Research Article

Identification of potential miRNA-mRNA regulatory networks contributing to Changthangi sheep hair follicle development

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ABSTRACT

Changthangi sheep, native to the high-altitude regions of Ladakh, are recognized for their fine fiber production and are distinguished by their grey skin and coats that can be either black or white. In this study, various non-coding RNAs (circRNAs, miRNAs, piRNAs, snRNAs, snoRNAs, and tRNAs) were mined from the RNA sequencing data of the skin tissue of four Changthangi sheep. Subsequently, the specific role of microRNAs was deciphered to uncover the molecular drivers and pathways that might contribute to fiber quality and resulting adaptation to the inimitable agroecological conditions. The miRNA–mRNA interactions at a genomic scale were examined utilizing miRNet and miRBD algorithms. Enriched KEGG pathways of the predicted genes werethe MAPK signaling pathway, PI3K-Akt signaling pathway, Ras signaling pathway, endocytosis and oxytocin signaling. Notably, NRAS gene was identified as a key participant in the prominent MAPK, Ras, and PI3K signaling pathways which play a significant role in melanogenesis and adaptation. Moreover, highly interconnected genes associated with fiber quality and development were identified through network analysis, including collagen genes (*COL1A2, COL3A1* and *COL15A1*), MAPK signaling pathway genes (*MAP3K1, MAP4K3* and *MAPK8*), and *HOX* genes in the Changthangi sheep from Ladakh. In conclusion, our findings provide a foundation for further research examining the role of microRNA-mRNA interactions in controlling hair development in Changthangi sheep.

Keywords: Fiber, mRNA, Non-coding RNAs, RNA sequencing, Sheep, Skin

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INTRODUCTION

Sheep (*Ovis aries*) play a pivotal role in the agricultural economy of India, particularly in arid, semi-arid, and hilly regions. Sheep are adapted to different climatic extremes and are influenced by physiological, behavioural, and morphological factors, but the most significant impact on acclimatization to diverse agro-climatic zones is exerted by the genetic landscape (Berihulay *et al.,* 2019). Sheep wool and fibre contribute significantly to the agricultural economy. The combination of genetic factors and environmental conditions influences the quality, quantity, and color of wool and fleece (Yao *et al.,* 2019). Wool is produced through various processes by hair follicles (HFs), which are embedded in the skin of sheep. These follicles play a central role in crafting wool through diverse biological mechanisms, collectively contributing to the unique characteristics of this valuable natural fiber (Yue *et al.,* 2015). Regulation of the hair cycle involves complex signaling interactions between MAPK (Mitogen-activated protein kinase), PI3k/Akt (phosphoinositide-3-kinase–protein kinase B/Akt), Ras and other signaling Pathways (Chen *et al.,* 2020; Ahlawat *et al.,* 2024).

The advent of RNA sequencing methodologies has transformed our ability to analyze comprehensive gene expression profiles in sheep. This facilitates the identification of molecular pathways, offering valuable insights into the genetic foundations of economically significant traits. Such knowledge contributes to refining breeding strategies and optimizing production efficiency in sheep (Arora *et al.,* 2021; Kaur *et al.,* 2020; Vasu *et al.,* 2024). Non-coding RNAs (ncRNAs) are a diverse family of RNA molecules such as circular RNAs (circRNAs), microRNAs (miRNAs), piwi-interacting RNAs (piRNA), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), ribosomal RNAs (rRNAs), and transfer RNAs (tRNAs). These ncRNAs do not encode proteins but play an important role in regulating gene expression, as well as biological pathways and processes. Recent studies expanding beyond protein-coding genes reveal the potential of non-coding RNAs (ncRNAs), including microRNA (miRNA), circRNA, piRNA, natural antisense transcripts (NAT), and long non-coding RNA (lncRNA) as more specific biomarkers for certain applications compared to protein-coding genes (Sulayman *et al.,* 2019; Wang *et al.,* 2019; Yue *et al.,* 2015). ncRNAs are not only abundant but also hold significant influence in the regulation of gene expression in various species such as sheep, goat, yak, cattle, buffalo, human and rabbit (Wang *et al.,* 2019; Yang *et al.,* 2021; Yue *et al.,* 2015). miRNAs are single-stranded non-coding RNAs (19-25 nucleotides) that modulate the expression of target genes post-transcriptionally by partially binding to the 3′-UTR region of target mRNA and hence play a crucial role in various biological activities. Among these non-coding RNAs (ncRNAs), a portion encoded by the sheep genome has been annotated (Kaur *et al.,* 2020). However, miRNAs, particularly those highly specific to skin tissue, have not undergone inclusive characterization.

This study aimed to examine the skin follicle non-coding RNAs and their potential roles based on advanced high-throughput sequencing technology. For this, the Changthangi sheep breed of India was selected as the model animal. Changthangi animals are adapted to the high-altitude hypoxic conditions prevailing in the highland Changthang region of Ladakh (India). They have a unique double coat, featuring down fibers, which safeguard them in harsh cold conditions and present an opportunity for producing fine luxurious fibers (Malik *et al.,* 2021). The study aimed to identify non-coding RNAs, and predict genes, various molecular pathways, and gene networks that contribute to fiber quality and hence adaptation to unique agroecological conditions.

MATERIALS AND METHODS

RNA sequencing data generation

RNA sequencing libraries were created from the skin tissue of four rams belonging to the Changthangi sheep breed utilizing the NEBNext Ultra Directional RNA Library Prep Kit compatible with Illumina (New England Biolabs, MA, USA). After preparing the libraries, the HiSeq 2500 Sequencing System (Illumina) was used to produce 150 bp paired-end reads. The quality of the raw sequences from the RNA sequencing run was assessed using FastQC software (v 0.11.5) (Leggett *et al.,* 2013). The data generated for this study has been submitted under NCBI project ID PRJNA995381, with accession numbers SAMN36468555 to SAMN36468558.

Quality control

The COMPSRA (Comprehensive Platform for Small RNA-seq Data Analysis) was conducted to assess the quality and reliability of RNA-seq data (https://github.com/cougarlj/COMPSRA). This involved evaluating the alignment statistics, detecting potential issues like adapter contamination or low-quality reads, and comparing the results from multiple alignments. Moreover, in order to remove adapter sequences within reads, randomized bases at ligation junctions and to filter out low-quality reads, a quality control filter was applied by using predefined parameters (-rh 20 –rt 20 –rr 20) (Li *et al.,* 2020).

Alignment and annotation

In the COMPSRA analysis, STAR serves as the default RNA sequence aligner for filtered reads to the reference genome. Initially, high-quality reads from the quality control modules are aligned to the human genome version hg19/hg38 (Dobin *et al.,* 2013). Moreover, COMPSRA employs various small RNA databases, including circBase for circular RNA, miRBase for miRNA, piRNABank, piRBase, and piRNACluster for piRNA, gtRNAdb for tRNA, and GENCODE for snRNA and snoRNA to extract various types of non-coding RNAs (Li *et al.,* 2020).

Prediction and analysis of target gene of potential miRNAs

miRDB (https://mirdb.org/), a web-based tool that uses a novel bioinformatics approach for miRNA target identification and functional annotation was used to retrieve the target prediction results produced by a new algorithm (Chen and Wang, 2020). Additionally, miRNet (https://www.mirnet.ca/), a user-friendly web tool designed for customization, visual exploration, and functional interpretation of interaction networks between miRNAs and their targets was used (Chang and Xia, 2022).

Functional annotation of the predicted genes

The analysis of the target genes aimed to uncover the biological functions and identify the molecular pathways was done through the utilization of DAVID software (Sherman *et al.,* 2022). This approach enabled a detailed investigation of the functional landscape of the genes, enhancing understanding of their significance and potential impact on various biological activities and enriched pathways.

Pathway analysis

Analysis of enriched pathways for the understanding of the biological processes associated with the investigated genes and their potential implications in various cellular activities was done using a comprehensive database called the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa *et al.,* 2023).

Over-representation analysis

ConsensusPathDB (CPDB) was used to visualize the different molecular concepts (pathways, neighborhood-based sets, gene ontology categories, and protein complexes), resulting from a particular over-representation or enrichment analysis (Kamburov *et al.,* 2011). CPDB generated a network graphic by manually selecting pathways of interest and organizing the nodes to effectively depict pathway interconnectivity data.

Network analysis

STRING software was used to study protein-protein interactions (PPIs) of miRNA-predicted genes. The network was built, focusing on reliable interactions with a high-confidence PPI score of 0.7 to ensure the incorporation of reliable molecular associations in this analysis (Szklarczyk *et al.,* 2023).

RESULTS AND DISCUSSION

RNA sequencing data

The RNA-seq data varied between 36.8 to 59.4 million raw reads and 36.5 to 67.9 million processed reads for different samples of Changthangi sheep. Alignment with the Oar v3.1 genomic.fna reference (GCA 000298735.1) revealed that 98.27% to 99.37% of reads were successfully mapped, indicating the data suitability for subsequent analysis. Any sequencing biases in the resulting dataset were removed by similarity in the proportion of mapped reads (Table 1).

Table 1: The read statistics of 4 libraries from the Changthangi sheep

Sample	Raw reads	Processed reads	High-quality data (%)	Aligned reads (%)
Changthangi1	3,68,27,512	3,65,94,148	99.36	99.37
Changthangi2	6,82,34,368	6,79,62,154	99.60	99.26
Changthangi3	5,61,09,072	5,58,66,399	99.56	99.30
Changthangi4	5,97,17,590	5,94,36,296	99.52	99.27

Candidate ncRNAs identification

The most abundant ncRNA molecules as identified in the skin of Changthangi sheep by using COMPSRA pipeline included 34,440 circRNAs, 22 miRNAs, 200 piRNAs, 59 snRNAs, 19 snoRNAs, and 27 tRNAs. miRNAs observed in Changthangi sheep were further analysed and the results are summarized in Table 2.

Table 2: List of miRNAsidentied in Changthangi sheep skin

S.No	Database	Name	Count	Accession number
1.	miRBase	hsa-miR-1244	29	LM380693.1
2.	miRBase	hsa-miR-374c-5p	26	LM382184.1
3.	miRBase	hsa-miR-3064-5p	8	FR772704.1
4.	miRBase	hsa-miR-1282	5	NR_031695.1
5.	miRBase	hsa-miR-196b-3p	3	LM381144.1
6.	miRBase	hsa-miR-196b-5p	3	LM379355.1
7.	miRBase	hsa-miR-374c-3p	3	NR_037511.1
8.	miRBase	hsa-miR-4444	1	LM382393.1
9.	miRBase	hsa-mir-5047	1	NR_039969.1
10.	miRBase	hsa-mir-4532	1	LM382443.1
11.	miRBase	hsa-let-7f-2-3p	1	LM380167.1
12.	miRBase	hsa-let-7f-5p	1	LM378754.1
13.	miRBase	hsa-miR-421	1	NR_030398.1
14.	miRBase	hsa-miR-3661	1	LM611188.1
15.	miRBase	hsa-mir-6879	1	NR_106939.1
16.	miRBase	hsa-miR-3074-3p	1	FR772707.1
17.	miRBase	hsa-miR-3074-5p	1	FR772708.1
18.	miRBase	hsa-mir-4481	1	NR_039701.1
19.	miRBase	hsa-miR-4680-5p	1	FR772934.1

20.	miRBase	hsa-miR-4680-3p	1	FR772933.1
21.	miRBase	hsa-miR-3120-5p	1	FR772713.1
22.	miRBase	hsa-miR-3120-3p	1	FR772712.1

Target prediction and analysis of candidate miRNAs

The top 10 potential miRNAs out of 22 miRNAs were used to predict the target genes by using miRBD. The analysis revealed 264 predicted target genes. To enhance the visualization of the data, miRNA-mRNA networks were constructed by using the miRNet database (Fig. 1). Among the predicted genes, hsa-miR-3074-5p showed the highest level of interaction, while hsa-let-7f-5p displayed the least interaction. The majority of miRNA interactions were linked to pivotal pathways such as MAPK signaling and Wnt signaling, playing a crucial role in hair follicle growth. Additionally, the involvement of genes in pathways like focal adhesion, Notch signaling, cell cycle, TGF-beta signaling, regulation of actin cytoskeleton, insulin signaling, RNA transport, GnRH signaling, and melanogenesis was observed.

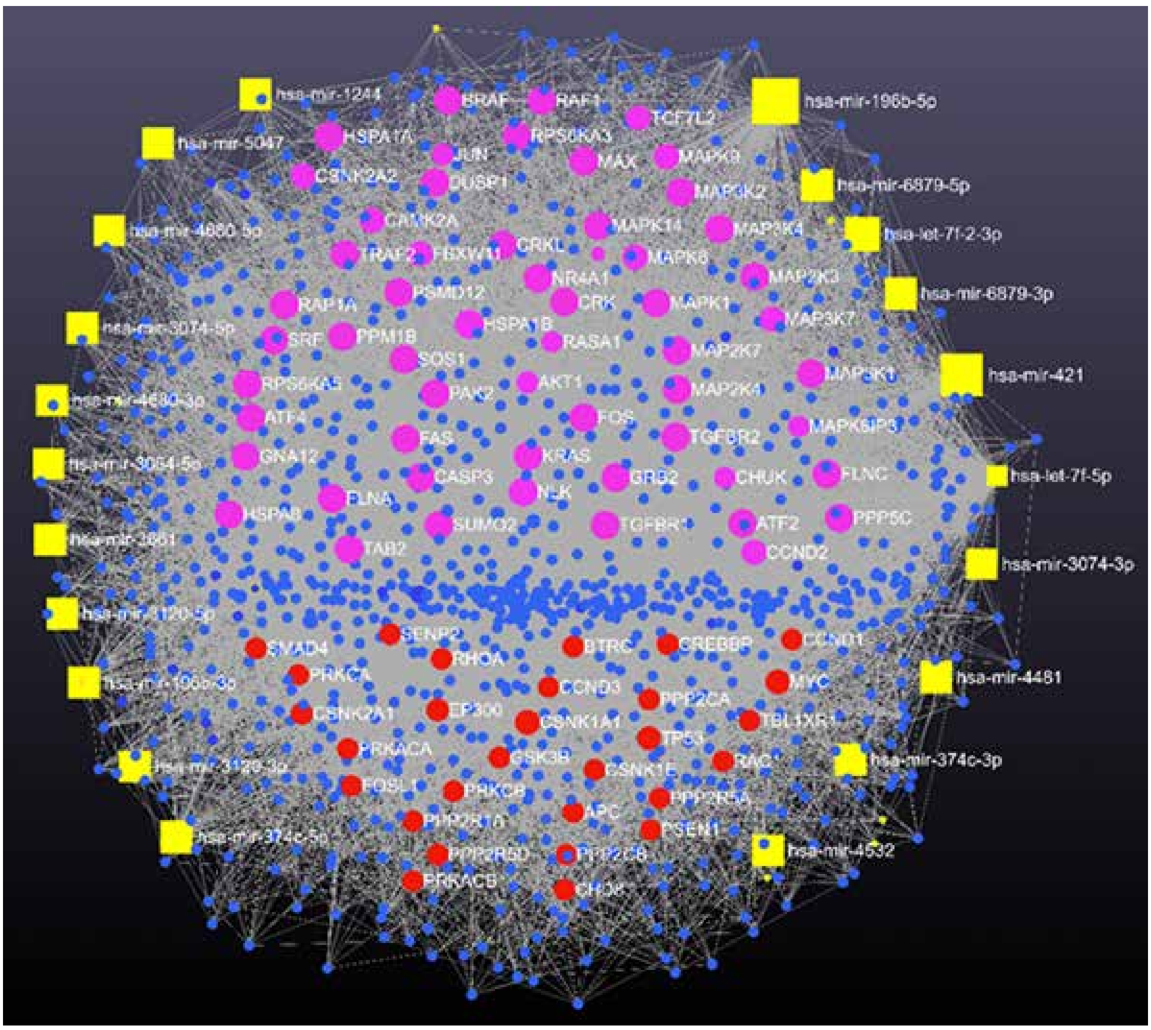

Fig. 1: *miRNA-mRNA gene networks constructed using the miRNet. Genes related to MAPK (pink) and Wnt (red) signaling pathways formed extensive networks. miRNAs are shown in yellow squares.*

Gene ontology analysis of predicted genes

Predicted genes were involved in various biological processes (BP), such as regulation of transcription from RNA polymerase II promoter, anterior/posterior pattern specification, extracellular matrix organization, stem cell differentiation, and cellular response to mechanical stimulus. Meanwhile, nucleus, cytosol, and nucleoplasm were the main GO terms in the cellular component (CC) category. The primary molecular functions (MF) were RNA polymerase II core promoter proximal region sequence-specific DNA binding, ATP binding, zinc ion binding, DNA binding etc (Fig. 2).

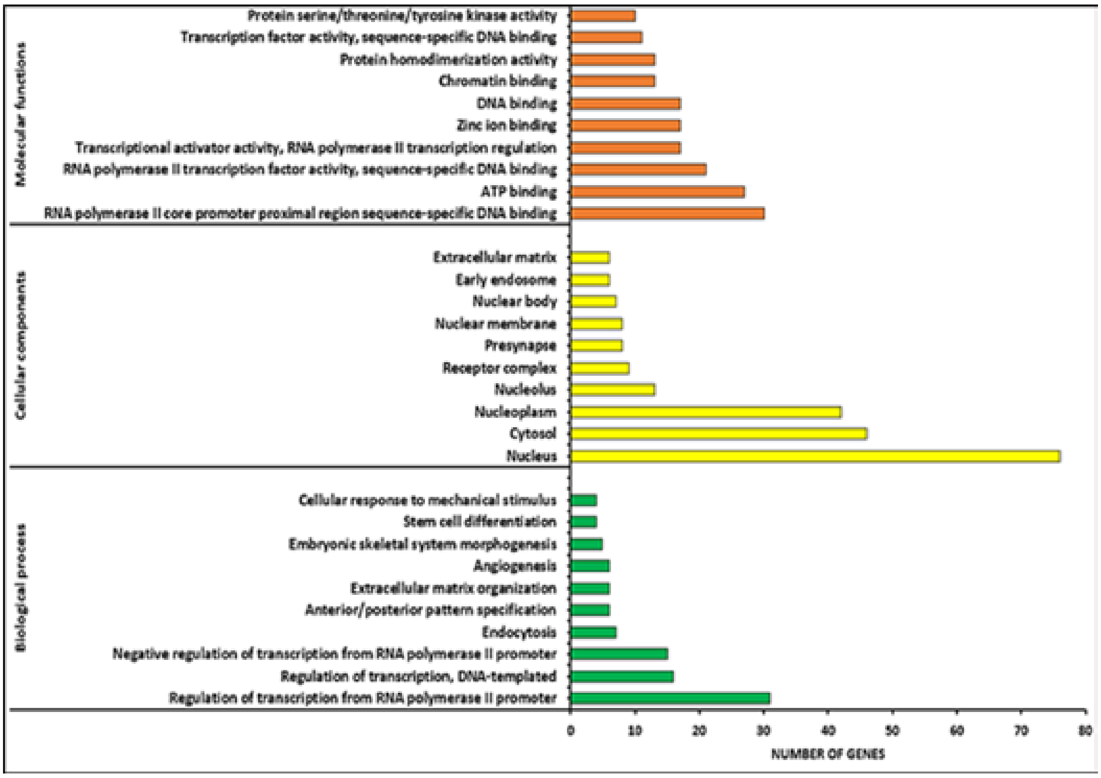

Fig. 2: *Functional classification of the predicted genes in Changthangi sheep*

Pathway analysis for the predicted genes

Based on KEGG pathway enrichment analysis, the predicted genes in Changthangi sheep were associated with several pathways including MAPK signaling pathway, PI3K-Akt signaling pathway, Rassignaling pathway, oxytocin signaling pathway etc (Fig. 3).

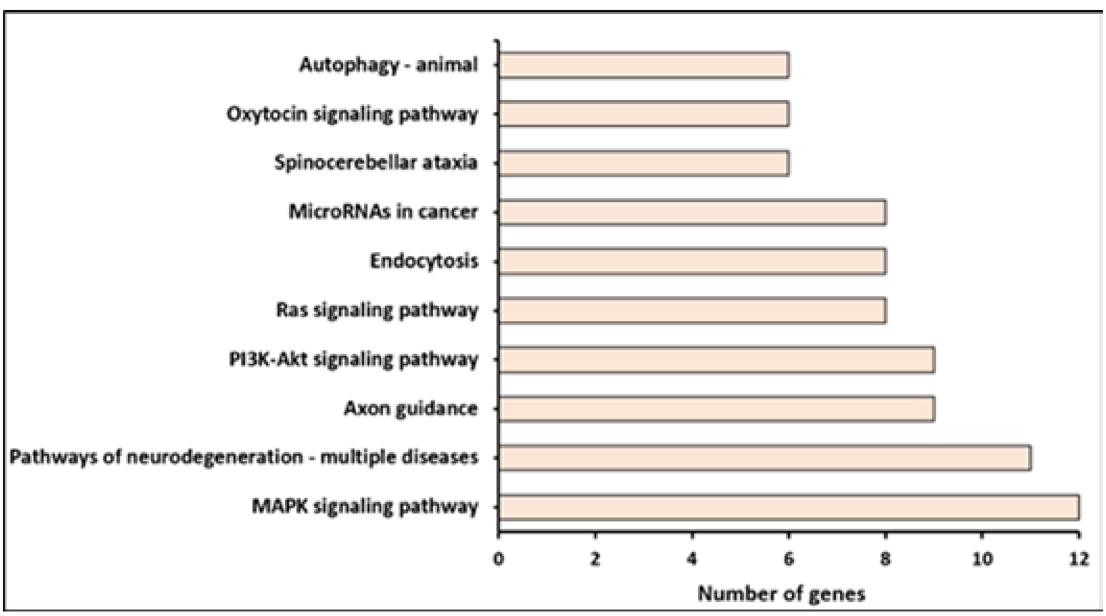

Fig. 3: *KEGG pathway enrichment analysis for predicted genes in Changthangi sheep*

Genes were majorly related to MAPK signaling pathway including *MAPK8, MAP3K1, MAP4K3, MAP3K21, NRAS, FAS, CACNB4, RET, PDGFRA, GDNF, CACNA2D1*, and *RASGRP1*. These play a crucial role in transmitting signals within the cell and also regulating cellular responses through the MAPK signalling cascade. Genes corresponding to PI3K-Akt signaling pathway were *RET, PDGFRA, NRAS, CDKN1B, COL1A2, IBSP, GDNF, OSMR*, and *PRLR* which play a crucial role in the *de novo* hair follicle regeneration. Genes associated Ras signaling pathway in the predicted dataset included *BRAP, PDGFRA, NRAS, MAPK8, ABL1, PLD1, RGL2,* and *RASGRP1* which regulate the cellular proliferation and differentiation, and hair follicle morphogenesis. Genes such as *NRAS, EEF2K, CACNB4, CACNA2D1, PPP1R12B*, and *KCNJ2* are related to oxytocin signaling pathway and play key roles in cell growth, differentiation, sodium excretion, and stress responses. *NRAS* gene was identified to be involved in MAPK, Ras, and PI3k pathways (Fig. 4).

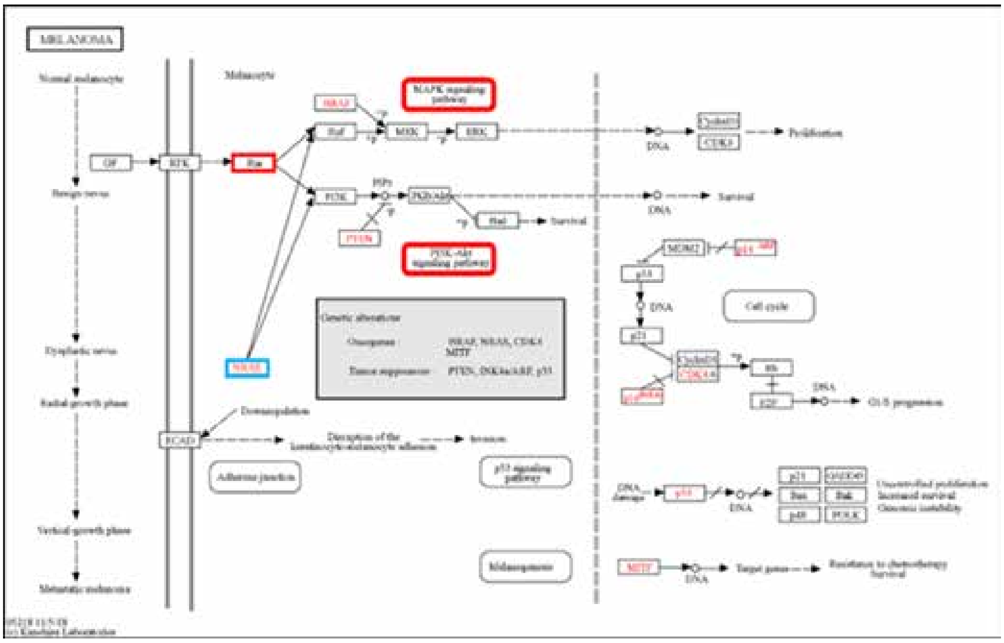

Fig. 4: *Pathways with red boundaries interacting with NRSA gene (blue boundary) in Changthangi sheep*

Moreover, CPDB generated a network graphic by manually selecting pathways of interest, and organizing nodes to effectively depict pathway interconnectivity data. Over-representation analysis was conducted, incorporating genes linked to mesodermal commitment pathways, MAPK signaling pathway, Rassignaling pathway, axon guidance, focal adhesion, PI3K/ Aktsignaling pathway, nervous system development, JAK-STAT pathway, keratinocyte differentiation, and VEGF signaling pathway (Fig.5).

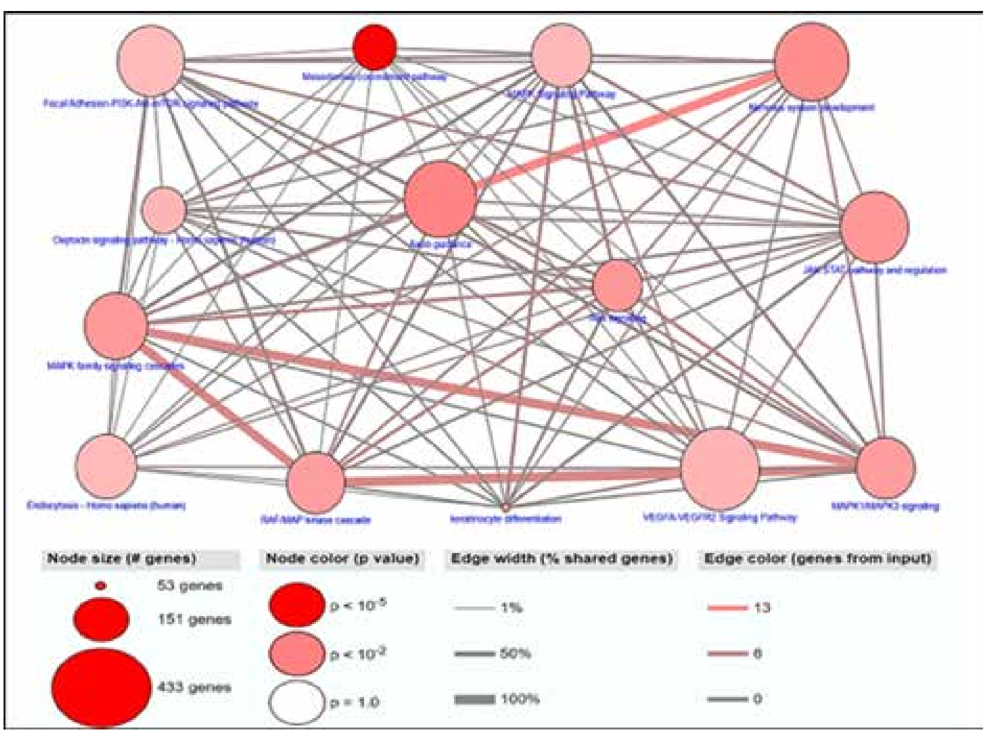

Fig. 5: *Network graphic of pathways created by CPDB*

Network construction

The protein-protein interactions within the predicted dataset of the Changthangi sheep were visualized utilizing the STRING database (Fig. 6). A network of collagen genes, such as *COL1A2, COL3A1*, and *COL15A1*, exhibited interconnected relationships, suggesting their potential involvement in the regulation of fiber production during development. Furthermore, conspicuous interactions associated with the MAPK signaling pathway (MAP3K1, MAP4K3 and MAPK8) were evident in the interaction network. Notably, interactions between MAPK proteins and NRAS were evident in the protein-protein network. Additionally, NRAS connection with the BRAP protein, an active participant in MAP kinase/ERK signaling pathway and subsequently associated with ribosomal proteins was evident. Through this analysis, potential interactions among HOX proteins specifically, HOXA5, HOXA7, HOXA9, HOXB6, HOXB7, HOXC8, PBX3 and PBX1 were observed.

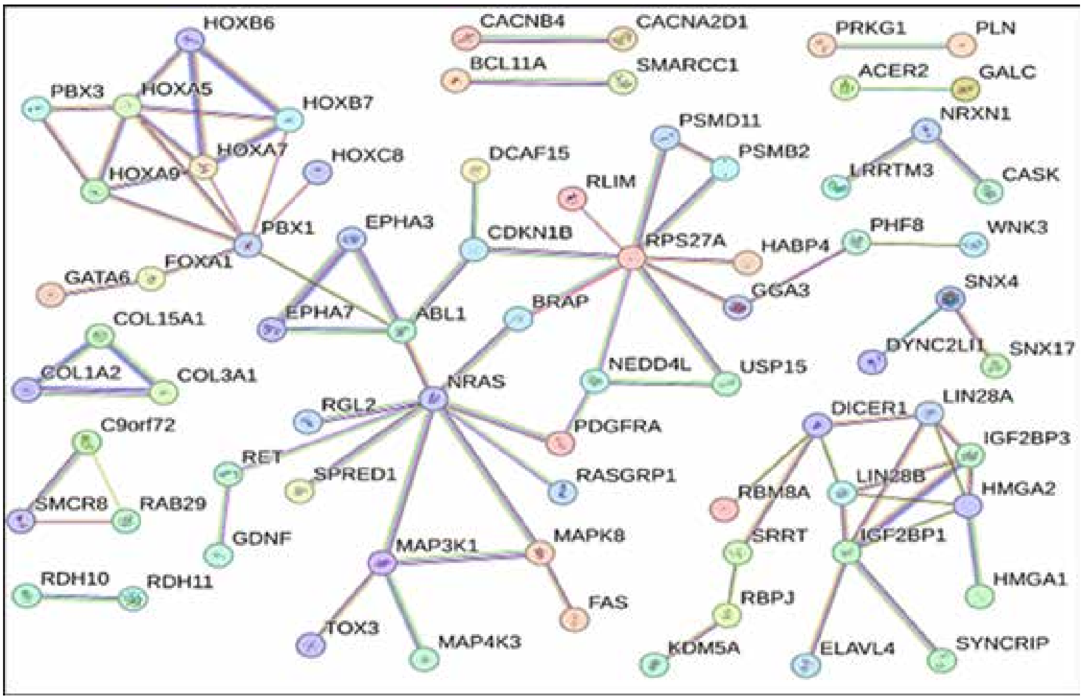

Fig. 6: *Protein-protein interactions of predicted genes in Changthangi sheep determined by STRING (https://string-db.org)*

Advanced high-throughput sequencing was utilized to explore the role of miRNAs in Changthangi sheep skin follicles. Firstly, various non-coding RNAs were identified including circRNAs, miRNAs, piRNAs, snRNAs, snoRNAs, and tRNAs. Subsequently, the specific role of microRNAs was deciphered so as to uncover the molecular drivers and pathways that might contribute to fiber quality and resulting adaptation to the inimitable agroecological conditions. The majority of the miRNA interactions were associated with key pathways like MAPK signaling and Wnt signaling, playing a vital role in the growth of hair follicles. Hsa-miR-3074-5p demonstrated the most significant level of interaction, whereas hsa-let-7f-5p exhibited the lowest interaction. Notably, a single miRNA can target numerous gene targets and influence hundreds of mRNAs with complementary binding sites. These targeted genes may constitute essential components of the same biological pathways.

The predominant pathways in Changthangi sheep were highlighted in KEGG pathway analysis (Fig. 3). These included MAPK signaling pathway, PI3K-Akt signaling pathway, Ras signaling pathway, endocytosis, oxytocin signaling pathways which play a crucial role in regulating cellular responses (Wang *et al.,* 2022), *de novo* hair follicle regeneration (Chen *et al.,* 2020; Ahlawat *et al.,* 2020), cellular proliferation, differentiation, hair follicle morphogenesis (Ahlawat *et al.,* 2024), stress responses (Kageyama *et al.,* 2023) and crucial for ensuring cell survival under hypoxic conditions (Dada *et al.,* 2003).

The complex mechanisms within skin follicles regulated by a multitude of proteins are prerequisites to the production of wool (Galbraith, 2010).In our investigation, the NRAS gene was notably identified as a key participant in the prominent MAPK, Ras, and PI3K signaling pathways (Fig. 4). The MAPK and PI3K-Akt pathways play crucial roles in melanogenesis, as they are consistently activated, contributing to the formation of melanocytes. Several of these genes regulate the formation of mammalian coat color by influencing the activity of melanocytes. While the NRAS (neuroblastoma RAS viral oncogene homolog) gene plays a pivotal role in melanoma research, its mechanistic involvement in the development of mammalian epidermal color remains unclear. NRAS gene is a member of the Ras gene family, and hydrolyses guanosine triphosphate (GTP) to guanosine diphosphate (Eisfeld *et al.,* 2014). Various cellular functions such as proliferation, differentiation, protein transport, and secretion are influenced by the active state of the Ras protein (Fernandez-Medarde and Santos,2011). The Ras protein encoded by the NRAS gene is actively engaged in the MAPK signaling pathway and plays a crucial role in transmitting signals within the cell, and is involved in regulating cellular responses (Hodis *et al.,* 2012). Additionally, phosphatidylinositol 3-kinase (PI3k) stands as a key effector pathway of RAS, regulation of cell growth, induction of the cell cycle, maintaining cell viability, organizing the cytoskeleton, and metabolism. Similar to the present findings, NRAS was identified to control melanocyte proliferation and apoptosis in a study designed to investigate mammalian fur color formation in rabbits (Bai *et al.,* 2022).

The network of collagen genes, including COL1A2, COL3A1, and COL15A1, displayed interconnected relationships, indicating their likely participation in regulating hair follicle development. Collagen gene family functioning in structural capacities, contribute to the mechanical characteristics, organization, and shape of tissues. These proteins exert an essential influence on tissue dynamics and functionality through their interactions with cells via various receptor families (Ricard-Blum, 2011). Consistent with our findings, COL1A2 and COL3A1 were observed to be associated with tissue remodelling and repair, detected by multiple probes showing similar expression trends across various stages of the cashmere growth cycle (Jiang *et al.,* 2014). Analysis of the skin transcriptome in short-haired and long-haired rabbits revealed variations in the expression of COL1A2 and COL3A1 genes (Ding et al., 2019). The substantial expression alterations observed in the COL1A2 gene across various developmental stages of hair follicles contributed to an enhanced comprehension of the internal mechanisms governing the growth and development of hair follicles in cashmere goats (Wang *et al.,* 2021). COL15A1 gene was characterized in hair follicles of coarse and fine wool of the Wan strain of Angora rabbits (Huang *et al.,* 2023).

Noticeable interactions were also identified between the genes of the MAPK signaling pathway (MAP3K1, MAP4K3 and MAPK8). Mitogen-activated protein kinase (MAPK) families play a crucial role in intricate cellular processes, including proliferation, differentiation, development, transformation, and apoptosis. Their involvement underscores their significance in orchestrating diverse cellular programs critical for the proper functioning and regulation of biological systems (Zhang and Liu, 2002). Notably, an interaction between MAPK and the NRAS gene was evident in the protein-protein network. Additionally, NRAS is connected with the BRAP protein, an active participant in the MAP kinase/ERK signaling pathway, and hence associated with ribosomal proteins. Hair follicle stem cell proliferation is regulated by MAP3K1 through the modulation of cell apoptosis and the ratio of cells in the S phase/G2 phase (Wang *et al.,* 2023). MAPK8 participates in the MAPK signaling pathway, exhibiting specific regulatory functions in the hair follicles of lamb skin (Lv *et al.,* 2016). Potential interactions among HOX proteins specifically, HOXA5, HOXA7, HOXA9, HOXB6, HOXB7, HOXC8, PBX3 and PBX1 genes were identified. HOX genes, members of a transcription factor family, are critical in specifying cell fate and identity in embryogenesis. Additionally, they regulate the regional characteristics of diverse tissues, contributing significantly to developmental processes (Jave-Suarez *et al.,* 2002; Vasu *et al.,* 2024). The role of HOX genes in skin development has not been extensively studied, but their importance in skin patterning has been established. This is evident from their expression in feathers in chickens and dermal papillae of hair follicles in mice (Yu *et al.,* 2018). Our findings corroborate earlier studies that have reported the identification of HOXA7 and HOXB7 expression in the suprabasal layer of normal adult human skin (Thomas *et al.,* 1989). HOXA5, HOXA7, and HOXB7 exhibit spatial and temporal variations in expression throughout human skin development (Stelnicki *et al.,* 1998), while HOXB6 is involved in epidermal development (Mathews *et al.,* 1993). Expression of HOXA7 and HOXC8 has been identified in the chick epidermis, although with distinct anterior-posterior restrictions (Reid and Gaunt, 2002). During keratinocyte proliferation, HOXA7 possibly in collaboration with HOXA5 suppresses differentiation-specific genes, which are subsequently liberated from inhibition in response to differentiation signals (La Celle and Polakowska, 2001). In addition, PBX1 stimulates the proliferation of mesenchymal stem cells derived from hair follicles, reduces cellular senescence and apoptosis, and augments the generation of induced pluripotent stem cells (Wang *et al.,* 2021). Given these observations, the identification of HOX genes in the skin of Changthangi sheep in our study presents an intriguing avenue for future research.

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