

EVALUATION OF THE TRANSCRIPTOMIC PROFILES OF CUMULUS CELLS ASSOCIATED WITH EUPLOID VERSUS WHOLE CHROMOSOME 21 ANEUPLOID EMBRYOS REVEALS DIFFERENTIALLY EXPRESSED GENES

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**EVALUATION OF THE TRANSCRIPTOMIC PROFILES OF CUMULUS CELLS
ASSOCIATED WITH EUPLOID VERSUS WHOLE CHROMOSOME 21 ANEUPLOID
EMBRYOS REVEALS DIFFERENTIALLY EXPRESSED GENES**

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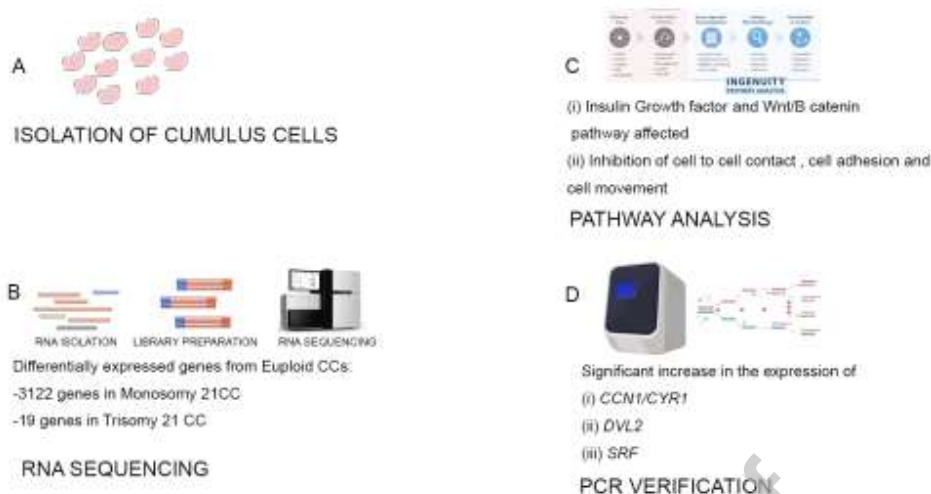
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Graphical Abstract



ABSTRACT

Research Question: Can cumulus cells be employed as a non-invasive target for the study of determinants of preimplantation embryo quality?

Design: Cumulus cells were collected from Monosomy 21, Trisomy 21 and Euploid embryos and subjected to RNA sequencing analysis and Real Time PCR assays. The differential gene expression was analyzed for different comparisons.

Result(s): A total of 3122 genes in Monosomy 21 CCs, and 19 genes in Trisomy 21 CCs were differentially expressed compared to euploid CCs. Thirteen of these genes were differentially expressed in both Monosomy and Trisomy 21, compared to euploid, including disheveled segment polarity protein 2 (*DVL2*), cellular communication network factor 1 (*CCN1/CYR61*) and serum response factor (*SRF*), which have been previously implicated in embryo developmental competence. In addition, IPA analysis revealed cell-cell contact function to be affected in both Monosomy and Trisomy 21 CCs.

Conclusions: These findings support the use of CC gene expression analysis for the development of biomarkers evaluating oocyte quality for patients undergoing fertility preservation of oocytes.

Key Words: cumulus cells; transcriptome; euploid; aneuploid; human embryos

INTRODUCTION

In assisted reproduction, the low proportion of retrieved oocytes yielding a live birth is demonstrative of the inefficiency of human reproduction (Fragouli et al., 2012). Investigations into factors impacting oocyte quality have been pursued to elucidate underlying mechanisms of this inefficiency. A target of these studies is the pre-ovulatory follicular environment, which has been shown to be critical in the development and condition of its corresponding oocyte (Ebner et al., 2006). Cumulus cells (CCs) associate with the oocyte via gap junctions and participate in the metabolism of glucose (Roberts et al., 2002), lipids (Singh et al., 2013)(Simerman et al., 2014), and amino acids (Seli et al., 2014) (Matzuk and Li, 2013)(Sutton et al., 2003)thereby providing essential nutrients to the oocyte. Additionally, CCs relay important signals supporting oocyte's cytoplasmic and nuclear maturation (Albertini et al., 2001)(Eppig et al., 2005)(Sutton-McDowall et al., 2010) (Salmon et al., 2004) (Feuerstein et al., 2007)(Cakmak et al., 2016)reported that regulatory factors (such as amphiregulin) act on CCs to promote secretion of factors (interleukin-7) from oocytes that in turn regulate cumulus cell proliferation and function, thus completing a feedback regulatory loop resulting in competent oocytes. Therefore, CC function is thought to be a major determinant of oocyte quality (Matzuk and Li, 2013); the oocyte, in turn, controls much

of an embryo's preimplantation development. As CCs are discarded after oocyte retrieval, they pose an attractive, non-invasive target for deeper study into the determinants of preimplantation embryo quality.

Studies have revealed an association between CC function and subsequent embryo development. For example, optimal CC metabolism is required to ensure the fertilizing capability of the oocyte (Eppig, 1991); (Russell and Robker, 2007)). A study evaluating genome-wide gene expression in CCs found seven genes, significantly expressed with respect to early cleavage (i.e., *CCND2*, *CXCR4*, *GPX3*, *CTNND1*, *DHCR7*, *DVL3*, *HSPB1* and *TRIM28*) (van Montfoort et al., 2008). Subsequently, several genes were found to be implicated in blastocyst stage development, including *ANG* (angiogenin), *PLIN2* (perilipin 2), *RGS2* (regulator of G-protein signaling 2) (Feuerstein et al., 2012), *TRPM7*, *ITPKA*, *STC2*, *CYP11A1*, *HSD3B1* (Wathlet et al., 2012), *CAMK1D*, *PTGS2* and *HAS2* (Scarica et al., 2019). Several other studies reported on CC gene expression with respect to embryonic competence. Reduced expression of *NFIB* and up-regulation of *BCL2L 11* and *PCK1* were associated with the CCs of embryos yielding live births (Assou et al., 2008). Two subsequent studies specifically evaluated genes known to be critical for effective cellular functioning. (Gebhardt et al., 2011) evaluated 13 genes and found *VCAN*, *PTGS2*, *GREM1*, *PFKP* to be up-regulated in CCs of embryos leading to successful pregnancy. Notably, gene expression patterns were not predictive of embryo morphology. (Wathlet et al., 2012) analyzed the expression of 11 genes, describing an association with pregnancy success with four genes (i.e., *EFNB2*, *CAMK1D*, *STC1* and *STC2*). The expression patterns of these genes were not related to cleavage stage morphology or subsequent

blastocyst development, however, other genes examined i.e., *TRPM7*, *ITPKA*, *STC2*, *CYP11A1* and *HSD3B1* did show a relationship with these parameters. Prediction model based on the gene expression pattern of CCs associated with embryos that resulted in live birth versus those that did not were later created. *FGF12*, *GPR137B*, *SLC2A9*, *ARID1B*, *NR2F6*, *ZNF132*, *FAM36A* were significantly up-regulated in the CCs of embryos that resulted in a successful pregnancy and several other were down-regulated (*ZNF93*, *RHBDL2*, *DNAJC15*, *MTUS1*, *NUP133*) (Iager et al., 2013). (Borup et al., 2016) identified another 30 genes with differential expression associated with embryos yielding live births. Unfortunately, these prior studies evaluated pregnancy outcomes after multiple embryo transfer and failed to control for embryo ploidy status, limiting the applicability of the findings to current practice.

(Papler et al., 2015) evaluated both fertilization and implantation rates after elective single embryo transfer in relation to CC gene expression. Microarray analysis followed by quantitative real-time polymerase chain reaction (PCR) validation, no significant difference in gene expression was identified according to these outcomes. (Green et al., 2018) utilized a paired design to evaluate the CC transcriptome of euploid embryos in double embryo transfers. Using PGT-A and embryo fingerprinting, authors identified which of the two embryos that were transferred implanted and resulted in a livebirth and which one did not. They then compared CC transcriptome of embryos that resulted in a live birth to the transcriptome of the CC transcriptome of their siblings that failed to implant. No difference was identified (Green et al., 2018)

Preimplantation genetic testing for aneuploidy screening (PGT-A) is utilized to choose an embryo for transfer amongst a cohort of embryos. Since its inception in the

early 1990s, PGT-A has undergone many iterations to reach its present-day form. Despite this, sustained implantation and live birth rates seem to have plateaued at approximately 65%, even for embryos tested using PGT-A and diagnosed as euploid (Jr et al., 2013) (Schoolcraft and Katz-Jaffe, 2013) (Shapiro et al., 2011). Given the non-invasive nature of analyzing — otherwise discarded — CCs as a marker of embryonic competence, is of interest. Overall, the extent to which the follicular environment correlates with subsequent embryonic ploidy status is largely unknown. A study evaluating CC gene expression and oocyte ploidy identified the under-expression of genes involved in DNA damage response (tumor protein p53 inducible protein 3 (TP5313)) and ubiquitination regulation (spla/ryanodine receptor domain and suppressor of cytokine signalling (SOCS) box containing 2 (SPSB2)) linked with the development of oocyte aneuploidy (Fragouli and Wells, 2012). A recent study evaluating CC gene expression according to the ploidy status of embryos found no difference between embryos diagnosed as euploid versus aneuploid by quantitative polymerase chain reaction (qPCR) (Scarica et al., 2019). Alternatively, reduced CC expression of *HSD3B* mRNA was found to be associated with a higher rate of euploidy in embryos in another recent investigation utilizing NGS-based PGT-A (Kordus et al., 2019)

Despite an overall consensus within the literature that CC function is vital to oocyte competence and, therefore, likely embryo competence, little consensus with regard to the specific genes implicated exists. Additionally, while many have evaluated genes associated with a successful pregnancy outcome, only a limited number of prior studies specifically evaluate the difference in gene expression between euploid and aneuploid embryos. The majority of these do not utilize a contemporary PGT-A platform,

and, therefore, such comparisons are less relevant to current practice. Additionally, the chromosomal aneuploidies (e.g., multiple versus single whole chromosome aneuploidies, as well as specification of the chromosomes involved) are not explicitly indicated in prior studies, possibly serving as an underlying etiology of the lack of consensus in genes implicated in aneuploidy. In humans the most common aneuploidies are trisomies, some of which are not compatible with life. Of all the trisomies known the most common human trisomy involves chromosome 21 (Rafii et al., 2019). As it is largely unknown that a diverse range of whole chromosome aneuploidies may be seen within a cohort of sibling embryos, evaluating the CC gene expression of embryos with one specific whole chromosome (in this case, chromosome 21) aneuploidy appears to be a logical starting point. Thus, the present proof-of-concept study sought to determine if the CC transcriptomic profile of embryos predicted to be euploid by NGS-based PGT-A differs from that of embryos with a single whole chromosome aneuploidy.

MATERIALS AND METHODS

Ethics Statement

Ethical approval was obtained by the Institutional Review Board (IRB) (Protocol RMA—2018—01) and registered with clinicaltrials.gov (NCT03604107). Eligible patients were contacted and counseled regarding the study procedures. Written informed consent was obtained from all participants prior to study enrollment.

Study population

Women 18 to 44 years of age with normal ovarian reserve (follicle stimulating hormone (FSH) ≤ 12 IU/ml and antral follicle count (AFC) ≥ 8) undergoing their first IVF/ICSI cycle were screened for eligibility. Patients were excluded if they had a body mass index (BMI) ≥ 35 kg/m² or possessed a single gene disorder or structural chromosomal rearrangement requiring preimplantation genetic testing for monogenetic disorders (PGT-M) or preimplantation genetic testing for structural chromosome rearrangements (PGT-SR), respectively.

Study Design

This study is evaluating the transcriptome of CCs from individual oocytes obtained prospectively from consenting patients undergoing their first in vitro fertilization (IVF) from August 2018 to November 2019 at Reproductive Medicine Associates of New Jersey (RMANJ), New Jersey, USA. CCs were collected as part of a larger prospective, blinded, non-selection trial evaluating the predictive value of an aneuploid PGT-A diagnosis using a targeted NGS-based assay (Tiegs et al., 2020). All oocytes and embryos were individually cultured in order to study CCs associated with a specific aneuploidy. The PGT-A diagnoses were un-blinded for all included embryos at the time of the present study.

Once PGT-A results became available, CCs from trisomy 21 and monosomy 21 embryos were selected and compared to samples from euploid embryos. Non-sibling embryos were used for analysis for two reasons. First, the statistical likelihood of having all three genotypes in a single cohort of embryos (generated from a single retrieval) is extremely low. Second, this study aimed to investigate clinically relevant differences

between aneuploidies and not differences related to individual variations without pathologic significance, such as genetic background, lifestyle and treatments.

In this study, instead of comparing a mixed group of trisomies and monosomies, we compared aneuploidies associated with chromosome 21. This was done in order to increase homogeneity within the groups and decrease the likelihood of a type II error (non-rejection of a false null hypothesis). Chromosome 21 was selected as its aneuploidies (trisomy 21 and monosomy 21) are among the most common aneuploidies observed in blastocysts generated through IVF (Shahbazi et al., 2020). In addition, trisomy 21 is the most common chromosomal anomaly observed in humans (Rafii et al., 2019)), whereas monosomy 21 is not compatible with life suggesting a significant difference between their associated transcriptomes.

Routine Clinical and Laboratory Procedures

All patients underwent controlled ovarian hyperstimulation with injectable recombinant or purified urinary gonadotropins in a protocol as per their primary physician's discretion. Stimulation protocols included gonadotropin-releasing hormone (GnRH) antagonist and micro-dose leuprolide flare cycles. Transvaginal ultrasound assessing for follicular growth as well as serum estradiol and progesterone levels were obtained every one to three days. When at least two follicles were $\geq 17\text{mm}$ in diameter, final oocyte maturation was induced by injection of either GnRH agonist, purified urinary human chorionic gonadotropin (hCG), or recombinant hCG. Approximately 36 hours later, all follicles were aspirated via transvaginal oocyte retrieval. Cumulus oophorus complexes were isolated from the follicular fluid and each was labeled. Maintaining

identity in relation to the oocyte, supernumerous CCs were removed prior to incubating the oocytes for later denudation by hyaluronidase in preparation for intracytoplasmic sperm injection (ICSI). Maintaining oocyte identity, the CCs were then aspirated from the media and centrifuged for 90 seconds at 15,000 revolutions per minute (rpm). The supernatant was removed from each set of cells, washed with phosphate-buffered saline (PBS) and again centrifuged at 15,000 rpm for an additional 90 seconds. The supernatant was removed, followed by freezing of the labeled pellet at -80°C.

Oocytes were individually cultured in order to ensure accurate correlation with embryo development and associated outcomes. The embryos were cultured in the IVF laboratory following standard protocols. Intracytoplasmic sperm injection (ICSI) was performed in all cases. After ICSI, oocytes were cultured in pre-equilibrated culture dishes with Quinn's Advantage Cleavage culture media drops, covered with OVOIL. The incubators were set with 37 degrees C, 5.0% CO₂ and 5.0% O₂. Routine laboratory procedures included laser-assisted hatching of the zona pellucida, culture to the expanded blastocyst stage, and daily morphologic assessment initiated on day 5. Additionally, all embryos considered usable (i.e., well-developed blastocoel cavity with adequate cellularity of the inner cell mass (ICM) and trophectoderm (TE)) underwent TE biopsy followed by vitrification.

PGT-A Assay: Library Preparation and Sequencing for PGTseq-A

PGT-A was accomplished using PGTseq-A. A two-step PCR strategy was used to incorporate sequencing library adapters and indexes for PGTseq-A, following the manufacturer's instruction (PGTseq Technology). The first PCR was performed with 1µl

of PCR1 primer pool, 5µl of PCR1 master mix, and 9µl of molecular biological grade water with 10µl of lysates. PCR cycling conditions were set to 10 min at 95°C, followed by 20 cycles of 15 sec at 95°C, and 4 min at 60°C. The second PCR was set up to add indexes to the amplified DNA by adding 2µl of first PCR product, 4µl of PCR2 master mix, and 16µl of molecular biological grade water to the index plate. The reaction was set to 10 min at 95°C, followed by 9 cycles of 15 sec at 95°C, and 4 min at 60°C. Seventy-two samples were quantified, pooled, and purified for one NextSeq 500/550 Mid Output Kit v2.5 (Illumina) using single 150 base pair (bp) reads. Copy number analysis was performed by PGTseq-A software (PGTseq Technology).

Messenger RNA (mRNA) Isolation and Sequencing

The selected CCs were thawed and assessed. Each sample was noted to contain approximately 100 to 200 cells. RNA were prepared using the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing, according to the user manual, to convert the mRNA into cDNA and amplify by Long Distance (LD) PCR (17 cycles for 100 cells). RNA integrity of samples was tested using Agilent Bioanalyser prior to sequencing. Samples meeting the stringent conditions for RNA quality as represented by the RIN number, and concentration were processed for sequencing. Complementary DNA (cDNA) was isolated using the Agencourt AMPure XP Kit (Beckman Coulter, Brea, CA) and quantified using the Agilent High Sensitivity DNA Kit on an Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA). The library preparation was performed using the Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA).

Samples were sequenced to depths of up to 44 million read pairs, 75 nucleotide (nt) length reads per sample using the Illumina Rapid v2 kit (75 cycles) on an Illumina HiSeq2500 Sequencing System. Image analysis, base calling, and generation of sequence reads, were produced using the HiSeq Control Software v2.0 (NCS) and Real-Time Analysis Software (RTA). Data was converted to FASTQ files using the bcl2fastq2 v1.8.4 software (Illumina Inc.).

Data Analysis

The reads were trimmed for quality, and aligned with the reference human genome hg38 with GENCODE annotation (GENCODE reference annotation for the human and mouse genomes; Nucleic Acid Research, October 24, 2018). The normal annotation has approximately 50,000 entries, but the gencode annotation has over 100,000 annotated regions on the genome. HiSAT2 was used for alignment and StringTie and BallGown for transcript abundance estimation (transcript level expression analysis of RNA-sequencing experiments with HISAT, String Tie and Ball gown (Pertea et al., 2016)). We used DESeq2 for differential analysis, which, for most data sets gives the highest estimate of power (Ching et al., 2014).

The genes were called as differentially expressed if the adjusted p -value was ≤ 0.05 . The statistical program R was used for downstream processing and visualization of data. Three comparisons of CC gene expression were made: euploid versus monosomy 21, euploid versus trisomy 21, and monosomy 21 versus trisomy 21.

Ingenuity Pathway Analysis (IPA)

Ingenuity Pathway Analysis (IPA) Ingenuity Systems (QIAGEN, content version: 51963813, 2020, Redwood City, CA, USA) was used to carry out pathway analysis for differentially expressed genes (DEG) across samples. Each gene symbol was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base (IPKB). DEGs used in pathway analysis were determined between experimental and control groups by using a filtering criteria fold change (FC) ≥ 1.5 and Benjamini-Hochberg (B-H FDR) ≤ 0.05 (Benjamini and Hochberg, 1995). IPA Core Analysis was used to generate a network showing the overlap between functions and differentially expressed genes (fold-change ≥ 1.5 , FDR $p \leq 0.05$) resulting from the comparison between the different groups, where FDR (or adjusted p-value) refers to the p-value (calculated using Fischer's exact test) that is used in the overrepresentation analysis. This analysis calculates the overlap ($p \leq 0.05$) between the list of DEGs and pathways in order to determine if subsets of genes associated with specific pathways are overrepresented (or enriched) among DEGs. The fold change refers to the cutoff used to identify a gene as differentially expressed (e.g. fold change modular value ≥ 1.5) and to be included in the pathway analysis. In addition, IPA calculates the z-score to infer the activation states (increased or decreased) of implicated pathways and biological functions. This inference is based on the experimentally observed causal relationships found in the biomedical literature between genes and those functions (Krämer et al., 2014).

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Real-time polymerase chain reaction (RT-PCR) was performed to confirm differential gene expression of any genes identified as altered between monosomy and

trisomy samples as compared to euploid samples. RNA was amplified, and cDNA was prepared using established protocol of SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing and gene expression analysis was done by real-time PCR using Syber Green on the ViiA7 real time PCR machine by Applied Biosystems. The PCR reaction was prepared using 5 μ L PowerUpTM SYBRTM Green Master Mix (2x), 1 μ L forward and reverse primers, 1 μ L of DNA template and 3 μ L of nuclease-free water. The components were mixed thoroughly and briefly centrifuged. PCR cycling conditions were 95°C for 5 min followed by 45 cycles of 10s at 95°C, 58°C for 15s, and 72°C for 15s. Hypoxanthine guanine phosphoribosyl transferase (*HPRT*) and β -Actin (*ACTB*) were used for normalization, based on a recent study published on human cumulus cells where these genes were used as the reference genes.(Chermuła et al., 2020) To our knowledge there are no other qRT-PCR studies performed on cumulus cells with these specific aneuploidies, assessing *HPRT* and *ACTB* expression. In our dataset there was no significant difference in the expression of these genes between the groups. $\Delta\Delta C_t$ method was used for the calculation of the difference in the expression of genes. A melt curve analysis was subsequently performed to confirm the specificity of products.

RESULTS

Study Population

A total of 24 cumulus cell samples collected from 24 unique, non-sibling oocytes from 23 different patients were evaluated. Of these, 8 samples were associated with euploid embryos, 8 associated with monosomy 21, and 8 associated with trisomy 21. One patient donated CCs from one euploid as well as one monosomy 21 embryo. All

included patients obtained more than one embryo (range: 2 to 26) during a single IVF cycle. Of note, all patients' embryo cohorts included both euploid and whole chromosome aneuploid embryos. RNA-sequencing alignment metrics of CCs from one euploid embryo and one monosomy 21 embryo were noted to be poor, so the remaining 22 CC samples used for analysis included 7 associated with euploid embryos, 7 associated with monosomy 21 embryos, and 8 associated with trisomy 21 embryos.

Baseline and IVF cycle characteristics of the patients associated with evaluated embryos are presented in Table I. There was no statistically significant difference between the mean age of patients from whom euploid, monosomy 21, and trisomy 21 were derived (33.6 ± 4.7 ; 35.5 ± 4.5 ; and 36.3 ± 4.0 , respectively). Similarly, there was no statistically significant difference between their BMIs (25.2; 25.1; and 24.6, respectively). Overall, the percentage of good quality embryos among euploids (75%) was higher than those among monosomic (12.5%) and trisomic (25%) embryos ($p < 0.05$). Similarly, euploid embryos were obtained from younger patients (33.6 ± 4.7) compared to monosomic and trisomic embryos (35.5 ± 4.5 and 36.3 ± 4.0 , $p < 0.05$). All other characteristics appeared to be similar between groups. Blastocyst quality is described according to the simplified Society for Assisted Reproductive Technology (SART) scoring system (Heitmann et al., 2013).

RNA-sequencing Defined Repertoire of Differentially Expressed Genes in CCs Associated with Aneuploid Embryos

Principal component analysis of RNA-sequencing data revealed distinct separation of gene expression in CCs of monosomy 21, trisomy 21, and euploid

embryos (Supplemental Figure 1A). 3D PCA was generated using the Qlucore Omics Explorer v3.6 software (QlucoreAB, Lund, Sweden). Using the differentially expressed genes (fold change $\geq |1.5|$, $\text{adj}p \leq 0.05$) three different groups were distinguished (Supplemental Figure 1B).

RNA-sequencing analysis revealed a total of 3122 genes that were significantly differentially expressed when comparing monosomy 21 versus euploid CC gene expression. Genes with significant difference in expression in the monosomy patients when compared to the euploid patients are listed in Supplemental Table 1. Of these 3122 identified genes, the associated protein products have been known to be found in all areas of the cell (i.e., cytoplasm, nucleus, plasma membrane, and extracellular space), however, the majority of the genes have not yet been associated with a protein product. Utilizing the regularized-logarithm transformation of gene counts, the Euclidean distances between euploid and monosomy 21 CC samples were evaluated. As demonstrated in the heatmap (Figure 1A), a differential pattern of gene expression is identified between the euploid and monosomy 21 CCs. The volcano plot (Figure 1B) shows the significantly increased expression of genes $-\log_{10}(p\text{-value})$ in red.

The comparison of euploid versus trisomy 21 CC gene expression revealed a total of nineteen significantly differentially expressed genes (Supplemental Table 2). Of these, six genes were associated with known protein products that have been localized to the cytoplasm ($n=2/6$), nucleus ($n=3/6$), and extracellular space ($n=1/6$). A heat map illustration shows the differences in expression of the identified genes according to ploidy status (Figure 2A). The volcano plot shows the significantly increased expression of genes $-\log_{10}(p\text{-value})$ in trisomy when compared to euploid embryos (Figure 2B).

Ingenuity Pathway Analysis Identified Pathways Over-Represented in DEGs in CCs Associated with Aneuploid Embryos

The differentially expressed genes (DEGs) were analyzed by overrepresentation using the Ingenuity Pathway Analysis Knowledge Base (IPA). Based on the expression pattern of the DEGs in the present data set, inhibition of several pathways, including the cyclic adenosine cyclic adenosine monophosphate (cAMP)-mediated pathway (Benjamini-Hochberg B-H $p=0.005$), was seen in the monosomy 21 samples based on the z-score value (Figure 1C). Significant changes in the expression of other pathways linked to embryonic developmental competence, such as the insulin-like growth factor 1 (IGF-1) (B-H $p=0.005$) and Wnt/ β -catenin-signaling pathways (B-H $p=0.009$), was seen in Trisomy 21 samples (Figure 2C). However, the activation status of some pathways could not be predicted due to the lack of data on the knowledge base for genes overrepresented in these pathways (i.e., bars without color in Figures 1C and 2C).

Based on the results of IPA Core Analysis, we generated a network showing the overlap between functions and differentially expressed genes (fold change ≥ 1.5 , FDR $p \leq 0.05$) in various groups. When comparing monosomy 21 aneuploid with euploid CCs, predictions show significant inhibition of important functions associated with embryo growth and development, such as cell-to-cell contact ($p=8.5e-161$), cell-to-cell adhesion ($p=4.89e-32$), and cell movement ($p=1.07e-18$) (Figure 3A). Interestingly, when trisomy 21 was compared to the euploid CC samples, the cell to cell adhesion process was also significantly affected, although the impact was less prominent (Figure 3 B).

Significantly Increased Expression of CYR61/CCN1, DVL2 and SRF in CCs Associated with Aneuploid Embryos

Thirteen genes were identified with significantly differential expression common to both the monosomy 21 versus euploid and trisomy 21 versus euploid comparisons (Figure 4A). In aneuploid as compared with euploid embryos, expression of three of these thirteen genes are implicated in embryo developmental competence: cellular communication network factor 1 (*CCN1/CYR61*), disheveled segment polarity protein 2 (*DVL2*), and serum response factor (*SRF*). Box plot from RNA sequencing shows increased expression of all three genes in the aneuploid samples when compared to the euploid samples (Figure 4B).

Quantitative RT-PCR for *CCN1/CYR61*, *DVL2*, and *SRF* was performed using euploid, monosomy 21, and trisomy 21 samples to validate the RNA-sequencing findings. Housekeeping genes *HPRT* and *ACTB* were used as the internal controls. qRT-PCR confirmed a significant increase in *CCN1/CYR61* ($p=0.01$) in monosomy and trisomy samples ($p=0.005$) as compared to euploid CCs. A significant increase of *DVL2* was also observed in monosomy ($p=0.02$) and trisomy ($p=0.06$) CCs as compared to euploid CCs. *SRF* showed increased expression approaching significant values in CCs associated with monosomy 21 embryos ($p=0.1$) and a significant increase was observed in trisomy 21 embryos ($p=0.007$) as compared with euploid embryos (Figure 4C). qRT-PCR results confirmed the sequencing findings. Due to the limited amount of RNA available from cumulus cell samples we were not able to include genes that showed a decreased or unchanged expression in individual aneuploidies compared to euploids, in

the validation analysis. We therefore verified the expression levels of the selected genes with potential biological relevance that were differentially expressed (increased) in both monosomy 21 and trisomy 21 samples compared to euploids.

DISCUSSION

The altered expression of several genes plausibly implicated in embryo development was identified upon comparison of CC transcriptomic profiles of euploid embryos versus embryos with whole chromosome aneuploidy for chromosome 21 (monosomy and trisomy). It is noteworthy that we found a high number of genes (3122) to be differentially expressed in CCs associated with monosomy 21, as compared to only 19 genes in CCs of oocytes that give rise to trisomy 21 embryos. Similarly, a recent study by (Sanchez-Ribas et al., 2019) which investigated differentially expressed genes in embryos with chromosome 21 aneuploidies (monosomy 21 and trisomy 21), found that 1232 genes were differentially expressed in monosomy 21 embryos compared to euploid embryos, whereas no gene was differentially expressed in trisomy 21 embryos. Their findings are in parallel with ours, as we observe a high number of genes (3122) that are differentially expressed in CCs associated with monosomy 21, as compared to only 19 genes in CCs of oocytes that give rise to trisomy 21 embryos.

Specifically, enhanced expression of *CCN1/CYR61*, *DVL2*, and *SRF*, were demonstrated in CCs of whole chromosome 21 aneuploid as compared to euploid embryos. These genes are known to be associated with protein products found in several different areas of the cell. Therefore, it is possible that the differentially expressed genes act in concert with one another to produce the associated aneuploidy,

implying that, unsurprisingly, a multitude of factors influence oocyte ploidy. Additionally, the fact that a single gene was significantly differentially expressed between monosomy and trisomy CCs as compared with the many significantly differentially expressed genes identified between euploid and both trisomy or monosomy 21 CCs lends credence to the possibility that CC gene expression may be implicated in the determination of ploidy status of the embryo, especially as it relates to the segregation of chromosome 21.

The possibility that the follicular environment may impact meiotic events within the oocyte has long been suggested. As reviewed by (Fragouli and Wells, 2012) optimal bi-directional communication between the oocyte and its surrounding CCs is critical to the competence of the oocyte. Additionally, the follicular environment may influence oocyte ploidy via expression of genes involved in cellular metabolism, transport, signaling and apoptosis. Specifically, an altered expression pattern of several of the genes implicated in these critical events was identified in the CCs in relation to euploidy, both in the present study as well as others.

These data reveal enhanced expression of three genes with previously described functions in the CCs of aneuploid embryos: disheveled segment polarity protein 2 (DVL2), cellular communication network factor 1 (CCN1/CYR61), and serum response factor (SRF). Studies show that when DVL2-GFP fusion proteins are overexpressed in single blastomeres of the 4-cell stage embryo, the progeny of the affected cells show reduction in cell adhesiveness and a rounded shape at the blastocyst stage. This suggests that perturbing DVL2 function interferes with cell-cell adhesion through the non-canonical Wnt pathway in blastocysts. (Wang et al., 2009) DVL2 encodes a protein precursor that becomes a cytoplasmic protein after posttranslational phosphorylation,

which possibly plays a role in the WNT signaling pathway. The WNT/ β –catenin signal transduction pathway is involved in the regulation of cell proliferation and thought to be critical in mediating normal folliculogenesis, as described by Wang *et al* (2009). *CCN1/CYR61* encodes for a protein localized within the extracellular space that promotes endothelial cell adhesion to integrins and heparan sulfate proteoglycans, and is required for angiogenesis and tissue repair. Other functions of this protein product include cell proliferation, differentiation, apoptosis, and extracellular matrix formation (Lee *et al.*, 2019). A previous study shows that SGHPL-5 trophoblast cells treated with the glycosylated forms of recombinant CCN1 and CCN3 decreased cell proliferation by bringing about G0/G1 cell cycle arrest (Kipkeew *et al.*, 2016) Finally, the translated protein of SRF is localized to the cell nucleus and instrumental in cell cycle regulation, acting via binding to target genes' serum response elements. SRF plays a role in apoptosis, cell growth, and cell differentiation (Posern and Treisman, 2006) (Franco and Li, 2009) (Taylor and Halene, 2015) its role in embryo development may be via its known function in mesoderm formation (Franco and Li, 2009). Increased expression of SRF promoted reprogramming in NPC cell lines and other cell lines like hepatoblasts and UBCs and promotes the loss of original cell identity(Ikeda *et al.*, 2018).SRF has also been found to be increased in the CCs of women with polycystic ovarian syndrome (Shi *et al.*, 2015), which may translate to altered IVF laboratory outcomes, although further study is needed. Additionally, decreased SRF has recently been described in malignant thyroid tumors (Jing *et al.*, 2020)and implicated in enhanced mortality after traumatic brain injury (Förstner and Knöll, 2020), further suggesting its important role in regulation of cell proliferation.

IPA analysis showed inhibition of multiple pathways, including the cAMP-mediated signaling pathway, which has demonstrated utility in trophoblast fusion (Gerbaud et al., 2015). Other pathways, such as insulin-like growth factor 1 (IGF-1) (Agrogiannis et al., 2014) and Wnt/ β -catenin signaling pathways (Sonderegger et al., 2007) have established roles in human embryo development and showed altered activity in CCs from trisomy 21 patients in the present study. Taken together, aberrant expression of the aforementioned genes and pathways would logically adversely impact embryonic development, perhaps including early meiotic events leading to aneuploidy.

Without further validation, there is limited utility of the present findings for clinical practice. However, several potential applications for this technology exist. For example, analysis of the CC transcriptome may better inform the quality of retrieved oocytes for patients undergoing oocyte vitrification for fertility preservation. Such information may encourage patients to undergo additional cycles if the transcriptomic profiles are associated with whole chromosome aneuploidy. Moreover, utilizing otherwise-discarded cumulus cell material for such a test does not require exposure of the oocyte to conditions outside of the incubator. Such practice contrasts with other proposed tests of oocyte quality, such as polar body biopsy (Fragouli et al., 2011) or polarized light microscopy of the oocyte spindle (Tilia et al., 2020). Additionally, an improved understanding of the CC transcriptome may lead to enhanced in vitro maturation techniques, potentially improving the efficiency of IVF cycles in women of all ages. Furthermore, although PGT-A via TE biopsy is currently the standard for preimplantation assessment of embryo ploidy, evaluation of CC gene expression has

the potential to serve as a non-invasive marker of ploidy, possibly allowing for enhanced embryo selection for patients choosing not to proceed with PGT-A.

There are several strengths of this study, including the identification of altered expression of CC genes with previously-described major regulatory functions in cell processes, lending relevance to the present findings. Confirmation of the identified genes using RT-PCR is an additional important step in ensuring the accuracy of the present analyses. Additionally, evaluation of the CC transcriptomes associated with euploid embryos versus embryos with a single whole chromosome aneuploidy is a unique assessment not previously reported with associated novel findings. In contrast to many prior investigations, a contemporary PGT-A platform was used for copy number assessment in the present study.

Limitations of the present analysis relate to its small sample size and design as a proof-of-concept study. As such, replication of these analyses in a larger cohort of embryos is necessary to confirm the findings. Additionally, evaluation of the CC transcriptome within and across patient cohorts is required to gain a deeper understanding of CC gene expression patterns associated with varying chromosome copy number. It is possible that the chromosome assessed in association with embryonic aneuploidy in the present study, chromosome 21, has a unique CC transcriptome. Analysis of other whole chromosomal aneuploidies is warranted. In addition, the comparison between the aneuploid embryos compatible and incompatible with birth is definitely worth pursuing. Unfortunately, that comparison was beyond the scope of the present study. Evaluating the CC transcriptome of embryos with PGT-A

predictions of mosaicism and segmental aneuploidies in correlation with clinical outcomes may also be of interest in future studies.

Although promising, transcriptomic profiling is presently costly of both time as well as financial resources. Additionally, many of the techniques and analyses involved require highly-trained personnel (Dumesic et al., 2015). As such, CC transcriptome profiling is not practical for every patient undergoing oocyte retrieval at the present time, but future technologies may reduce the cost and increase the ease with which such procedures are performed. With respect to the findings of the present study, further research is needed to evaluate whether whole chromosome 21 aneuploidy and/or euploidy can be reliably determined via the differential expression of the genes identified in the present investigation. Notably, a non-selection study, in which the CC transcriptome is analyzed after the clinical outcome of the embryo transfer is known, will need to be performed for clinical validation of the identified CC gene expression patterns associated with ploidy status prior to utilization of this technology in clinical practice.

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CONFLICT OF INTEREST

E.S. is a consultant for and receives research funding from the Foundation for Embryonic Competence; he is also cofounder and a shareholder of ACIS LLC and coholds patent US2019/055906 issued for utilizing electrical resistance measurement for assessing cell viability and cell membrane piercing.

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Table I. Baseline and in vitro fertilization (IVF) retrieval cycle characteristics of included patients creating embryos with evaluated cumulus cells. Oocyte retrievals were performed at a single, large infertility center from 2018 to 2019. Means are expressed with (\pm) standard deviations. Medians are expressed with interquartile ranges (IQR).

Patient Baseline and Cycle Characteristics	Patients associated with Euploid Embryos (n=7)	Patients associated with Monosomy 21 Embryos (n=7)	Patients associated with Trisomy 21 Embryos (n=8)
Mean Female Age (years)	33.6 \pm 4.7	35.5 \pm 4.5	36.3 \pm 4.0
Median BMI (kg/m²)	25.2 (IQR 21.4 – 28.7)	25.1 (IQR 21.7 – 29.5)	24.6 (IQR 23.3 – 28.4)
Median Antral Follicle Count	22.0 (IQR 13.7 - 24.6)	19.5 (IQR 12.4 - 22.8)	20.0 (IQR 16.8 – 23.8)
Indication for IVF (% (n))			
Diminished ovarian reserve	25% (2) 0% (0)	25% (2) 25% (2)	0% (0) 12.5% (1)

Male factor	0% (0)	12.5% (1)	12.5% (1)
Tubal factor	25% (2)	37.5% (3)	12.5% (1)
Ovulatory Dysfunction	50% (4)	0% (0)	37.5% (3)
Unexplained	0% (0)	0% (0)	25% (2)
Same Sex or Single			
Mean Total Motile Sperm Count (Million)	75.8 ± 55.7	60.1 ± 58.3	75.8 ± 18.1
Median Number of Usable Embryos Obtained	7.0 (IQR 3.7 – 8.2)	6.5 (IQR 4.4 – 9.6)	8.0 (IQR 5.3 – 11.2)
Mean Proportion of Euploid Embryos Obtained per Cohort	53.9% ± 18.5	56.8% ± 10.5	54.1% ± 17.7
Proportion of Blastocysts Deemed:			
Good Quality (% (n))	75% (6)	12.5% (1)	25% (2)
Fair Quality (% (n))	25% (2)	75% (6)	62.5% (5)
Poor Quality (% (n))	0% (0)	12.5% (1)	12.5% (1)

FIGURE LEGENDS

Figure 1. Heatmap, Volcano plot and Over represented pathways of comparison between cumulus cells of euploid versus monosomy 21 embryos

(A) Heatmap of the sample-to-sample Euclidean distances displaying the significantly differentially expressed genes when comparing cumulus cells of euploid versus monosomy 21 embryos (n=3122 genes). The color spectrum ranging from red to blue indicates normalized levels of gene expression from high to low. The dendrogram demonstrates hierarchical sample clustering. A list of the significantly differentially expressed genes is found in Supplemental Table 1. **(B)** Volcano plot compares the expression of genes in euploid versus monosomy 21 cumulus cell samples. Red represent genes that reached statistical significance ($-\log_{10}(p\text{-value})$). **(C)** Major pathways found to be significantly altered in monosomy 21 cumulus cell samples. The over-represented pathways are ranked according to the calculated Benjamini-Hochberg FDR, $p < 0.05$.

Embryos are identified by their study number. E= euploid embryo; M= monosomy 21 embryo.

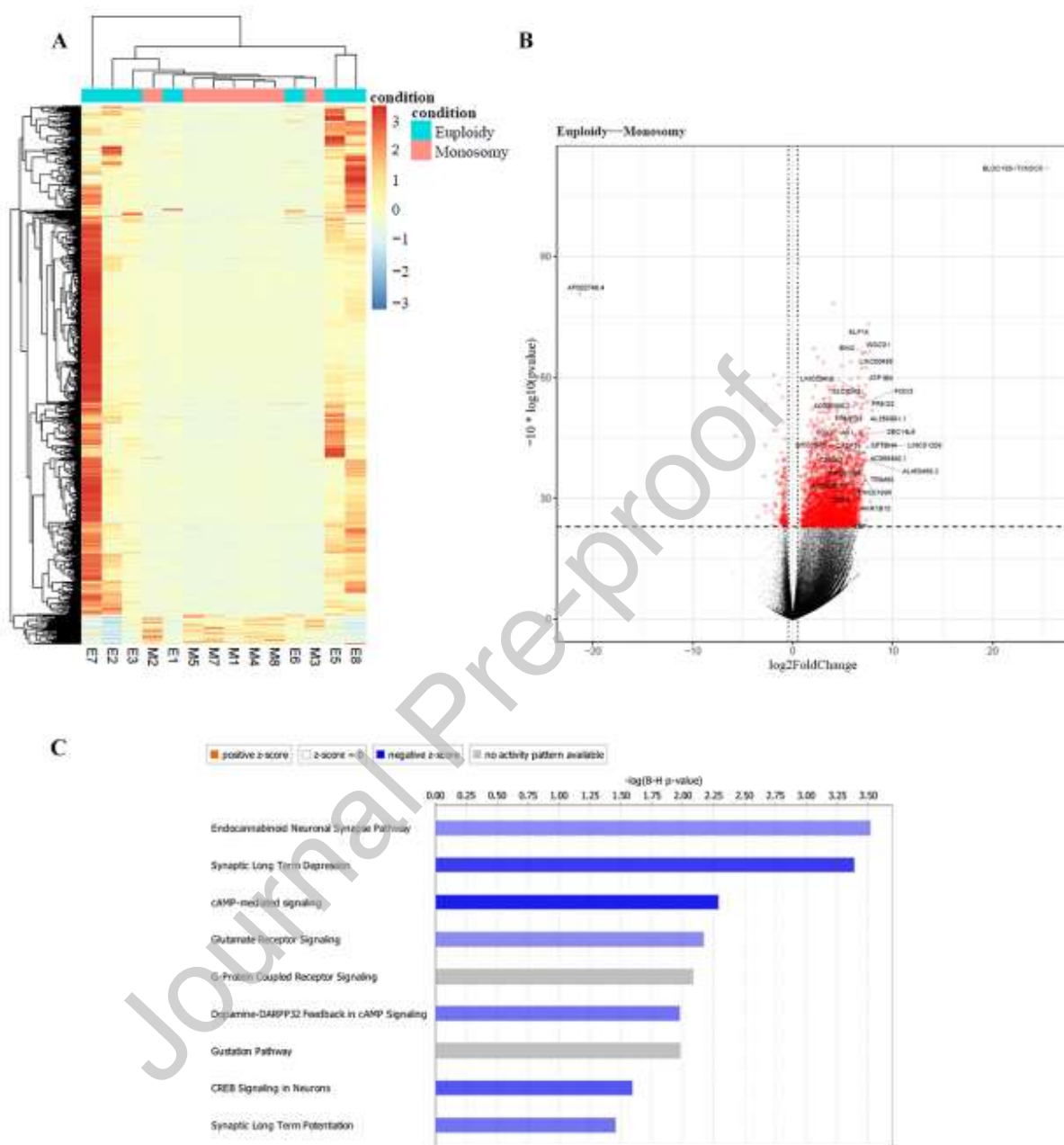


Figure 2. Heatmap, Volcano plot and Over represented pathways of comparison between cumulus cells of euploid versus trisomy 21 embryos

(A) Chermuła, B., Kranc, W., Jopek, K., Budna-Tukan, J., Hutchings, G., Dompe, C., Moncrieff, L., Janowicz, K., Józkowiak, M., Jeseta, M., Petite, J., Mozdziak, P., Pawelczyk, L., Spaczyński, R.Z., Kempisty, B., 2020. Human Cumulus Cells in Long-Term In Vitro Culture Reflect Differential Expression Profile of Genes Responsible for Planned Cell Death and Aging-A Study of New Molecular Markers. Cells. <https://doi.org/10.3390/cells9051265>

Heatmap of the sample-to-sample Euclidean distances displaying the significantly differentially expressed genes when comparing cumulus cells of euploid versus trisomy 21 embryos (n=19 genes). The dendrogram demonstrates hierarchical sample clustering.

(B) Volcano plot compares the expression of genes in Euploidy versus monosomy 21 samples. Red represent genes that reached statistical significance ($-\log_{10}(p\text{-value})$).

(C) Ingenuity Pathway Analysis showed major pathways that were found to be significantly altered in trisomy 21 cumulus cell samples. The over-represented pathways are ranked according to the calculated Benjamini-Hochberg FDR, $p < 0.05$. E= euploid embryo; T= trisomy 21 embryo.

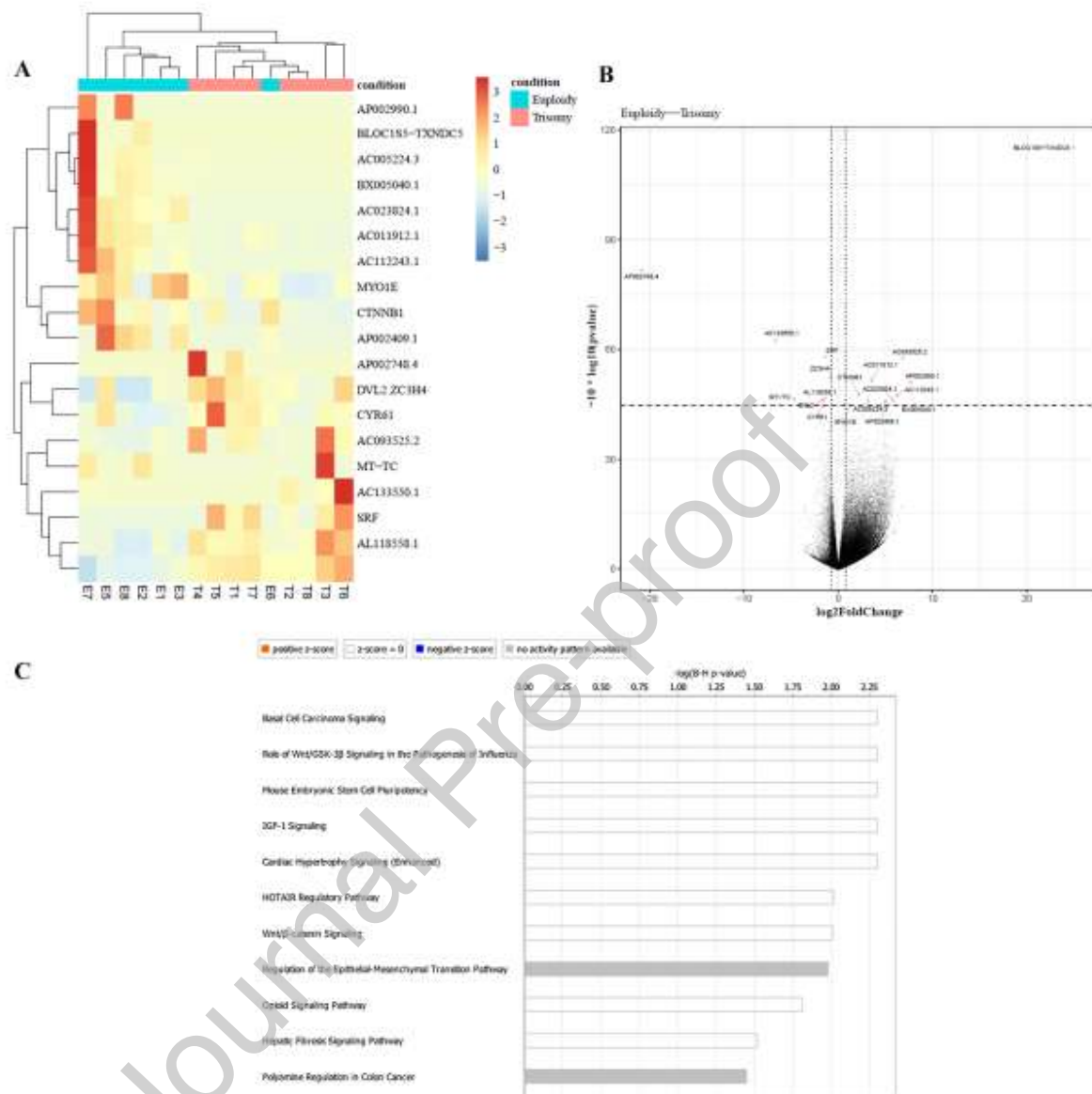
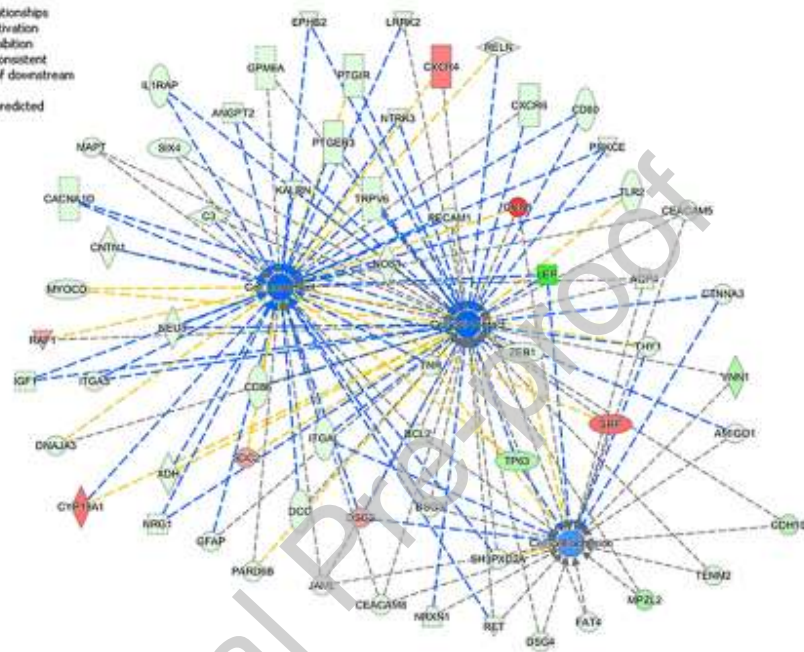
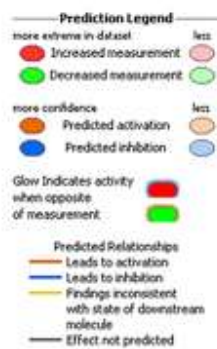


Figure 3. Ingenuity Pathway Analysis of differentially expressed genes in monosomy 21 (M), trisomy 21 (T) versus euploid (E) groups

(A) Ingenuity Pathway Analysis Core Analysis-generated network showing the overlap between functions and differentially expressed genes (fold change ≥ 1.5 , FDR $p \leq 0.05$)

resulting from the comparison between monosomy 21 (M) versus euploid (E) groups. *P*-value of the overlap is calculated by the fisher's exact test. **(B)** Ingenuity Pathway Analysis Core Analysis-generated network showing the overlap between functions and differentially expressed genes (fold change ≥ 1.5 , FDR $p \leq 0.05$) resulting from the comparison between trisomy 21 (T) versus euploid (E) cumulus cell samples. *P*-value of the overlap is calculated by the fisher's exact test. Green represents differentially expressed (downregulated) genes that passed the cutoff fold-change ≥ 1.5 (FDR $p \leq 0.05$) when comparing M versus E groups.

A



Cell cell contact

 $p=8.56E-161$

Cell movement

 $p=1.07E-18$

Cell cell adhesion

 $p=4.89E-32$

B

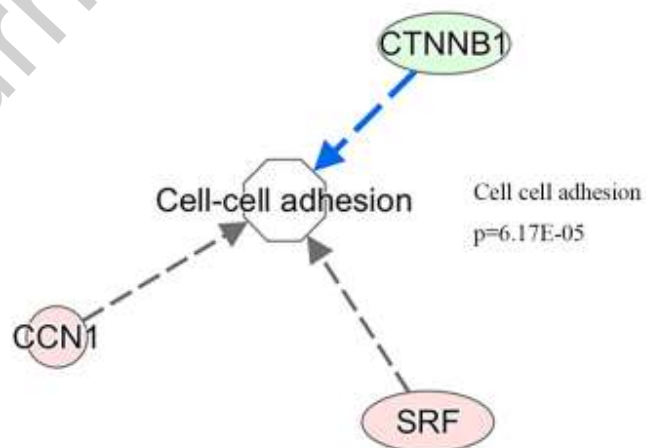
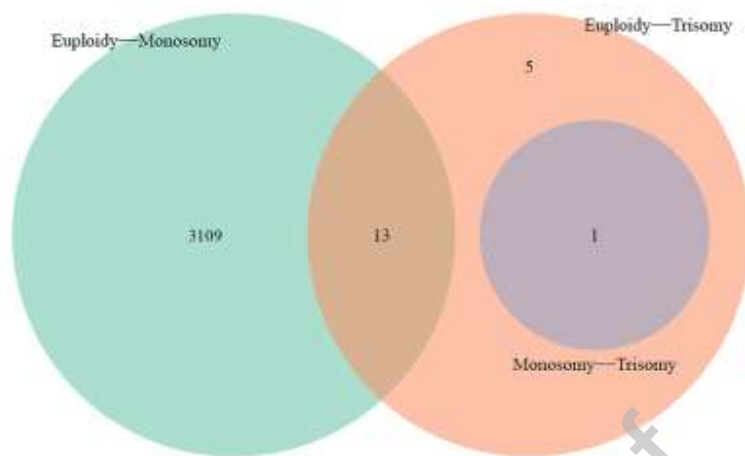
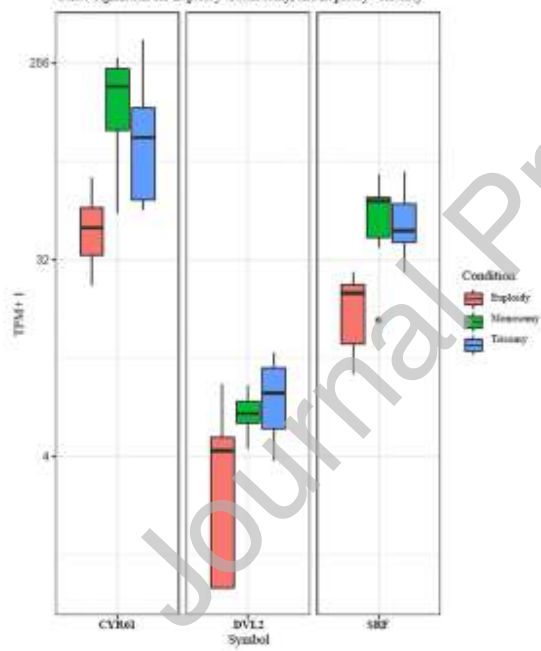


Figure 4. Venn diagram representing the overlapping genes in the comparison of euploid vs monosomy vs trisomy samples. Box plot and Real time PCR graphs of the 3 of the overlapping genes.

(A) Venn diagram demonstrating the number of significantly differentially expressed cumulus cell genes across all comparisons identified via RNA Seq analysis (i.e., euploid versus monosomy 21, euploid versus trisomy 21, and monosomy 21 versus trisomy 21). Numbers indicate the number of genes significantly differentially expressed in the indicated comparison. **(B)** Box plot representation of three genes (of the thirteen total overlapping genes differentially expressed in the euploid-monosomy and euploidy-trisomy comparisons as seen in (A)) that significantly increased in expression in both the monosomy 21 and trisomy 21 samples. DESeq2 uses the Wald's test (modified chi-squared test) to determine statistical difference. **(C)** Quantitative RT-PCR of *DVL2*, *CYR61/CCN1*, and *SRF* was performed on euploid (n=12), monosomy 21 (n=9), and trisomy 21 (n=9) cumulus cell samples to confirm RNA sequencing data. PCR results were consistent with RNA sequencing analysis, demonstrating significant increase in *CCN1/CYR61* ($p=0.01$ monosomy vs euploid; $p=0.005$ trisomy vs euploid), *DVL2* ($p=0.02$ monosomy vs euploid; $p=0.06$ trisomy vs euploid). *SRF* showed increased expression approaching significant values in CCs associated with monosomy 21 embryos ($p=0.1$) and a significant increase was observed in trisomy 21 embryos ($p=0.007$) as compared with euploid embryos. PCR= polymerase chain reaction.

A**B**

Genes Significant for Euploidy-Monosomy, and Euploidy-Trisomy

**C**