RESEASRCH ARTICLE

Gene Regulatory Pathways Associated with Maintenance of Acrosomal Integrity in Bovine Sperm

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Abstract

The objective of this study was to identify the sperm-expressed genes and their pathways in maintaining acrosomal integrity for elucidating the regulatory genes involved in determining bull fertility. Semen samples collected from Holstein-Friesian bulls (n=12) were analyzed for acrosomal integrity and were grouped into high acrosomal integrity (n=6) and low acrosomal integrity (n=6). In these sperm samples, total RNA was isolated and subjected to whole transcriptome profiling using Illumina platform. Bioinformatic analyses led to the identification of 1601 up-regulated *(TMCO2, GTSF1, SWI5, CCDC174* and *CABS1*) and 567 down-regulated *(MYCB, RPS21* and *PPIA*) genes. The study also validated the expression levels of *CCDC174* and *TMCO2* using real-time PCR. The pathways [$\log_2$ (*p*-value)] such as spermatid development (12.17), protein transport (9.57) and mitochondrial electron transport (10.20) were enriched in the up-regulated genes. These identified pathways and their associated transcripts in high acrosome integrity group suggest that these pathways are essential for maintaining acrosome integrity and sperm fertility in bulls.

Key words: Acrosome integrity, Acrosome pathways, Acrosome related transcripts, Bull fertility, *CCDC174*.

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Introduction

Productive efficiency of the dairy cows is determined by the reproductive performance. However, low conception rate especially after artificial insemination affects economy of the dairy farms. Though cow's and bull's fertility are of paramount importance for improving conception rate in the dairy farms, selection of fertile bulls assumes greater significance as single bull produces thousands of insemination doses during its life-time. Bulls are selected for breeding based on the breeding soundness evaluation, which comprises of phenotypic and semen functional analysis, mainly sperm motility and membrane integrity. Earlier studies from this laboratory documented that acrosomal integrity and functional membrane integrity influence the bull fertility rate (Selvaraju *et al.,* 2021a).

Quality of the sperm and their fertility status are influenced by many factors, including genetics, nutrition, environment, etc. Defects in spermiogenesis lead to abnormal sperm morphologies including acrosomal defects. A significant decrease in acrosomal integrity has also been observed in teratozoospermia, asthenozoospermia and oligoasthenospermia (Sreenivasa *et al.,* 2012). Cryopreservation, routinely employed to preserve bovine sperm also impact acrosome integrity. Since acrosomal integrity is essential for the sperm to penetrate through the cumulus cells and zona pellucida, loss in acrosomal integrity leads to fertilization failure. So far, no study has been conducted in cattle to establish the genes regulating acrosomal biogenesis and acrosomal membrane integrity. Understanding the regulatory genes and their associated pathways involved in acrosomal quality may help to design the strategies for improving sperm quality and conception rate. Thus, the present study was aimed to elucidate genes regulating acrosomal integrity in bulls based on whole transcriptome profiling of sperm RNA.

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Materials and Methods

Semen Sample Procurement

Fresh semen samples of Holstein Friesian bulls (n=12) were procured from Nandini sperm station, Karnataka Milk

Federation, Hessarghatta, Bengaluru, Karnataka. A minimum of three ejaculates were collected from each bull using an artificial vagina. The collected sample was divided into two aliquots. An aliquot of semen sample (0.3 mL) was centrifuged at 200 g for 5 min at 37°C. The supernatant was discarded and the sperm pellet was resuspended in 1X phosphate buffered saline (PBS). The semen samples were then immediately snap frozen in liquid nitrogen. The second aliquot was used for the evaluation of acrosome integrity.

Sperm Acrosomal Integrity and Sperm RNA Isolation

The acrosome integrity (%) of the semen samples was assessed using Giemsa staining (Selvaraju *et al.,* 2008). The percentage of acrosome intactness was calculated relative to the total sperm counted. The bulls were classified into two (high and low) groups based on acrosome integrity.

Sperm RNA isolation was carried out using a double-lysis method followed by silica membrane-based extraction (Parthipan *et al.,* 2015). Briefly, 30-40 million sperm were lysed using Trizol and RLT lysis buffer, followed by the PureLink RNA mini kit (Invitrogen, USA) extraction according to the manufacturer's instructions. The RNA obtained was quantified using Qubit (Invitrogen, USA) fluorometer and the quality was assessed using Nanodrop (Thermo Scientific, USA). The RNA samples were subjected to DNase treatment using a DNA-free kit (Ambion Life Technologies, USA) to remove the gDNA. Further, the samples were checked for contamination from germ cells, epithelial cells, immune cell, gDNA using cell-specific primers of genes *KIT*, *CDH1*, *PTPRC* and *PRM1*, respectively (Table 1). Samples devoid of contamination were taken for further analysis.

Differentially Expressed Transcripts

In order to identify the regulatory genes involved in acrosomal integrity, the data generated as a part of previous studies from the Reproductive Physiology Lab, ICAR-NIANP, Bangalore (Selvaraju *et al.,* 2021a) were used. Briefly, the RNA-Seq libraries were prepared using SMARTer Ultra-low input kit for Illumina sequencing-HV (CloneTech Laboratories, USA) and subjected to 2X 75 paired end sequencing. Approximately, 20 million reads per sample were generated using the Illumina NextSeq 500 (Illumina, USA). The read quality was checked using FastQC, trimmed using Cutadapt (version 2.3), aligned to bovine genome (Ensembl UMD 3.1) using STAR alignment (version 2.6.0a). The differentially expressed genes (DEGs) were identified using NOISeq (version 2.18.0) (Table 2). The up-regulated genes in the high acrosomal integrity group were taken for downstream analysis.

Gene Enrichment and Pathway Analysis

The up-regulated genes from the high acrosome integrity group were subjected to enrichment analysis using Database for Annotation, Visualization, and Integrated Discovery (DAVID). The significantly enriched ($p<0.05$) biological processes, cellular components, molecular functions, and pathways were obtained from the DAVID.

RT-qPCR Validation

cDNA synthesis was carried out using SuperScript IV first strand synthesis system with a mixture of random hexamers and oligodT (Invitrogen, USA). The expression levels of transcripts were studied in real-time PCR (Step One Plus, Applied Biosystems, USA). Gene expression analyses were calculated for the transcripts *TMCO2* and *CCDC174* which were randomly chosen from the up-regulated transcripts. Each 10 µL reaction consists of the SYBR Green Master mix (TB Green Premix Ex Taq II, Takara Bio, Japan), 125 nM each of forward and reverse primers and 1.2 ng of cDNA. The PCR cycle conditions were 95°C for 30s, 40 cycles of 95°C for 5s and 60°C for 1 min followed by the default melt curve settings. The specificity of each product was checked using melt curve in q-PCR software as well as using agarose gel electrophoresis. *RPL23* was used as a housekeeping gene

Table 1: List of primers used for the RNA quality control and RT-qPCR validation

Primer ID	Primer	Primer sequence (5' to 3')	Primer length (bp)	Product size (bp)	NCBI Accession No
PRM1	Forward	AAGATGTCGCAGACGAAGGAG	21	222	NM_174156.2
	Reverse	GTGGCATTGTTCGTTAGCAGG	21		
KIT	Forward	GAATAGCTGGCATCAGGGTG	20	224	AF263827.1
	Reverse	CCAGATCCACATTCTCTCCATC	22		
CDH1	Forward	CTGCATTCCTGGCTTTGGTG	20	171	NM_001002763.1
	Reverse	GTAAGCACGCCATCTGTGTG	20		
PTPRC	Forward	TGGACGAAATTGCATCCCTCAGGA	24	237	NM_174156.2
	Reverse	RTGGTCAGGACGTTTACAGCTCACA	24		
RPL23	Forward	CAGCGGTGGTAATTCGACAAC	21	116	NM_001035014.2
	Reverse	GGCGGAACCTTTCATCTCG	19		
TMCO2	Forward	ACTACTGCAGCTGCTCTGACTG	22	122	NM_001077077.2
	Reverse	CCACCGCACACACAAGGAAA	20		
CCDC174	Forward	GGGTACTGGTCGAAGAGGCA	20	112	NM_001075578.1
	Reverse	CCTGGCTGCTCAGAGACTCA	20		

(Parthipan *et al.,* 2017). The expression levels of the genes were calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

All the sperm function data were normally distributed and hence the student's t-test was used for calculating the significance. All the values were represented as mean ± SEM and the significance was set at $p < 0.05$.

Animal Ethics

All the experiments were conducted as per the approval of the Institute Animal Ethics Committee (IAEC approval, vide: NIANP/IAEC/1/2020/12). All the methods were performed in accordance with the relevant guidelines and regulations.

Results and Discussion

Grouping of Bulls based on Sperm Acrosome Integrity

The high acrosome integrity group had significantly higher percentages of sperm head with intact acrosome (88.87±1.4 Vs. 78.99 ± 2.15) than the low acrosome integrity group (Fig. 1).

Differentially Expressed Transcripts

The number of significantly ($q < 0.05$) differentially expressed transcripts were 2659 in the acrosome integrity group (Fig. 2). The top 5 up-regulated transcripts (fold change, p-sig) in the high acrosome integrity group were *GAPBPB2* (8.16, 1.65E-05), *LIN54* (8.07, 0.00E+00), *CCDC174* (7.85, 0.00E+00), *PTP4A2* (7.41, 1.242E-02) and *RPL27A* (7.37, 3.99E-02). LIN family of genes are regulators of cell cycle genes and LIN54 is a paralog of TESMIN. TESMIN (Testis expressed Metallothionein like protein) is involved in the cell growth and regulation during spermatogenesis (Sugihara *et al.,* 1999). Coiled-coil (CCDC) family of proteins are involved in gametogenesis events like nuclear condensation, acrosome biogenesis, etc. and embryo function (Priyanka and Yenugu, 2021). Earlier study from this laboratory also revealed higher expression levels of *CCDC174* associated with good quality semen and high fertile bulls (Selvaraju *et al.,* 2021b). Decreased expression of *RPL27A* when exposed to infrared radiation, triggers apoptosis of sperm and hence, the higher expression of *RPL27A* in high acrosome intact sperm suggests that it might act as an anti-apoptosis factor (Li *et al.,* 2020).

Table 2: Differentially expressed genes in the acrosome integrity group and their functions

S.No	Gene Name	Fold- Change	p-sig	Function
				Up-regulated transcripts
1	*GABPB2*	8.16	1.65E-05	Positive regulation of transcription by RNA polymerase II [GO:0045944]
2	*LIN54*	8.07	0.00E+00	Regulation of transcription, DNA-templated [GO:0006355]
3	*CCDC174*	7.85	0.00E+00	mRNA export from nucleus [GO:0006406]
4	*PTP4A2*	7.41	1.24E-02	Regeneration [GO: 0031099]
5	*RPL27A*	7.37	3.99E-02	Translation [GO:0006412]
6	*KDM3B*	7.31	2.88E-02	Histone H3-K9 demethylation [GO:0033169]
7	*ADGRL3*	7.16	0.00E+00	Cell surface receptor signaling pathway [GO:0007166]
8	*TMEM67*	7.05	0.00E+00	Ubiquitin-dependent ERAD pathway [GO:0030433]
9	*ZNF106*	6.87	1.27E-02	Insulin receptor signaling pathway [GO:0008286]
10	*TRIP12*	6.70	0.00E+00	Ubiquitin-dependent protein catabolic process [GO:0006511]
				Down-regulated transcripts
1	*MYCBP*	-5.32	2.93E-02	Regulation of transcription, DNA-templated [GO:0006355]
2	*VDAC3*	-5.25	4.27E-02	Regulation of cilium assembly [GO:1902017]
3	*RPS21*	-5.01	3.24E-02	Endonucleolytic cleavage in ITS1 to separate SSU-rRNA from 5.8S rRNA and LSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA) [GO:0000447]
4	*PPIA*	-4.96	4.18E-02	Protein refolding [GO:0042026]
5	*NDUFB5*	-4.94	2.84E-02	Mitochondrial respiratory chain complex I assembly [GO:0032981]
6	*RPL38*	-4.84	3.99E-02	Translation [GO:0006412]
7	*ARMC12*	-4.78	3.98E-02	Regulation of mRNA splicing, via spliceosome [GO:0048024]
8	*SCGB1D*	-4.60	1.12E-02	Sterol metabolic process [GO:0016125]
9	*SLIRP*	-4.33	4.12E-02	Germ cell development [GO:0007281]
10	*HBE1*	-4.20	1.87E-05	Hydrogen peroxide catabolic process [GO:0042744]

The genes *GABPB2, LIN54* and *CCDC174* were up-regulated and *MYCBP, VDAC3* and *RPS21* were down-regulated in the high acrosome integrity group.

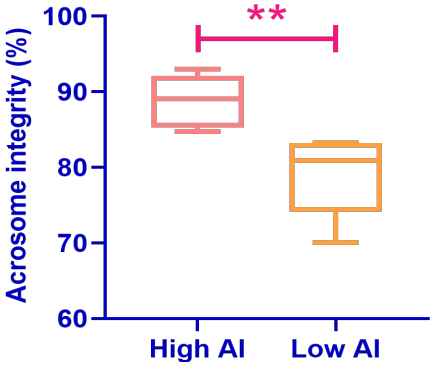

Fig. 1: Grouping of bulls based on acrosome integrity. Bulls were grouped into two groups based on the acrosome integrity and the two groups differ significantly (p<0.01). ** denotes p value less than 0.01

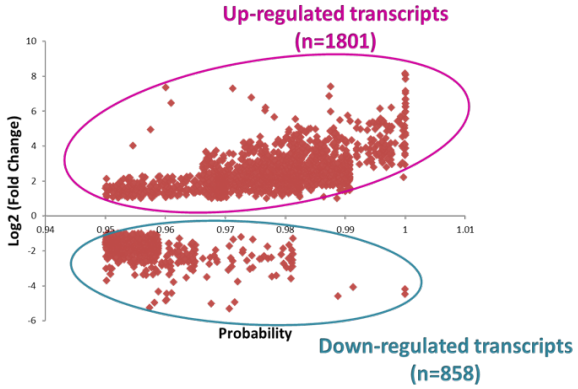

Fig. 2: Differentially expressed transcripts in the acrosome integrity group. There were 1801 significantly up-regulated and 858 significantly down-regulated transcripts.

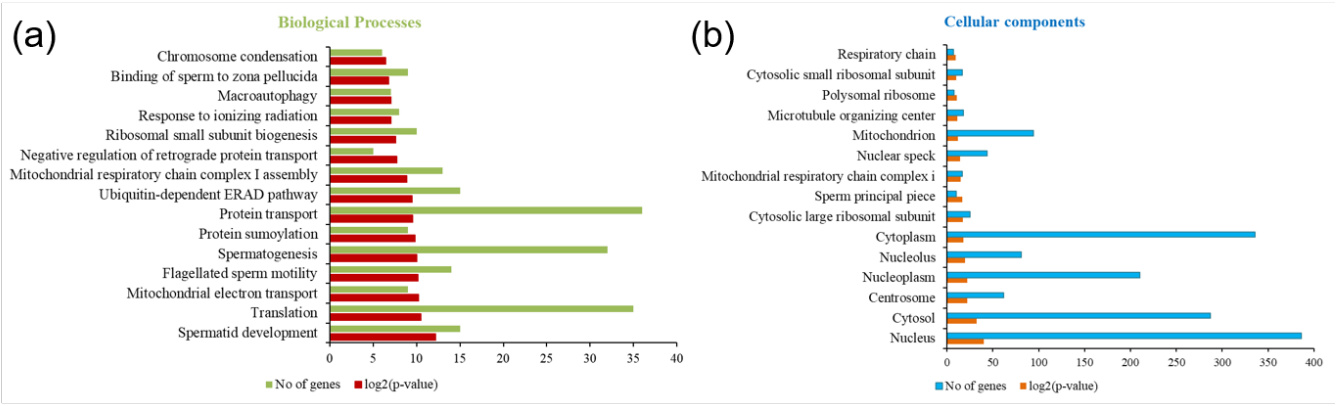

Fig. 3: Enrichment analysis of the up-regulated transcripts in high acrosome integrity sperm group. The gene ontology [-$\log_2$(*p*-value)] analysis revealed that (a) Biological process - spermatid development (12.17), (b) Cellular component – Nucleus (40.24) and (c) Molecular function – structural constituent of ribosome (25.77) were the top enriched ontologies in their respective categories.

In the same group, the top 5 down-regulated transcripts (fold change, p-sig) were *MYCBP* (-5.32, 2.93E-02), *VDAC3* (-5.25, 4.27E-02), *RPS21* (-5.01, 3.24E-02), *PPIA* (-4.96, 4.18E-02) and *NDUFB5* (-4.96, 2.84E-02). *MYCBP* is a c-myc binding protein and is specifically expressed during spermatogenesis (Yukitake *et al*., 2002). c-myc binding protein may control the transcriptional activities of c-myc. In addition, c-myc is involved in capacitation and acrosomal reaction (McReynolds *et al*., 2014). Hence downregulation of MYCBP may affect the c-myc activity during spermatogenesis and affect gamete function. Similarly, voltage dependent anion channels (*VDCA3*) is an indicator of reactive oxygen species (ROS) levels in the cells. *VDCA3* levels were increased when the ROS levels increases (Reina *et al*., 2016). Hence, these transcripts were downregulated in the high acrosome integrity group to counteract the effects of ROS.

Functional Enrichment of Up-Regulated Transcripts

Functional enrichment analysis of the up-regulated transcripts revealed that the biological processes

[-$\log_2$ (*p*-value)] such as, spermatid development (12.17), protein transport (9.57), mitochondrial electron transport (10.20), protein sumoylation (9.83) and binding of sperm to zona pellucida (6.74) were enriched in the acrosomal integrity group (Fig. 3a). One of the major steps in acrosome biogenesis is the trafficking of proteins to the newly forming acrosome (Teves *et al.,* 2020). Enrichment of the protein transport and protein sumoylation processes suggests that high acrosome intact bulls had an organized transport system. Such an orchestrated protein transport is required for the trafficking of proteins such as ZPBP1, which is involved in fertilization (Lin *et al.,* 2007).

Cytosol (32.11), centrosome (22.40), microtubule organizing center (11.33), cytosolic large ribosomal subunit (17.38) and sperm principal piece (16.45) were the enriched cellular components [-$\log_2$ (*p*-value)] of the up-regulated transcripts of acrosome integrity group (Fig. 3b).Whereas the enriched molecular functions [-$\log_2$ (*p*-value)] of the up-regulated transcripts were a structural constituent of ribosome (25.77) and associated with metal ion binding (8.89), ubiquitin protein ligase binding (8.32), ubiquitin protein transferase activity (7.31) and unfolded protein binding (6.96) (Fig. 3c).

Pathway Enrichment of Up-Regulated Transcripts

The enriched pathways [-$\log_2$ (*p*-value)] of the up-regulated transcripts were SRP dependent co-translational protein targeting to membrane (14.83), translation (13.54), non-sense mediated decay (12.58), eukaryotic translation initiation (12.27) and metabolism of proteins (5.31) (Fig. 4). The transcripts associated with high acrosome integrity group pathways were ribosomal proteins - RPS7, RPS24, etc., signal recognition particles - SRP54, SRP14 and UBB. The top enriched pathway was the transport of transcripts to the membrane, which is similar to the findings of the enriched biological processes.

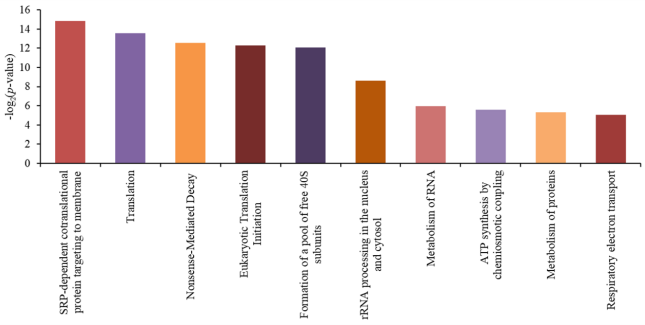

Fig. 4: Pathway enrichment analysis of up-regulated transcripts in high acrosome integrity sperm group. Pathway enrichment analysis carried out using DAVID tool. Ribosome, oxidative phosphorylation, thermogenesis, and reactive oxygen species were the enriched pathways of the up-regulated transcripts.

Validation of Up-Regulated Transcripts

The transcripts *TMCO2* (Fold change - 1.16) and *CCDC174* (Fold change -5.88) were up-regulated in the high acrosome integrity group (Fig. 5). The qPCR results showed a similar trend as that of RNA-seq findings. Importantly, the coiled-coil domain containing transcript (*CCDC42*) was present in the sperm manchette and associated with the infertility of mice (Tapia Contreras and Hoyer-Fender, 2019). Similarly, transmembrane, and coiled-coil family of the gene (*TMCO5*) were involved in the vesicle transport across the manchette (Yamase *et al.,* 2019). In earlier studies also we reported that *CCDC174* can be a potential marker of bull fertility (Selvaraju *et al.,* 2021b).

Though the direct evidence on the functions of *CCDC174* and *TMCO2* were not known, from the transcripts of coiled-coil domain families, we hypothesize that both *CCDC174* and *TMCO2* might be involved in the transport of RNA or proteins through the manchette to the acrosome. Sperm manchette serves as an essential structure for the transport of proteins during acrosome biogenesis. So, it can be inferred that any compromise in the manchette organization creates defects in the acrosome. Hence, there was a higher expression of manchette associated transcripts in the high acrosome integrity group.

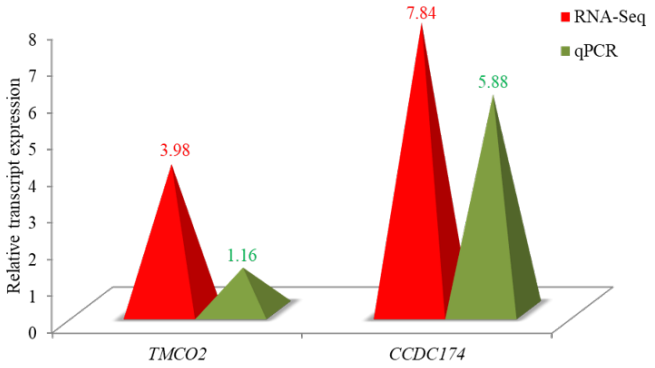

Fig. 5: qPCR validation of up-regulated transcripts in high acrosome integrity sperm group. The transcripts TMCO2 (1.16) and CCDC174 (5.88) were up-regulated in the high-acrosome integrity group. The fold-change values were in trend with the RNA-seq.

Conclusion

The present study suggests that, SRP dependent co-translational protein targeting to membrane (14.83), translation (13.54) and eukaryotic translation initiation (12.27) were significantly enriched pathways in the high acrosome integrity group. Further, modulation of expression levels of these genes might improve the quality of the semen from bulls having abnormal sperm such as globozoospermia and for maintaining acrosome integrity during preservation.

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