

***In Silico* Gene Expression and Pathway Analysis to Explore the Link Between Hes1 and Adipocyte Stem Cell Differentiation in Obesity**

ABSTRACT

Aim: Hairy/enhancer of split homologue-1 or Hes1 is a transcription factor with bHLH domains and participates in controlling proliferation and differentiation of various stem cell progenitors. The study aims to analyze pathways and genes up regulated and co-expressed with Hes1 to examine their linkage with adipocyte stem cell differentiation in obesity.

Methodology: ~~In this in-~~ *In silico* analysis, Gene Expression Omnibus Dataset GDS5056 was used to shortlist 23 genes differentially up regulated and co-expressed along with Hes1 during adipocyte stem cell differentiation in obese patients. Then, these genes along with their interactor genes were submitted to Reactome pathway analysis database and were statistically analyzed.

Results: Total 12 enriched pathways were obtained which majorly belonged to two categories: neuronal cell differentiation and signaling; and ~~Inflammatory-inflammatory~~ response. ~~While Since~~ Hes1 is known to regulate such pathways as a master transcription factor and repressor, its level of expression determines the result as proliferation or differentiation. Thus, these *in silico* findings may help in designing future experiments to determine role of Hes1 in deciding fate of adipocyte differentiation or proliferation in obesity.

Keywords: Hairy enhancer of split homologue-1, Hes1, pathway, stem cells, obesity

1. INTRODUCTION

Stem cells require stringently controlled regulation of gene and protein expression for self-renewal, growth, differentiation, division and signalling. Transcription factors act as master “regulator switches” to control this expression by binding to promoters and interacting with other co-regulators. In the same way, adipose stem cells residing in white adipose tissue also utilize these factors to regulate self-renewal and repair. ~~Hairy/enhancer of split homologue-1 or Hes1 is one such transcription factor.~~ Hes1 protein belongs to a family of basic helix-loop-helix Hairy and Enhancer of split proteins and is key target protein of Notch signalling pathway, which is one of the main pathways regulating proliferation and differentiation of haematopoietic stem cell progenitors [1, 2]. Human Hes1 gene is present on long arm of chromosome 3 and encodes a 268 amino acid long protein having less conserved Orange and WRPW in addition to bHLH [3].

Increasing and accumulating experimental evidences indicate that deregulated high expression of Hes1 is associated with occurrence of tumorigenesis and metastasis of colon cancer [4], neuroendocrine [5], pancreas [6], non-small cell lung cancer [7] and adenoid cystic carcinoma [8]. In all these cases, altered developmental and differentiation pattern is

often linked to cancer stem cells. Hes1 has been found to be involved in tumor invasiveness and morphogenetic changes from epithelial to mesenchymal cell type along with self renewal characteristic of cancer stem cells [9]. Its expression has been found to be increased in rather poorly differentiated tumors.

Enhanced expression of Hes1 via Notch signalling was observed to be associated with proliferation, differentiation and adipogenesis of mouse adipose-derived stem cells [10]. On the other hand, Hes1 was reported to inhibit Adipogenesis process in mesenchymal stem cells derived from pig [11]. Thus, an understanding of regulation of different pathways by Hes1 is still required in adipocyte regulated stem cell differentiation.

~~Here in In~~ this study, an attempt has been made to perform pathway analysis using *in silico* mining of genes differentially co-expressed along with Hes1 in adipocyte differentiation using publicly available gene expression GEO datasets. The Gene Expression Omnibus (GEO) database of NCBI (National Centre of Biotechnology Information) stores gene expression data from microarray and RNA-Seq experiments in the form of datasets, their series and individual expression profiles of each gene in them [12]. The publically available data from a previous investigation made by Oñate et al. [13] has been being used here in this *in silico* study as a third party to identify effect of obesity on stemness of adipocytes and its relation to Hes1 expression. Dataset GDS5056 has been being meta-analysed to find pathway associations of Hes1 with its neighbouring genes having similar expression patterns and their interactors to their link with obesity and stemness at molecular level.

2. METHODOLOGY

2.1 Gene list Retrieval from GEO dataset of NCBI

Differential expression profiles of Hes1 protein, 232 out of 9071 were searched to find terms related to obesity and stem cell throughout Gene Expression Omnibus datasets. As a result, one GDS5056 and series GSE48964 was shortlisted to proceed further. The main purpose of this study was to perform transcriptome profiling using microarray GPL6244 platform derived from Adipose stem cells isolated from subcutaneous white adipose tissue. The expression data considered for study, was derived from transcriptome of Subcutaneous white adipose tissues stem cell RNA samples (n = 6) processed and taken from morbidly obese patients as against the non-obese cases [13]. Genes which had similar differentially regulated profiles as of Hes1 gene were downloaded using Profile neighbours option to obtain the gene list.

2.2 Protein-Protein Interactions and Pathway Analysis

The proteins interact with each other to operate various signalling, drug and metabolic pathways. The list of differentially co-expressed genes obtained in previous section acted as an experimental dataset and submitted to execute a pathway over-representation analysis in Reactome database. Some genes were added to the current list based on protein-protein interactions reported in IntAct database, to expand the pathway results for increasing the number of interactors to be integrated with Reactome database [14, 15]. This database contains all possible interconnected and interwoven networks and pathways imported from different public databases. These have been made on the basis of experiments and studies, in which various signalling events, metabolic, drug and other pathways, including all possible annotations of proteins and molecules participating in them are included.

2.3 Statistical Analysis

The final gene list was compared with each and every pathway using statistical hypergeometric distribution test for enrichment to evaluate over-represented values in the data. Benjamini–Hochberg Method was used to calculate corrected *P* values in form of false discovery rate to test the significance. Thus, final result was then obtained after filtering the output using FDR (or corrected *P* values $P < .05$).

3. RESULTS AND DISCUSSION

Since *Hairy and Enhancer of Split 1* (Hes1) is linked to self-renewal and differentiation in stem cells. Thus, our aim in this study was to perform pathway analysis to find influence of Hes1 and its co-expressed proteins which might interact with each other to participate in changing adipocyte stem cell fate. Hes1 was found to be highly expressed in adipose stem cells obtained from morbidly obese samples as compared to those from non-obese individuals. This indicates that Hes1 being a target gene of NOTCH signalling may be playing a role in stemness regulation. This role however, in the experimental study producing dataset GDS5056 has not been mentioned and unnoticed probably due to higher overall expression of other genes. Hence, in order to conduct pathway analysis, 22 genes as profile neighbours bearing similar pattern of expression were taken as input for further analysis. The list and details of these genes are shown in Table 1.

Table 1: Gene symbols and IDs of Hes1 and associated differentially regulated genes submitted as gene list for analysis

| Gene ID | Gene symbol | Gene title |
|----------------------|-----------------------|---|
| 8707 | B3GALT2 | beta-1,3-galactosyltransferase 2 |
| 64651 | CSRNP1 | cysteine and serine rich nuclear protein 1 |
| 2920 | CXCL2 | C-X-C motif chemokine ligand 2 |
| 3576 | CXCL8 | C-X-C motif chemokine ligand 8 |
| 1958 | EGR1 | early growth response 1 |
| 1959 | EGR2 | early growth response 2 |
| 1960 | EGR3 | early growth response 3 |
| 2353 | FOS | Fos proto-oncogene, AP-1 transcription factor subunit |
| 2354 | FOSB | FosB proto-oncogene, AP-1 transcription factor subunit |
| 2898 | GRIK2 | glutamate ionotropic receptor kainate type subunit 2 |
| 8349 | HIST2H2BE | histone cluster 2, H2be |
| 3553 | IL1B | interleukin 1 beta |
| 3976 | LIF | leukemia inhibitory factor |
| 102724428; 150094 | LOC102724428; SIK1 | serine/threonine-protein kinase SIK1; salt inducible kinase 1 |
| 9208 | LRRFIP1 | LRR binding FLII interacting protein 1 |
| 50804 | MYEF2 | myelin expression factor 2 |
| 3164 | NR4A1 | nuclear receptor subfamily 4 group A member 1 |
| 4973 | OLR1 | oxidized low density lipoprotein receptor 1 |
| 6335 | SCN9A | sodium voltage-gated channel alpha subunit 9 |

| | | |
|-------|---------|-----------------------------|
| 57698 | SHTN1 | shootin 1 |
| 7128 | TNFAIP3 | TNF alpha induced protein 3 |
| 10221 | TRIB1 | Tribbles_pseudokinase 1 |

Pathway analysis was performed against Reactome version 73, according to which, total 21 entities with 3 IntAct list interactors as gene list were recognised by software and processed further. In total, 388 pathways were obtained as an output, however, only 50 pathways were considered for further analysis after applying P value filters for test of significance ($P < .05$). Furthermore, some of the pathways were removed in which less than 2 interactors were found. As revealed by analysis, figure 1 represents the significant general clusters of pathways or biological processes, as visualised in database with corrected P value $< .05$. These include Developmental biology, Neuronal system, Immune System, Signal transduction, Chromatin organisation, DNA repair, Cell cycle, Cellular response to External stimuli, Gene expression, Diseases and Metabolism of proteins (highlighted by Golden copper colour branches or nodes).

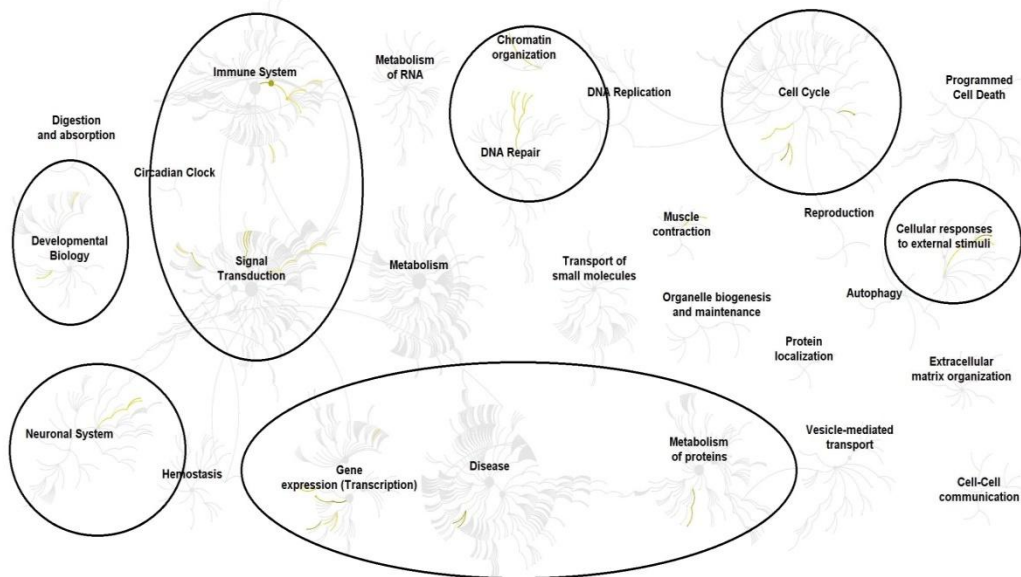


Fig. 1: Significant clusters of pathways or biological processes

($p < 0.05$ as visualised in Reactome Database).

Golden copper colour highlights branches or nodes of sub-pathways.

Further, overrepresentation or enrichment analysis was carried out to find specific sub – pathways. Total 10 significant pathways pathways (sub-pathways) after enrichment analysis were obtained as shown in Table 2. Signalling by NTRKs, Signalling by NTRK1, Nuclear Events (kinase and transcription factor activation), NGF-stimulated transcription, Interleukin-4 and Interleukin-13 signalling and Interleukin-10 signalling, which show around 9 to 10 genes or entities up regulated in them.

Table 2: Details of the enriched pathways and the genes or entities involved in them

| Pathway identifier | Pathway name | #Entities found | #Entities total | #Interactors found | #Interactors total | Entities FDR | Submitted entities found |
|--------------------|---|-----------------|-----------------|--------------------|--------------------|--------------|-------------------------------|
| R-HSA-166520 | Signaling by NTRKs | 9 | 166 | 1(NR4A1) | 877 | 0.035 | EGR1;EGR2;EGR3;FOSB;FOS;TRIB1 |
| R-HSA-187037 | Signaling by NTRK1 (TRKA) | 9 | 143 | 1(NR4A1) | 659 | 0.006 | EGR1;EGR2;EGR3;FOSB;FOS;TRIB1 |
| R-HSA-198725 | Nuclear Events (kinase and transcription factor activation) | 9 | 80 | 0 | 319 | 4.24E-05 | EGR1;EGR2;EGR3;FOSB;FOS;TRIB1 |
| R-HSA-9031628 | NGF-stimulated transcription | 9 | 56 | 0 | 296 | 2.02E-05 | EGR1;EGR2;EGR3;FOSB;FOS;TRIB1 |
| R-HSA-6785807 | Interleukin-4 and Interleukin-13 signaling | 9 | 211 | 0 | 143 | 2.02E-05 | CXCL8;IL1B;LIF;FOS |
| R-HSA-6783783 | Interleukin-10 signaling | 10 | 86 | 1 (NR4A1) | 91 | 6.22E-09 | CXCL8;IL1B;LIF;CXCL2 |
| R-HSA-380108 | Chemokine receptors bind chemokines | 4 | 57 | 2 (CXCL8; CXCL2) | 70 | 0.006 | CXCL8;CXCL2 |
| R-HSA-2559582 | Senescence-Associated Secretory Phenotype (SASP) | 6 | 91 | 0 | 46 | 8.92E-05 | CXCL8;IL1B;FOS;HIST2H2BE |
| R-HSA-5660668 | CLEC7A/inflammasome pathway | 3 | 8 | 0 | 25 | 0.004 | IL1B |

| | | | | | | | |
|--------------|--|---|----|---|----|-------|-------|
| R-HSA-448706 | Interleukin-1 processing | 2 | 8 | 0 | 15 | 0.045 | IL1B |
| R-HSA-451308 | Activation of Calcium-permeable Kainate Receptor | 2 | 13 | 0 | 6 | 0.045 | GRIK2 |
| R-HSA-451306 | Ionotropic activity of kainate receptors | 2 | 14 | 0 | 6 | 0.045 | GRIK2 |

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These pathways are part of neuronal stem cell differentiation, signalling and inflammation, such as one signalling by NTRKs (R-HSA-166520) shown in figure 2. It shows an overview of nerve growth factor and Brain-derived neurotrophic factors like neurotrophins participate and significantly regulate differentiation and survival of neurons in the nervous system by binding and activating to their receptors such as, receptor tyrosine kinases activated by ligand binding to their extracellular domain [16].

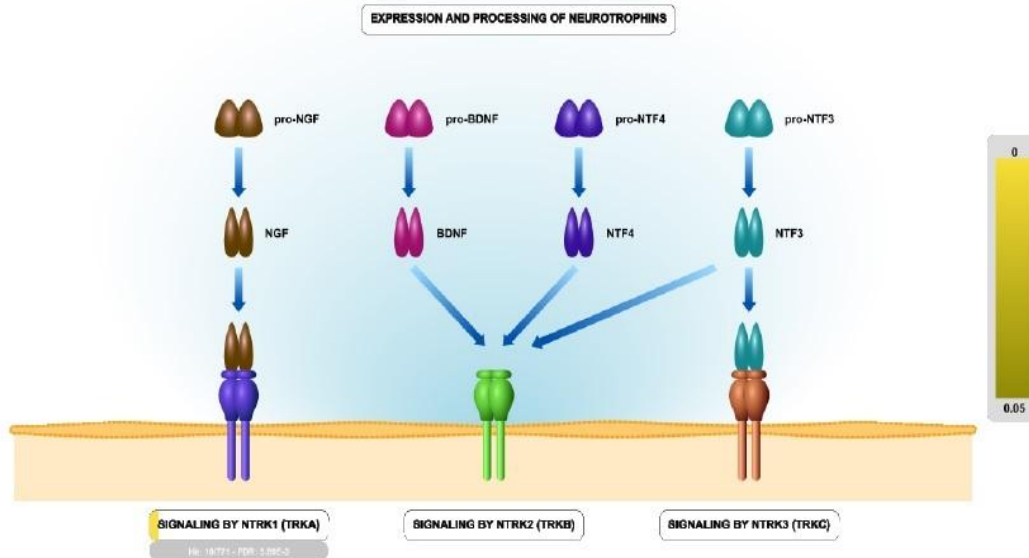


Fig. 2: Neurotrophins and Enriched Reactome Signaling pathway of NTRKs (R-HSA-166520)

Hes1 is actively involved in Notch signalling and plays dual role in maintaining the quiescent or active/proliferative state of neural stem cells depending on expression levels [17]. This is also true for control of developing and regenerating muscle stem cells regulated by Hes1 by deciding between occurrence of events of proliferation and differentiation [18]. Hes1 is well known to inhibit the inflammatory response by controlling B cell and T cell differentiation [19]; inflammatory molecule release from macrophages in presence of its co-repressors and even recruitment of neutrophil through regulating transcription elongation complexes [20, 21].

Scanty of resources and specific experimental data are available against effect of Hes1 on obesity and adipocyte stem cell migration, differentiation and proliferation. This study provides a new direction and scope to perform experimental work or knock down assays to prove association of Hes1 mediated physiological and molecular action on adipocyte stem cells in obese individuals.

4. CONCLUSION

This study gave an insight into the probable pathways and protein interactions occurring in conjunction with stem cell differentiation mediated by Hes1. The involvement of Hes1 mediated neuronal networks and inflammatory pathways found in obesity were related to adipocyte development and differentiation. This *in silico* analysis may later be confirmed by using experimental set up.

REFERENCES

1. Kunisato A, Chiba S, Nakagami-Yamaguchi E, Kumano K, Saito T, et al. HES-1 preserves purified hematopoietic stem cells ex vivo and accumulates side population cells in vivo. *Blood*. 2003; 101(5): 1777–83.
2. Lampreia FP, Carmelo JG, Anjos-Afonso F. Notch Signaling in the Regulation of Hematopoietic Stem Cell. *Curr Stem Cell Rep*. 2017; 3(3):202-209.
3. O'Leary NA, Wright MW, Brister JR, Ciufu S, Haddad D, McVeigh R, Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. *Nucleic Acids Res*. 2016; 44(D1):D733-45.
4. Weng MT, Tsao PN, Lin HL, Tung CC, Change MC, Chang YT, Wong JM, Wei SC. Hes1 Increases the Invasion Ability of Colorectal Cancer Cells via the STAT3-MMP14 Pathway. *PLoS One*. 2015; 10(12):e0144322
5. Cui M, Cai Z, Awadallah A, Xin W. Uniform and Robust Nuclear Expression of HES1 in Neuroendocrine Neoplasms. *Int J Surg Pathol*. 2019; 27(8):844-851.
6. Perron A, Nishikawa Y, Iwata J, Shimojo H, Takaya J, Kobayashi K, Imayoshi I, Mbenza NM, Takenoya M, Kageyama R, Kodama Y, Uesugi M. Small-molecule screening yields a compound that inhibits the cancer-associated transcription factor Hes1 via the PHB2 chaperone. *J Biol Chem*. 2018; 293(21):8285-8294.
7. Yuan Q, Chen X, Han Y, Lei T, Wu Q, Yu X, Wang L, Fan Z, Wang S. Modification of α 2,6-sialylation mediates the invasiveness and tumorigenicity of non-small cell lung cancer cells in vitro and in vivo via Notch1/Hes1/MMPs pathway. *Int J Cancer*. 2018; 143(9):2319-2330.
8. Huang XY, Gan RH, Xie J, She L, Zhao Y, Ding LC, Su BH, Zheng DL, Lu YG. The oncogenic effects of HES1 on salivary adenoid cystic carcinoma cell growth and metastasis. *BMC Cancer*. 2018; 18(1):436.
9. Liu ZH, Dai XM, Du B. Hes1: a key role in stemness, metastasis and multidrug resistance. *Cancer Biol Ther*. 2015; 16(3):353-9.
10. Ba K, Yang X, Wu L, Wei X, Fu N, Fu Y, Cai X, Yao Y, Ge Y, Lin Y. Jagged-1-mediated activation of notch signalling induces adipogenesis of adipose-derived stem cells. *Cell Prolif*. 2012; 45(6):538-44.
11. Lei T, Bi Y, Gao MJ, Gao SM, Zhou LL, Zheng HL, Chen XD. HES1 inhibits adipogenesis of porcine mesenchymal stem cells via transcriptional repression of FAD24. *Domest Anim Endocrinol*. 2013; 45(1):28-32.
12. Clough E, Barrett T. The Gene Expression Omnibus Database. *Methods Mol Biol*. 2016; 1418:93-110. doi: 10.1007/978-1-4939-3578-9_5. PMID: 27008011; PMCID: PMC4944384.
13. Oñate B, Vilahur G, Camino-López S, et al. Stem cells isolated from adipose tissue of obese patients show changes in their transcriptomic profile that indicate loss in stem cellness and increased commitment to an adipocyte-like phenotype. *BMC Genomics*. 2013; 14:625.
14. Fabregat A, Sidiropoulos K, Garapati P, Gillespie M, Hausmann K, Haw R, et al. The Reactome pathway knowledgebase. *Nucleic Acids Research*, 2016; 44: D481–D487.
15. Fabregat A, Sidiropoulos K, Viteri G, Forner O, Marin-Garcia P, Arnau V, D'Eustachio P, Stein L, Hermjakob H. Reactome pathway analysis: a high-performance in-memory approach. *BMC bioinformatics*. 2017; 18(1):142.
16. Skaper SD. The neurotrophin family of neurotrophic factors: an overview. *Methods Mol. Biol.*, 2012; 846, 1-12.
17. Harris L, Guillemot F. HES1, two programs: promoting the quiescence and proliferation of adult neural stem cells. *Genes Dev*. 2019; 33(9-10):479-481.

18. Lahmann I, Bröhl D, Zyrianova T, Isomura A, Czajkowski MT, Kapoor V, Griger J, Ruffault PL, Mademtzoglou D, Zammit PS, Wunderlich T. Oscillations of MyoD and Hes1 proteins regulate the maintenance of activated muscle stem cells. *Genes & development* **Development**. 2019; 33(9-10):524-35.
19. Shang Y, Coppo M, He T, Ning F, Yu L, Kang L, Zhang B, Ju C, Qiao Y, Zhao B, Gessler M, Rogatsky I, Hu X. The transcriptional repressor Hes1 attenuates inflammation by regulating transcription elongation. *Nat Immunol*. 2016; 17(8):930-7.
20. Shang Y, Coppo M, He T, Ning F, Yu L, Kang L, Zhang B, Ju C, Qiao Y, Zhao B *et al*. The transcriptional repressor Hes1 attenuates inflammation by regulating transcription elongation. *Nat Immunol* 2016; 17: 930–937.
21. Zhang, X., Li, X., Ning, F. *et al*. TLE4 acts as a corepressor of Hes1 to inhibit inflammatory responses in macrophages. *Protein Cell* 2019; 10: 300–305.

ABBREVIATIONS

Hairy and Enhancer of Split 1 (Hes1)
Basic helix-loop-helix (bHLH)
Gene Expression Omnibus (GEO)
National Centre for Biotechnology Information (NCBI)
Nerve Growth Factor (NGF)
Neurotrophic Tropomyosin Related Kinase (NTRK)

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