Variants in *Hdac9* Intronic Enhancer as Candidates for Skin Tumor Susceptibility Locus

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Abstract

Non-melanoma skin cancers (NMSC) are the most common forms of cancer in the world accounting for nearly half of all cancer diagnoses. Rates of NMSC are on the rise with an over 300% increase in diagnosis of these cancers in the last 20 years. While environmental risk factors for skin cancers are well understood, little is known about genetic risk factors for these cancers. Mouse linkage studies have identified several loci housing skin cancer susceptibility genes. Human tumors show evidence of preferential allelic imbalance for polymorphisms in *Hdac9*, a gene mapping to one of the linkage regions, *Skts5*. One intron in the *Hdac9* gene between exons 8 and 9 contains an enhancer for *Twist1*, affecting limb development and phenotypes in the skin. *Twist1* is a known regulator of skin differentiation and has a documented role in cancer. The hypothesis of this study is that this enhancer locus plays a role in the differential risk for NMSC between the cancer susceptible *NIH/Ola* and cancer resistant *Spretus/EiJ* mice. Sequence analyses identified several polymorphisms between these strains in this intron which are predicted by *in silico* methods to disrupt transcription factor binding. To investigate *in vitro* effects of these variants, intron fragments from both *NIH/Ola* and *Spret/EiJ* murine DNA were cloned into an enhancer reporting PGL3 vector and transfected into both normal keratinocyte C5N and squamous cell carcinoma A5 cells. Luciferase assay and real-time PCR data suggest these variants are responsible for changes in gene expression, specifically in the *Twist1* gene. Chromatin Immunoprecipitation studies are being performed to test whether transcription factors, Oct1 and Gata3 that are predicted to differentially bind the NIH/Ola and Spret/EiJ enhancer , are involved in the differential *Twist1* expression. This project has the potential implication of discovering the role a specific gene locus, *Skts5*, plays in NMSC risk.

Hypothesis and Specific Aims

We hypothesize that variants in the *Hdac9* intron 8 Enhancer Locus play a role in the differential risk for Non-melanoma Skin Cancers observed between the cancer susceptible *NIH/Ola* and the cancer resistant *Spretus* mouse strains.

The Specific Aims of this project include the following:

1. Determine if variants exist between *NIH* and *Spretus* DNA within *Hdac9* intron 8.
2. Identify and characterize any enhancer sites in the murine *Hdac9* intron 8 sequence
3. Determine which gene(s) are targets of the enhancer(s) and demonstrate via qPCR
4. Identify specific transcription factor/DNA binding interactions potentially associated with enhancer activity using Chromatin Immunoprecipitation Assays

Identification of Polymorphisms Between *NIH & Spretus* Murine DNA at *Hdac9* Intron

- Custom primers were designed to break up *Hdac9* intron 8 into smaller fragments capable of being sequenced
- In total, 43 polymorphisms were found between cancer susceptible *NIH/Ola* and cancer resistant *Spretus* mice DNA

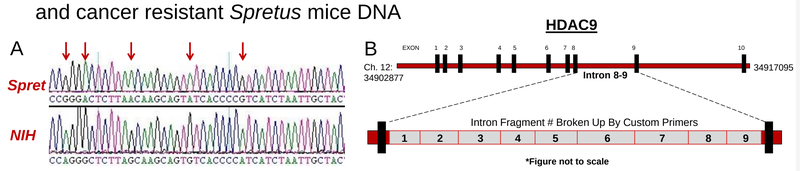

Figure 1: A) Examples of sequence variants within *Hdac9* intron 8 between *NIH* & *Spret* B) Map of *Hdac9* gene with Intron 8 magnified. Custom primer inserts shown visually.

Enhancer Activity Demonstrated in *Hdac9*

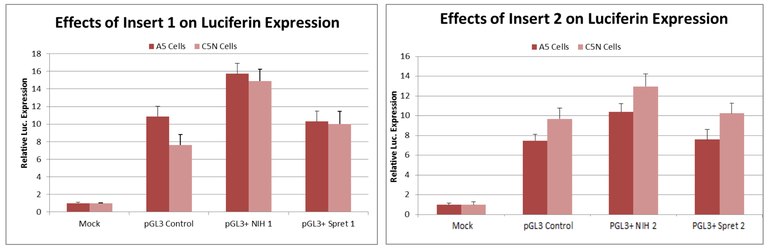

Figure 2: Cells transfected with pGL3 + *NIH* Inserts 1 & 2 show significantly higher Luciferin expression than those transfected with pGL3 + *Spret* Inserts 1 & 2 in both C5N normal keratinocyte and A5 SCC cell lines. Insert 1: p<0.01**, Insert 2: p<0.05*

- Enhancer activity exists in the Insert 1 & 2 region of *Hdac9* Intron 8
- This enhancer activity is unique to the *NIH* mouse strain, not found in *Spretus*
- Enhancer activity does not extend to later portions of the intron (i.e. Insert 7)

Hdac9 Enhancer Affects *Twist1* Expression

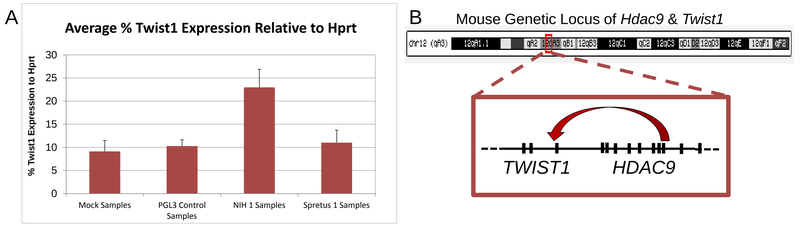

Figure 3: A) Real-Time PCR Data indicating cells transfected with pGL3+*NIH* Insert 1 display a near 2 fold increase in *Twist1* expression compared to pGL3 control and pGL3+*Spretus* Insert 1 in A5 cells (p<0.05). B) Map of proposed genetic interaction

- *Twist1* identified as a target gene of the *Hdac9* Intron 8 enhancer activity
- Similar study looking at *Hdac9* did not see significant differences in expression

Transcription Factor Binding Predictions

Transcription Factor	DNA Binding Site	Polymorphism	Binding Strain	Significance Score
Oct1	ATATACACT	C/G	Spretus	89.7
Gata3	AATCACG	C/T	NIH	85.9
CdxA	ATATAG	G/C	NIH	85.0
Lyf-1	TGGGAT	A/G	Spretus	85.7
Gfi-1	ATAGTTGTGAT	A/G	Spretus	85.9
Nkx-2	TCAAGTG	C/A	Spretus	89.9
AP-1	GTGATTAA	A/G	NIH	86.4

Table 1: *In silico* predictions of transcription factors that will differentially bind *NIH* and *Spretus* DNA at the *Hdac9* Enhancer locus. Databases utilized include TFsearch, DBD, PROMO(Transfac) and TFSiteScan. Cutoff score of 85.0 used for significance.

Oct1 and Gata3 Transcription Factors Differentially Bind *NIH & Spretus in vitro*

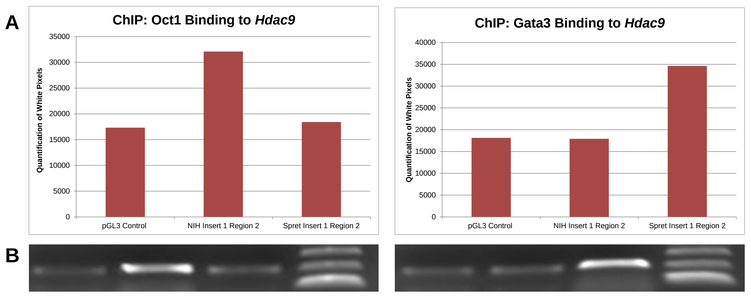

Figure 4: A) Quantification of ChIP results for Oct1 and Gata3 Transcription Factors (TF). B) Gel images of ChIP results for the respective TFs (C5N cells)

- Oct1 shown to preferentially bind *NIH* Insert 1 in comparison to *Spretus* Insert 1
- Gata3 shown to preferentially bind *Spretus* Insert 1 in comparison to *NIH* Insert 1
- Both *in silico* predictions validated through *in vitro* ChIP studies
- Implications for these factors in the functionality of the *Hdac9* enhancer

Conclusions and Impact

- An intronic enhancer in the *Hdac9* gene was identified in mice
- This intronic enhancer was shown to be present in cancer susceptible *NIH/Ola* mice but not in cancer resistant *Spretus* mice
- A relation between this enhancer and *Twist1* upregulation was demonstrated
- Two transcription factors, Oct1 and Gata3, were shown to differentially bind one of the two mice DNA strains, suggesting a possible mechanism for SCC risk at *Skts5*
- One of the first studies analyzing the role of intronic enhancers in skin cancer risk

Acknowledgements

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