MITF Isoforms- Insights from an RNA-Seq study

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Abstract

Isoforms play an essential role in enhancing evolutionary diversity. Microphthalmia inducing transcription factor (MITF) is known to be a master regulator of pigmentation response and a lineage survival oncogene in melanoma. MITF is also known to have several functional isoforms, the –M form is known to be essential for the melanocyte lineage and is the most widely studied in the context of pigmentation biology and melanoma development and progression. This study uses an RNA seq approach in order to examine changes in the transcriptional profile of Skmel28 cells when treated with double strand break inducing compound and/or after knockdown of MITF. Intriguingly, we observe an enrichment of MITF-Mdel isoform on both MITF knockdown as well as on induction of DNA damage. Closer examination revealed that the siRNA used for the study targets all MITF isoforms except MITF-Mdel. We also observe a protein band at the size predicted for the protein. We therefore hypothesize a potential redundant role for the MITF-Mdel isoform and a potential novel role in DNA double strand beak response.

Introduction

The Microphthalmia associated transcription factor (MITF) is essential for development and regulation of various cell types like melanocytes, osteoclasts, mast cells, retinal pigment epithelial (RPE) cells of the eye and cardiomyocytes (Levy & Fisher, 2011; Steingrímsson, Copeland, & Jenkins, 2004; S. Tshori et al., 2007). Studies have also reported the importance of MITF in olfaction (Atacho et al., 2020). The MITF isoforms are known to be transcribed from several alternative promoters with distinct transcriptional start sites (Goding & Arnheiter, 2019; Vu, Dilshat, Fock, & Steingrímsson, 2020). To this date, nine distinct mouse isoform mRNAs have been identified (MITF-A, -B, -C, -D, -E, -H, -J, -M, and -MC). These are generated via the alternative splicing of the first exon of MITF. Exons 2–9 of all MITF isoforms are identical (Bharti et al, 2008). Further, MITF-M shows the presence of two variants referred to as the + and – forms, with the – form missing a six amino acid sequence in exon 6 (residues 187–192) (Pogenberg et al., 2012). A novel splice variant of MITF-M, the MITF-Mdel has also

been identified, this variant apart from lacking the six amino acid sequence of the MITF – variant also lacks an additional 54 amino acids (residues 32-87) leading to the elimination of the serine 73 (Ser73) phosphorylation site present in the exon 2B of MITF (Wang et al, 2010). Studies in mice have shown that deletion of domains in the exon 2B does not affect the function of MITF (Bauer et al., 2009). Previous studies on MITF isoforms in cell lines have highlighted their regulatory roles. For example, MITF-A which is the longest transcript has been shown to interact with HDAC and TFE3 to regulate the expression of TGF-α in chronic kidney disease (Laouari et al., 2012). Similarly, heart specific MITF-H is known to be essential in cardiac growth and hypertrophy (Sagi Tshori et al., 2006). Other cell lines like osteoclasts show the presence of MITF-E isoform that is regulated by RANKL transcription factor(Lu, Li, & Lin, 2010). Isoform MITF-MC has been shown to play an important role in mast cell biology (Takemoto, Yoon, & Fisher, 2002). Of all the isoforms MITF-M is the shortest and the most widely studied. It is expressed abundantly in neural crest derived melanocytes and melanoma cells. MITF-M is known to be a lineage survival oncogene and plays an essential role in tumor cell survival and progression (Garraway et al., 2005). Along with being involved in various processes like melanosome biogenesis, melanosome transport and melanocyte dendricity (Cheli et al, 2010), MITF has also been shown to be involved in autophagy and DNA damage repair (Katrín Möller, 2017; Strub et al., 2011). Furthermore, the link between UV damage and pigmentation response via regulation by MITF has been well studied (D'Orazio, 2013; Fell et al, 2014; Seoane et al., 2019; Xia et al., 2017). However, the MITF-mediated DNA damage response other than to ultraviolet radiation has not been fully examined.

Results and Discussion

We wished to explore the global transcriptional response of the Skmel28 melanoma cell line to the double strand break inducing compound, Neocarzinostatin (NCS) in the presence and absence of MITF. We used siRNA mediated knockdown of MITF followed by treatment with 2 µM of NCS for a duration of 1hr and 4hr in order to identify the DNA damage genes that might be regulated by MITF. The control scrambled siRNA and siMITF cells with and without the NCS treatments were harvested and RNA isolation

was carried out. The RNA samples were analyzed using a bioanalyzer and all samples were found to have an RNA integrity of 9.8 and higher. Paired end library sequencing was carried out on the Illumina platform. The RNA-seq data was analysed using the Kallisto (v 0.43) program (Bray et al; 2016) with the Ensembl human transcriptome release-94 as a reference and using 30 bootstrap samples. The abundance estimates of each transcript was then analysed using Sleuth (v 0.3) (Pimentel et al., 2017) and likelihood ratio test used to estimate differential expression and to obtain q-values for each transcript. The log2fold change was calculated using the beta estimate value.

The expression values of the MITF transcripts were examined under all conditions. We observed that along with the expected reduction in gene expression for the MITF-M isoform in the MITF knockdown sample, there was also a reduction in the MITF-A isoform (Table 1) A recent study also reports the presence of MITF-A in melanocytes (Flesher et al., 2020). Interestingly, we observed the increased expression of MITF-Mdel isoform upon knockdown of MITF (Table 1). We examined in detail all the Refseq sequences identified by the three silencer select siRNAs targeting MITF from Thermofisher (S8790, S8791 and S8792) and observed that although all the siRNAs target the isoforms –M, -A, -C, -H, transcript variant 5, and transcript variant 7, the siMITF S8792 that was used for the RNA-seq study did not target the MITF-Mdel isoform (Sup Table 1). We validated the RNA-seq results by performing RT-qPCR using the MITF and MITF-Mdel specific primers (Wang et al., 2010) in the RNA-seq samples and observed a threefold increase in the expression of the MITF-Mdel mRNA upon knockdown of MITF (Fig. 1). The expression levels of MITF mRNA using RT-qCR primers recognizing isoforms–M, -A, -H, -C, -Mdel, Variant 5, and Variant 7 (Sup Table 2) showed ~50% decrease in expression after being treated with siMITF S8792 (Fig.1). Interestingly, another study using the same siRNA for MITF knockdown in Skmel28 has also shown ~50% knockdown efficiency at the mRNA level (Katrin Möller et al., 2019). The presence of an enriched MITF-Mdel fraction in cells treated with siMITF S8792 could be one reason for observing low knockdown efficiency at the mRNA level on using the S8792 siRNA and carrying out RT-qPCR using primers recognizing all isoforms. An unpublished RNA-seq study from our center using this siRNA on the 501mel melanoma cell line also showed similar results, with MITF-M and MITF-A

showing a decreased expression with log2 fold change of -3.27 and -3.52 respectively and MITF-Mdel showing an increased expression with log2 fold change of 1.35. This leads us to believe that the increased expression of MITF-Mdel on MITF knockdown with S8792 is not a cell line specific artifact.

The previous study that identified the MITF-Mdel variant had predicted its protein size to be 357 aa (Wang et al., 2010). However, the study lacked any confirmatory experiments. Given that most of the available MITF antibodies identify all isoforms, it has been notoriously difficult to examine specific isoforms. However, when examining our MITF immunoblots, we indeed observed a band at 40kDa in 501mel cells treated with siMITF-S8792. Further, in accordance with the RNA-Seq data the band was enriched in the knockdown sample (Fig. 1B). This 40kDa band might go unnoticed in immunoblot experiments using beta actin (42kDa) as control, with both protein bands migrating closely. Interestingly, a recent study using the (CRISPR)-Cas9 technique in order to generate MITF knockout Skmel28 cell lines also shows a 40KDa truncated protein in one of the knockout cell lines targeting a region in exon2 (Dilshat et al., 2020) . Further investigations are required to confirm if these truncated proteins are indeed MITF-Mdel and its functional role.

Our RNA-seq study also showed increased expression of the MITF-C and the MITF-Mdel isoform under conditions of DNA damage (Table 1). Considering that the siMITF S8792 also targets the –C isoform it is interesting to note that its expression levels are nevertheless increased on MITF knockdown followed by double stand break induction. This hints at the possible enrichment of the existing pool of the MITF-C transcript under conditions of DNA damage. The MITF-Mdel shows a further additive increase in expression on both MITF knockdown and induction of DNA damage (Table 1).

In summary, we find the MITF-Mdel variant was enriched upon depletion of the other MITF isoforms in two melanoma cell lines. Additionally, we also observe the increased expression of MITF-Mdel and MITF-C on inducing double strand break. A recent review states that little is known about the regulation of the upstream promoters of non-melanocyte isoforms (Goding & Arnheiter, 2019). It is therefore useful to identify these isoforms in the context of biological processes like DNA damage, in order to allow for

their focused study in melanoma as well as non-melanoma cell lines. These kinds of studies can be facilitated by using RNA-seq data analysis programs that incorporate transcript-based abundance estimates.

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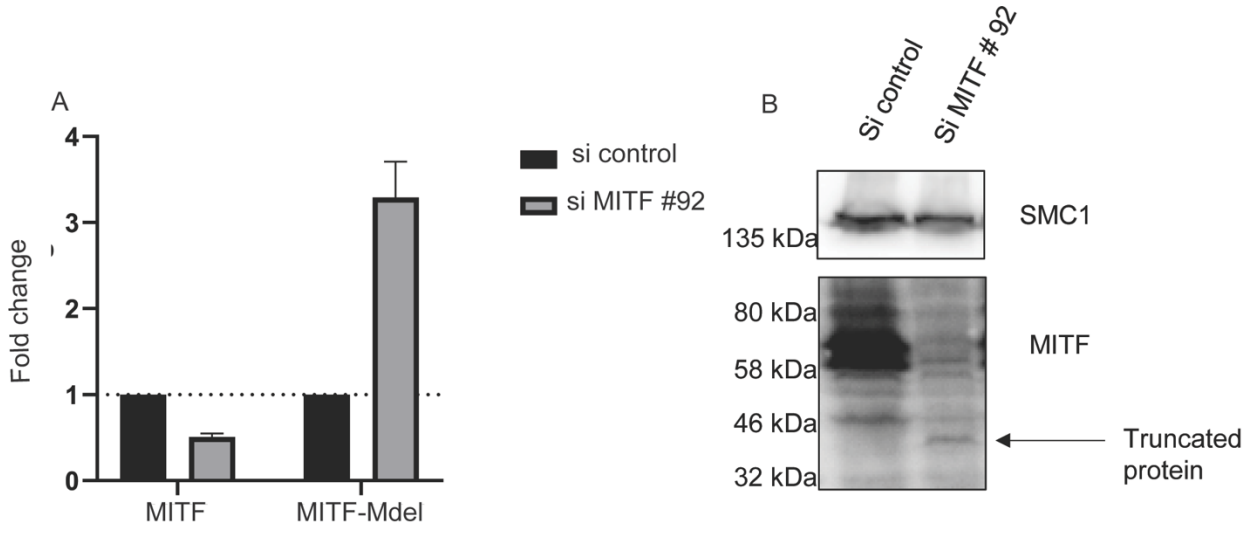
Table 1. **MITF Transcripts identified from RNA-seq under different conditions**

Transcript_ID	Isoform	log2 Fold change
	siMITF	
ENST00000314557.10	MITF-M	-1.72
ENST00000352241.8	MITF-A	-1.58
ENST00000531774.1	MITF-Mdel	1.83
	NCS 1h	
ENST00000328528.10	MITF-C	1.45
ENST00000314557.10	MITF-M	0.71
ENST00000531774.1	MITF-Mdel	1.33
	siMITF+ NCS 1h	
ENST00000531774.1	MITF-Mdel	2.45
ENST00000352241.8	MITF-A	-1.46
ENST00000328528.10	MITF-C	1.03
ENST00000394348.2	Variant 8	0.70
ENST00000314557.10	MITF-M	-1.24
	siMITF+ NCS 4h	
ENST00000352241.8	MITF-A	-2.36
ENST00000314557.10	MITF-M	-2.76
ENST00000531774.1	MITF-Mdel	1.20
ENST00000328528.10	MITF-C	-0.77

Figure legend

Figure 1(A). RT qPCR validation for RNAseq data was carried out on the siMITF S8792 treated RNA that was converted to cDNA using manufacturer's protocol. The decrease in MITF level and increase in MITF-Mdel is statistically significant over three technical replicates with with p-value< 0.001. The geometric mean of beta actin and HPRT housekeeping genes was used as endogenous control for normalization. Fold change was calculated using the 2^-ddct method **(B)** western blot image of MITF protein in sicontrol vs siMITF in lysates from 501Mel cells, siRNA used against MITF is S8792. SMC1 is used as the loading control (SMC1 antibody- abcam, ab9262, MITF antibody-Thermo Scientific, MS771).



Supplementary information:

Supplementary Table 1 Refseq identified by siRNAs against MITF (Thermofisher.com)

s8790	s8791	s8792	Isoform
NM_000248.3	NM_000248.3	NM_000248.3	MITF-M
NM_001184967.1	NM_001184967.1	NM_001184967.1	Transcript variant 7
NM_006722.2	NM_006722.2	NM_006722.2	MITF-C
NM_198158.2	NM_198158.2	NM_198158.2	Transcript variant 5
NM_198159.2	NM_198159.2	NM_198159.2	MITF-A
NM_198177.2	NM_198177.2	NM_198177.2	MITF-H
NM_198178.2	NM_198178.2	NM_001184968.1	S8790, 8791(MITF-Mdel) S8792 (transcript variant 8)

Supplementary Table 2 Primers used for qRT-PCR

Primer	Sequence 5'-3'	Isoforms identified
F1	CGACAGAAGAAACTGGAGCAC	NM_198159.3 (MITF-A)
		NM_198177.3 (MITF-H)
		NM_006722.2 (MITF-C)
		NM_000248.4 (MITF-M)
R1	AAATCTGGAGAGCAGAGACCC	NM_198158.3 (Variant 5)
		NM_198178.3 (MITF-Mdel)
		NM_001184967.2 (Variant 7)
F2	TTATAGTACCTTCTCTTTGCCAGTCC	
R2	CTTATAAAATCCCTGCCGTTGG	NM_198178.3 (MITF-Mdel)