines overexpressing IRF4 at both 24hr and 48hr time

points post-UVR as determined by crystal violet (CV) staining (Fig. 4B-C) or MTT assay (Fig. S2B-C). Together these data suggest that elevated levels of IRF4 negatively impact on cell viability after UVR in melanocytic cells.

Effect of *IRF4* rs12203592 genotype on endogenous protein expression and pigmentation-related gene expression in melanocytic cells

We have previously reported reduced IRF4 expression in primary human melanocytes homozygous for the *IRF4* rs12203592*T allele, however to date reduced protein levels have only been shown for two independent strains and have yet to be reported in melanoblasts. Accordingly we performed immunoblot analysis of five MB cell strains homozygous for each intronic variant and found that differential IRF4 expression based on genotype concurred with our previous observations that IRF4 protein expression is considerably lower in T/T homozygous strains in comparison to C/C strains (Fig. 5A). Furthermore, a similar differential trend was observed whereby other melanocytic proteins MITF ($P<0.05$) and TYR ($P<0.01$) were both expressed at significantly lower levels in T/T strains (Fig. 5A-B).

As an indicator of melanogenic potential the TYR enzymatic activity was determined in the panel of melanoblasts by the rate of L-DOPA oxidation. All T/T homozygous MB strains exhibited a lower rate of L-DOPA oxidation across the different time points tested (Fig. 5C). The difference in average oxidation rate at 120 minutes was greater than two fold (C/C>T/T) ($P<0.0001$) consistent with lower TYR levels observed in T/T strains (Fig. 5A-B). To further evaluate the role of IRF4 levels in pigmentation we performed immunoblot experiments on four C/C MB strains subjected to pooled IRF4 knockdown. TYR expression was found to decrease in response to IRF4 siRNA knockdown across all the strains tested (Fig. 5D). These data suggest that higher IRF4 levels regulate pigmentation by increasing TYR levels. Moreover, the data provides a mechanistic basis for the association of the lower IRF4 protein expression rs12203592*T allele with lighter skin pigmentation.

Effect of *IRF4* siRNA knockdown and *IRF4* rs12203592 genotype on the IFN γ response of melanocytic cells

Interferon-gamma (IFN γ) signalling has emerged as a key component of the interplay between UVR responses and melanocyte-immune cell cross-talk in the process of UVR induced melanomagenesis (Zaidi et al., 2011). IRF4 as part of the Interferon Regulatory Family, has a long established role in the propagation or modulation of interferon signalling in most cell types. Accordingly it is important to determine if IRF4 expression and genotype influences interferon signalling in human melanocytes. In order to examine the effect of IRF4 on IFN γ signalling in primary melanocytic cells, we compiled a candidate gene list based on previously reported IFN γ responsive genes in melanocytes (Zaidi et al., 2011) which included CCL3, CCL5 and CTLA4 in addition to PD-L1 which has recently been shown

to be IFN γ -responsive (Garcia-Diaz et al., 2017). Importantly, we confirmed the IFN γ responsiveness of both MB and MC primary cell strains by performing a 24hr dose-response analysis whereby we determined the induction of the hallmark IFN γ target gene *IDO1* (Sarkhosh et al., 2003; Taylor and Feng, 1991) by qPCR with both strains producing a robust induction of *IDO1* even at the lowest dose tested (Fig. S3B).

Firstly we tested the effect of siRNA-mediated IRF4 knockdown on basal expression of our selected target genes, *CCL3*, *CCL5*, *CTLA4* and *PD-L1* in both primary cells cultured as melanoblasts or melanocytes. Reducing IRF4 expression by siRNA knockdown was sufficient to significantly downregulate the endogenous levels of *CTLA4* ($P<0.01$), *PD-L1* ($P<0.01$), *CCL3* ($P<0.01$) and *CCL5* ($P<0.01$) in both MB (Fig. 6A) and MC strains (Fig. S4A and C).

Next we evaluated if IRF4 knockdown could modulate IFN γ induction of these genes in the primary cell strains. Accordingly, in parallel the siRNA treated MB or MC cultures, 24hr post siRNA transfection were subjected to a 2hr treatment with IFN γ at 1ng/ml (equivalent to 20IU/ml) or vehicle and RNA extracted after a further 24hr. Quantitative RT-PCR analysis indicated that in addition to reducing the basal level expression of our gene set, a decrease in IRF4 levels was sufficient in ablating IFN γ -induced *CTLA4* ($P<0.01$) and *CCL5* ($P<0.001$) expression and reducing *CCL3* ($P<0.01$) expression in MB strains in comparison to the negative control cells (Fig. 6B). While basal *PD-L1* ($P<0.05$) expression was significantly reduced by IRF4 siRNA knockdown, the effect on IFN γ induction did not reach significance (Fig. 6B). A similar outcome was observed in MC following IRF4 knockdown with ablation of *CTLA4* ($P<0.05$) and *CCL3* ($P<0.05$) expression but reduction in *CCL5* ($P<0.05$) induction following IFN γ treatment (Fig. S4B). This indicates that IRF4 plays a key role in determining the extent and selection of genes that are IFN γ -mediated in melanocytic biology.

Evidence demonstrating the role of IFN γ signalling in mediating the function of cutaneous melanocytes as part of a UV induced melanomagenesis mechanism (Zaidi et al., 2011), together with altered signalling responses observed when we manipulated IRF4 expression in melanocytic cells prompted us to determine if IFN γ responses are altered in primary human melanocytes of defined IRF4 genotype. Differentiated MC cell strains homozygous for C/C and T/T were subjected to an acute IFN γ treatment and total RNA extracted at different time intervals over a 72hr time course. *CTLA4* and *CCL5* levels increased gradually after initial treatment attaining high significance at 72hr for C/C genotype strains showing a 15-fold ($P<0.0001$) and 18-fold increase ($P<0.0001$) in *CTLA4* and *CCL5* levels (Fig. 6C) respectively. A similar trend was evident for *CCL3* expression with significantly higher levels evident in C/C strains from 24hr ($P<0.01$) onwards to 72hr ($P<0.0001$) (Fig. 6C). Notably, *CCL3* and *CCL5* expression in T/T strains was essentially undetectable all time points

tested. An induction in *PD-L1* expression was evident in all strains tested, displaying an early response from 8hr onwards after IFN γ treatment, however the level of response in T/T strains was significantly lower ($P<0.01$) than that detected in MCs harbouring C/C alleles (Fig. 6C). The maximum *PD-L1* induction observed in T/T strains at 72hr was still lower than 8hr induction observed in C/C strains. It is clear from these expression analyses that IFN γ responses in C/C strains occur earlier and are also of much higher magnitude than those observed in T/T strains.

Discussion

A SNP in intron4 of the *IRF4* gene, rs12203592*C/T, associated with pigmentation traits, nevus count and melanoma susceptibility has been reported to form a melanocyte specific enhancer region that is regulated by the MITF and TFAP2 α transcription factors (Praetorius et al., 2013; Visser et al., 2015). Further fine-mapping of the *IRF4* enhancer region reported here identified a regulatory site located downstream of the SNP that was as critical for the cis-regulatory activity of the region as sites found to be targeted by MITF and TFAP2 α . The striking sequence conservation of this region across 31 mammalian species coupled with significant loss of cis-activity when mutated suggests that this sequence is integral to the function of the 3' regulatory element. EMSA analysis indicates the potential for BRN2 to interact within this region with the potential of IRF4 as a BRN2 target gene further underscored by consistent correlation between IRF4 and BRN2 levels in melanoma cell lines, primary melanoblasts, and over the course of melanocyte differentiation.

Using primary MB strains homozygous for C/C and T/T *IRF4* genotypes we observed a positive correlation between higher IRF4 expression levels directed by the rs12203592*C allele with endogenous TYR levels, a finding further supported by reduction in TYR levels following IRF4 knockdown in primary melanocytic cells. Higher TYR expression matched a higher activity based on the L-DOPA oxidation assays and can potentially explain the basis of rs12203592*T allele association with lighter pigmentation traits (Duffy et al., 2010b; Praetorius et al., 2013), solar lentigines (SL) (Laville et al., 2016) and skin aging (Law et al., 2017) as a consequence of lower IRF4 expression in this context. Together these data corroborate earlier reports of synergy between MITF and IRF4 in regulating TYR expression (Praetorius et al., 2013; Visser et al., 2015) and reveals that this regulation also extends to less differentiated melanoblast cells.

Given that pigmentation phenotype and UVR responses are known predictors of melanoma susceptibility, we examined the effect of UVR on the different MB cell strains chosen based on their *IRF4* genotype. Firstly, the basal growth rate of the variant strains was evaluated and demonstrated that strains homozygous for the C-allele had a significantly higher proliferation rate than T-allele homozygous strains, an observation further supported by increased proliferation rates of melanoma

cells expressing exogenous IRF4. Similar proliferative effects of IRF4 have been previously reported in non-melanocytic cell types (Aldinucci et al., 2011; Iida et al., 1997; Shaffer et al., 2009; Wang et al., 2014). Contrary to the increase in proliferation, increased levels of IRF4 resulted in significantly higher cell death in primary MB cultures following UV-B irradiation. Physiologically, exposure of the skin to UVR has been shown to result in dose-dependent direct DNA damage (CPDs) and oxidative lesions (8-OHdG) in both keratinocytes and melanocytes (Lee et al., 2013). Interestingly, IRF4 overexpression has been reported to repress DNA repair following damage induced by chemotherapeutic agents and mutagens including UVR in Jurkat cells (Mamane et al., 2005) but if such an effect occurs in melanocytic cells owing to IRF4 level variability remains to be evaluated.

In addition to the strong germline effect of *IRF4* rs12203592 on pigmentation and naevi (Duffy et al., 2010a), numerous studies have associated germline variance in *IRF4* intron4 to melanoma development (Gibbs et al., 2016). While many studies report an association of the C-allele with higher melanoma rates (Duffy et al., 2010a; Newton-Bishop et al., 2011; Pena-Chilet et al., 2013) a number of studies have reported the T-allele to be positively associated with melanoma, squamous cell carcinoma (SCC) and basal cell carcinoma (Chahal et al., 2016; Han et al., 2011; Kvaskoff et al., 2011). More recently, the *IRF4* SNP has been shown to have independent yet contrasting associations with melanoma subtypes, occurring due to high levels of cumulative sun exposure or those arising from nevi, indicating involvement of divergent pathways in melanoma development (Gibbs et al., 2016) substantiated by survival analysis and primary tumour localisation from two independent melanoma patient sets (Potrony et al., 2017) which together may explain the inconsistent association of the *IRF4* intronic SNP with melanoma risk in numerous GWAS studies reported to date. Despite these various associations, the biological basis of IRF4 contribution to melanocytic cell function, nevogenesis and melanoma development remains poorly understood.

The role of the Interferon Regulatory Factor family in modulating interferon signalling responses in the context of the immune system is well recognised. Interestingly, interactions between melanocytes and macrophages have been reported to be a crucial component of UV-induced melanomagenesis in a process involving IFN γ signalling (Zaidi et al., 2011). Central to the hypothesis proposed by Zaidi and colleagues is the promotion of an IFN γ driven immuno-evasion gene expression signature that allows UV-B damaged melanocytes to avoid immune surveillance allowing survival and subsequent transformation of these damaged melanocytic cells, we have shown here that differential *IRF4* expression mediated by genotype or manipulation of IRF4 levels directly influences expression of a subset of IFN γ -inducible genes known for their role in tumourigenesis (*CCL3*, *CCL5*) or immuno-evasion (*CTLA4*, *PD-L1*). The expression of these secreted factors have been implicated in models of melanomagenesis where they contribute to melanocyte-immune cell cross talk (Zaidi et al., 2011) and have been linked to higher CD8⁺ T-cell infiltration in tumours (Harlin et al., 2009). Interestingly, the

decrease in nevus count with age is significantly higher in the context of *IRF4* rs12203592*T allele homozygosity and may represent a differential interaction with the immune system over time. Accordingly, the effect we have observed here with lower expression of secreted cytokines such as *CCL3* and *CCL5* and altered *IFN* γ responses may contribute to an altered nevus-immune cell interaction that impacts on the stability of nevi with age.

Importantly, our data has revealed that *IRF4* levels in primary melanocytic cells influences the basal level expression of both *CTLA4* and *PD-L1* and accordingly reduces the induction of these genes in response to *IFN* γ . This divergence is evident through both the manipulation of endogenous *IRF4* expression by siRNA-mediated knockdown in melanocytic cells and also in primary MCs which were stratified solely on their rs12203592 genotype. *CTLA4* and *PD-L1* expression was robustly induced by *IFN* γ treatment in cells homozygous for the C-allele that we have demonstrated to express high levels of *IRF4* protein, while the low expressing T/T homozygous cells exhibited significantly lower responses.

Targeting of immune checkpoints has recently emerged, together with molecular targeted therapies, as exciting new avenues to treat a broad range of cancer types including melanoma and shown to confer dramatic tumour regression with extended survival in a proportion of patients (Sharma and Allison, 2015). Blockade of T-cell inhibitory pathways includes the *CTLA4*, *PD-1* and its ligand *PD-L1*, and while significant survival benefit is observed in some patients, the reason for a lack of clinical response in many patients and the potential development of resistance remains unclear. Crucially, several reports underscore an association between *IFN* γ signalling and the response of tumours to immune checkpoint targeting revealing that *CTLA4* and *PD-1* blockade lead to an increase in *IFN* γ production (Chen et al., 2009; Garcia-Diaz et al., 2017; Liakou et al., 2008; Peng et al., 2012). Moreover, the efficacy of *CTLA4*/*PD-1* combinatorial therapy is abrogated when *IFN* γ signalling is perturbed (Shi et al., 2016). Notably, recent evidence argues that *IFN* γ signalling defects will have a negative impact on anti-*CTLA4* therapy, with genomic analysis of clinical samples revealing a high frequency of disruption of *IFN* γ signalling genes (Gao et al., 2016). Furthermore, resistance to *IFN* γ signalling manifesting through acquired mutations in the *JAK1* or *JAK2* have been shown to correlate with acquired resistance to therapy in the context of *PD-1* blockade. In light of our findings here, our observations that variable *IRF4* expression in melanocytic cells based on genotype promote a divergent *IFN* γ signalling response may ultimately impact on patient responses to melanoma immune checkpoint blockade. Accordingly, it will be important to examine the genotype of responders and non-responders to determine if the *IRF4* intronic SNP segregates with patient therapeutic response.

Methods

Cell Isolation and culture conditions

Human primary melanoblasts (MBs) were cultured from neonatal foreskin tissue as previously described (Cook et al., 2003). All cultures were propagated as MBs and subsequently differentiated to melanocytes (MCs) where indicated by maintaining in MC growth medium for 7 days as previously described (Cook et al., 2009), however cholera toxin was removed from media for the final three days of the differentiation process. The study was conducted according to the Declaration of Helsinki Principles, and procurement of foreskin tissue samples for medical research was approved by the University of Queensland Institutional Human Research Ethics Committee with parental consent to the use of tissue. The pigmentation genotype of MB cell strains used was restricted to European wildtype *TYR*, *MATP* and *NCKX5* haplotypes (Table S1) to provide consistency in the genetic background in which to assay the melanogenic effects of homozygosity for rs12203592 C/C and T/T (Jagirdar et al., 2014).

Established melanoma cell lines A2058, MM96L and HT144 were cultured in RPMI-1640 (Life Technologies) supplemented with 2mM Gluta-MAX (Life Technologies), antibiotics (100IU penicillin and 100µg streptomycin, Life Technologies) and 5% Foetal Bovine Serum (FBS). Non-melanoma cell lines HEK293 and HELA were cultured in DMEM (Life Technologies) supplemented with 10% FBS. All cell lines were maintained in humidified 5% CO₂ incubator at 37°C.

Lentivirus transduction and RNA interference

IRF4, BRN2 and empty vector lentivirus were generated using the Lenti-X HT packaging system (Clontech) according to the manufacturer's protocol. A2058 and HT144 melanoma cells were transduced with lentivirus as previously described (Yin et al., 2017) and cells were selected in media containing 0.75µg/ml puromycin and expression confirmed by western blotting. MB cell strains and established melanoma lines were transfected with 20nmol/l of non-targeting siRNA (scramble/control, Dharmacon) and siRNAs targeting IRF4 (GE Dharmacon), BRN2 (Shanghai GenePharma) and MITF (Shanghai GenePharma) using Lipofectamine 2000 (Invitrogen) according to manufacturer's guidelines. The efficacy of knockdown was confirmed by western blot analysis.

Statistical analysis

Densitometric quantitation was performed on immunoblots using ImageJ software. Values were normalised to GAPDH and represented as arbitrary units. Statistical analyses were performed using GraphPad-PRISM software based on a minimum of three independent experiments using student's t-test for comparison between two groups or one-way ANOVA test followed by a Tukey's post-test or two-way ANOVA followed by a Bonferroni post-test. Significance was scored as: ****, p<0.0001; ***, p<0.001; **, p<0.01; *, p<0.05.

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Conflict of interest

The authors have no conflict of interests to declare.

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Figure Legends

Figure 1: Characterisation of *IRF4* intron4 enhancer and binding activity. (A) Essential cis-regulatory elements flank the *IRF4* intron4 rs12203592 SNP. Luciferase assay in MM96L cell line transfected with deletion constructs 5' (upper panel) and 3' (lower panel) of the rs12203592*C allele. Two crucial region identified by this analysis is highlighted by blue and yellow boxes respectively and are analysed further in subsequent experiments. (B) EMSA was performed using MM96L nuclear extracts using radiolabelled probes for the human Tyrosinase M-Box and the IRF4 M-box wild type (wt) or mutant (mut) form with sequences indicated in panel D below. FP denotes Free Probe. (C) An intact M-Box is essential for *IRF4* enhancer activity. Schematics and luciferase assay in A2058 and MM96L cells transfected with intact (wt) and mutated (mut) M-Box constructs in both C- and T-alleles. Data presented as mean $\pm$ standard error of mean (SEM) with statistical analysis ****P<0.001, **P<0.01, performed by student t-test. (D) MITF regulation of IRF4 occurs at mRNA and protein expression level. A2058 cells subjected to pooled siRNA directed against MITF, IRF4 or NEG (scramble control) are analysed by qPCR for *MITF* and *IRF4* expression represented as fold change relative to NEG (normalised to *B2M*) (E) and immunoblotting. (F) Schematic of location and comparative sequence alignment of *IRF4* intron4 30bp (Δ 30) region indicating a conserved region (TGT) within the TTGT-C/G-C motif. Luciferase assay in A2058 and MM96L cells transfected with wild-type TGT and mutated CAG (red) constructs. Data represented as fold luciferase activity relative to empty pGL2-Promoter set as 1 with the values presented as mean $\pm$ SEM from four independent experiments. (G) BRN2 interacts with the Δ 30 region within *IRF4* intron4. MM96L nuclear extracts were assayed for BRN2 (N-Oct-3) DNA-binding activity using OA25 (Lane 1), IRF4 Δ 30-TGT (Lane 2), and IRF4 Δ 30-CAG mutant (Lane 3) probes respectively. FP denotes Free Probe. All data are expressed as mean $\pm$ SEM with statistical significance indicated as ****P<0.0001, ***P<0.001, **P<0.01, using One-way ANOVA test unless specified. All luciferase data representative of six independent experiments and represented as fold luciferase activity relative to empty pGL2-Promoter set as 1. All other data is representative of three independent experiments.

Figure 2: BRN2 regulation of IRF4. (A) Immunoblot analysis of lysates from 4 primary MB strains and paired MC indicative of endogenous BRN2 and IRF4 levels. (B) Immunoblot on a MB strain induced to differentiate into MC. Lysates collected each day of the 7-day differentiation protocol. (C) Immunoblot of melanoma cell lines A2058, MM96L and MB strain QF1566 following siRNA-

mediated knockdown of BRN2. (D) Immunoblot of A2058 and MM96L cell lines stably overexpressing BRN2 compared to Empty vector. GAPDH was used for normalisation of loading across all panels, and numbers underneath represent expression ratios relative to MB samples (in panel A), MB Day 0 (in panel B), Neg-siRNA (in panel C) and Empty vector (in panel D) were set to 1. All data representative of three independent experiments.

Figure 3: *IRF4* intron4 rs12203592 SNP and IRF4 protein levels effect melanocytic cell proliferation. (A) Pooled MTT assay on MB strains (n=5/genotype) genotyped as rs12203592 C/C or T/T seeded at equal cell densities (on Day 0) and subjected to MTT assay at Day 1, 3 and 7 post-seeding. (B) DAPI stained nuclei at Day 3 and 7 in representative MB strains genotyped C/C and T/T (left panel) and graphical representation of DAPI count in random fields of view by genotype (right panel) analysed by Two-way ANOVA test, n=5/genotype seeded at same cell densities. (C) BrdU incorporation in C/C and T/T MB strains seeded at same cell densities and quantitated by immunofluorescence at Day 3 post-seeding (left panel). Pooled BrdU incorporation analysis by genotype using student-t-test is shown (right panel), n=5/genotype. (D) IRF4 overexpression promotes proliferation in melanoma cells. A2058 and HT144 stably overexpressing IRF4 or Empty vector control seeded at similar cell densities and counted at Day 2, 4, 6 and 8 by trypan blue exclusion. (E) Clonogenic cell assay for A2058 and HT144 stably overexpressing IRF4 or Empty vector control using crystal violet staining. All data representative of three independent experiments and expressed as mean $\pm$ SEM with statistical significance indicated as ***P<0.001; **P<0.01; *P<0.05, using One-way ANOVA test unless specified.

Figure 4: *IRF4* intron4 rs12203592 SNP and IRF4 protein level effects viability post-UVR. (A) Primary MBs grouped into C/C (solid line) or T/T (dashed line) rs12203592 homozygous genotypes subjected to 50mJ/cm² UV-B. Cell viability was determined by MTT incorporation assay at 24hr, 48hr and 72hr post-UVR between the genotypes, with n=5/genotype normalised to respective mock (B) A2058 and (C) HT144 melanoma lines stably overexpressing IRF4 and Empty vector control subjected to 50mJ/cm² UV-B. Total cell viability was estimated by crystal violet staining 24hr and 48hr post-UVR between UVR-treated Empty and IRF4 transduced cells. All data representative of three independent experiments and expressed as mean $\pm$ SEM with statistical significance indicated as ***P<0.001; **P<0.01; *P<0.05, using One-way ANOVA test.

Figure 5: *IRF4* intron4 rs12203592 SNP effects pigmentation-related genes. (A) Primary melanoblasts MBs homozygous for C/C or T/T rs12203592 genotype subjected to immunoblotting. (B) Densitometric quantitation of expression normalised to GAPDH levels is represented, with n=5/genotype. (C) The rate of L-DOPA oxidation in extracted cell lysates of C/C (black) and T/T (white) MB strains performed at specified time intervals. A pooled analysis of L-DOPA oxidation rate

at 120 minutes is shown with n=5/genotype. (D) Immunoblot of four C/C MB cell strains subjected to pooled IRF4 siRNA and Neg siRNA (scramble control) for endogenous levels of TYR and IRF4. GAPDH was used as loading control and numbers underneath represent normalised ratio of TYR expression with Neg siRNA treatment set as 1. A pooled analysis of relative TYR levels in Neg siRNA and IRF4 siRNA MB strains (n=4) is shown in right panel. All data generated from at least three independent experiments and graphical plots represented as mean $\pm$ SEM, with statistical significance indicated as ****P<0.0001; ***P<0.001; **P<0.01, *P<0.05 using student-t-test.

Figure 6: Effect of IRF4 levels on basal and IFN γ response in melanocytic cells. (A) RNA extracted from three rs12203592 C/C MB strains subjected to pooled IRF4 siRNA or siNEG (scramble/control). qPCR analysis for endogenous *CTLA4*, *PD-L1*, *CCL3* and *CCL5* expression indicating fold change relative to NEG siRNA and analysed by student-t-test. (B) MB strains subjected to siRNA treatment (as above) treated with IFN γ (1ng/ml) or PBS (vehicle) for 2hr. RNA extracted 24hr post-treatment (and 48hr post-transfection) and analysed by qPCR. Graphs represent fold change relative to NEG siRNA treated with PBS. (C) qPCR analysis on MC strains homozygous for C/C and T/T genotype subjected to IFN γ (50ng/ml) or PBS (vehicle) for 2hr. RNA was extracted at specified time points and analysed for *CTLA4* (n=5/genotype), *PD-L1* (n=3/genotype), *CCL3* (n=3/genotype) and *CCL5* (n=3/genotype) represented as relative gene expression. All genes normalised to *GAPDH* and graphs represented as mean $\pm$ SEM with statistical analysis by One-way ANOVA test unless specified (****P<0.0001; ***P<0.001; **P<0.01, *P<0.05).

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