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Bone Marrow Stroma-induced Transcriptome Signatures of Multiple Myeloma as Modulated by JUNB

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**Bone Marrow Stroma-induced Transcriptome Signatures of Multiple Myeloma as
Modulated by JUNB**

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Thesis submitted

to School of Medicine at West Virginia University

in partial fulfillment of the requirements for the degree of

Master of Science in Biomedical Sciences

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Abstract

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Jasleen Gandhi

The bone marrow (BM) microenvironment acts as a breeding ground for drug resistance in multiple myeloma (MM). The interaction with bone marrow stromal cells (BMSCs) confer environment-mediated drug resistance (EMDR) to multiple myeloma. We investigated BM stroma-induced transcriptome signatures of MM cells through a sophisticated analysis of gene expression. In particular, we defined transcription program modulated by JunB, an emerging regulator of MM pathogenesis and a member of the transcription factor superfamily activator protein 1 (AP-1), in response to BM stimulation. The data and results lay down a foundation for future studies to illustrate the regulatory role of JunB in the *de novo* drug resistance of multiple myeloma.

Abbreviations

MM	Multiple Myeloma
MRD	Minimal Residual Disease
BM	Bone Marrow
BMM	Bone Marrow Microenvironment
BMSCs	BM Stromal Cells
ECM	Extracellular Matrix
ATCC	American Type Culture Collection
EMDR	Environmental Mediated Drug Resistance
CAM-DR	Cell Adhesion-Mediated Drug Resistance
SM-DR	Soluble Factor-Mediated Drug Resistance

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Background

Multiple myeloma (MM) is a plasma cell cancer that has led to an estimated 34,920 new cases and an estimated 12,410 deaths in the United States in 2021 (Dziadowicz et al., 2022). Amongst hematologic cancers, MM is the second most common malignancy (Kane, 2020). MM survival, proliferation, and progress mainly occur in the bone marrow (BM). Current treatment options for MM include drug therapy, corticosteroids, radiation therapy, stem cell transplant, CAR T-cell therapy, and surgery (Kane, 2020; Palumbo and Anderson, 2011). Despite innovative therapeutic options, there is a chance that tiny amounts of myeloma cancer cells still persist in the bone marrow after treatment, which is referred to as minimal residual disease (MRD) (Ding et al., 2021). MM cells hijack the BM niche for immune invasion and to gain resistance to cytotoxic agents (Chen et al., 2020).

The bone marrow consists of various types of accessory cells such as BM stromal cells (BMSCs), myeloid cells, endothelial cells, and immune effector cells (Hideshima and Anderson, 2021). The BM microenvironment (BMM) also consists of the extracellular matrix (ECM) that confers to the tumor progression (Chen et al., 2020). While oncogenes and genetic mutations drive MM progression, the BM microenvironment sanctuaries play an integral role in the therapeutic drug aversion and act as a breeding ground for drug resistance (Chen et al., 2020; Meads et al., 2008). Recent studies have established the importance of the BM microenvironment on cell sensitivity to drugs, cell migration, and the presence of sanctuary sites that contribute to the evolution of drug resistance (Chen et al., 2020; Fu et al., 2015).

The BM acts as an environmental factor for MM cells to gain *de novo* multi-drug resistance via dynamic interactions in a reciprocal pro-survival loop (Anderson and Carrasco, 2011; Di Marzo et al., 2016). MM cells develop resistance to chemotherapy in two ways: acquired drug resistance and *de novo* drug resistance (Hazlehurst and Dalton). Acquired drug resistance can be through chronological genetic and epigenetic changes that vests the tumor into a drug-resistant phenotype (Di Marzo et al., 2016). The *de novo* drug resistance is omnipresent before the drug exposure but is selected during the drug treatment (Hazlehurst and Dalton). The protective effect of BM on MM belongs to environment-mediated drug resistance (EMDR), a form of *de novo* resistance (Chen et al., 2020; Meads et al., 2008). EMDR can be bifurcated into soluble factor-mediated drug resistance (SM-DR) and cell adhesion-mediated drug resistance (CAM-DR) (Chen et al., 2020; Di Marzo et al., 2016).

SM-DR confides in growth factors, cytokines, chemokines (Di Marzo et al., 2016; Meads et al., 2008). The development of drug resistance is mediated by the soluble factors secreted during the interaction of MM cells with BM stromal cells (BMSCs) (Chen et al., 2020). Soluble

factors activate intercellular signaling cascades in the BMM (Hideshima and Anderson, 2021). The soluble factor-mediated drug resistance in MM is associated with activation of various signaling pathways including MAPK(ERK1/2) Signaling Pathway, JAK2-STAT3 Signaling Pathway, PI3K-Akt Signaling Pathway, NF- κ B Signaling Pathway, and Wnt β -Catenin Signaling (Hideshima and Anderson, 2021) which are activated by receptor IL-6 (Di Marzo et al., 2016).

The CAM-DR mechanism is an outcome of the adhesion of MM cells to BMSCs or the ECM components (Di Marzo et al., 2016). CAM-DR was originally characterized as a mechanism that suppresses drug-induced apoptosis (Damiano et al., 1999), and has since been associated with integrins and fibronectin receptors (Di Marzo et al., 2016). MM, cells-BM stromal cells adhesion has been known to trigger IL-6 secretion, NF- κ B activation in stromal cells, and the up-regulation of many signaling pathways evolving in MM cell proliferatdrug-inducedival (Di Marzo et al., 2016; Hideshima et al., 2004).

Both SM-DR and CAM-DR occur quickly and are reversible once the environment simulations are relieved, supporting epigenetic factors as potential regulators of the gained drug resistance. Epigenetic factors such as transcription factors (TFs) and chromatin regulators (CRs) frequently mutate cancers (Lu, 2021). Identification of gene targets of TFs is a critical step in the epigenetic approach to understanding the role in disease pathophysiology. While traditional drug therapeutics and conventional treatment options are heavily studied, the investigation of TFs as new potential drug targets using epigenetic approaches remains under-researched.

The transcription factor superfamily activator protein 1 (AP-1), composed of the JUN, FOS, ATF, and MAF multigene families (Fan and Podar, 2021), is an all-pervasive collection of protein complexes known to regulate transcription in response to environmental stimuli. Recent discoveries have investigated the members of the AP-1 family as new potential therapeutic targets for myeloma (Fan and Podar, 2021; Fan et al., 2017) lymphoma (Wu et al., 2021),(Garces de Los Fayos Alonso et al., 2018), and leukemia (Gazon et al., 2017). For instance, the AP-1 TFs c-Maf, c-Jun, and c- JunB implicate tumorigenesis and MM pathogenesis and are associated with MM cell proliferation, survival, drug resistance, and bone disease (Fan and Podar, 2021). Our recent work indicates JUNB as a key regulator of transcription response of MM in coculture with BMSCs through modulating chromatin landscape. However, validation of the prediction through loss-of-function is absent with target genes regulated by JUNB in MM remain largely undefined.

One of the current ongoing projects in our research lab focuses on unraveling the importance of JunB. Dziadowicz et al., (2022) characterized BMSC-induced transformations of the transcriptome and regulome in MM cells. One of the prominent transcription regulator inferred from the work was JunB, also for its connection to drug response and overall clinical

prognosis in MM. This thesis aims to uncover the role of JunB in transcription regulation in MM cells in the context of BM microenvironment. To this end, we have knocked out JunB in MM cells and evaluated the impact on gene expression before and after BMSC stimulation.

Methods and Materials

Cell lines

The American Type Culture Collection (ATCC, Manassas, VA, USA) supplied the lab with RPMI8226 (CCL-155). JunB CRISPR knockout in RPMI8226 cell lines was conducted by Synthego with 94% of the deletions presented as out-of-frame. The guide sequence for the knockout cells was UUUGAGACUCCGGUAGGGGU. MSP-1, a MM patient-derived BMSC cell line, was from Cellatrix (Cat#: 0007).

Cell Culture

The growth media in which MSP-1 cells were cultured in contained RPMI-1640 complete media with L-Glutamine (Corning, Waltham, MA, USA, 10-040-CV) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, Waltham, MA, USA, 10082-147) and 1% penicillin/streptomycin (Gibco, Waltham, MA, USA, 15140122). The growth media was maintained at 37 °C and the equipment used was Heracell™ VIOS 160i CO2 Incubator with 5% CO₂

A 6 well transwell plate with 2mL of growth media was used to seed 2.5×10^5 MSP-1 BMSC cells overnight. The following day, the media was replaced with fresh 2mL of growth media as well as 2.5×10^5 multiple myeloma cells added to the upper transwell (Thermo Sci, Waltham, MA, USA, 140640). For the monoculture, a 6 well plate with 2mL of growth media was used to seed 2.5×10^5 multiple myeloma cells. The cells extracted from transwell and monoculture were then subjected to RNA-Sequencing and OMNI-ATAC (OmniSeq, Inc., Buffalo, NY).

RNA-Seq

The Qiagen RNeasy Plus Mini Kit (74134) was used for RNA isolation according to the original instructions. Nanodrop/qubit was then used to quantify the RNA extracted, as well as Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) to assess the RNA integrity. For the RNA-Seq library prep, a starting material containing 250–500 ng of total RNA was used by the WVU Genomics Core using the KAPA mRNA HyperPrep Kit (Roche Diagnostics, Wilmington, MA, USA, 08098123702) following original instructions. The

Genomics Core Facility at Marshall University sequenced the RNA libraries using Illumina Nextseq2000.

RNA-Seq Data Analysis

The paired-end RNA-Seq reads were mapped to the human reference genome using the Subread aligner (Liao et al., 2013). RNA-Seq reads were quantified and summarized after alignment to transcripts at the gene level using featurecounts from Rsubread (Yang Liao et al., 2019) that included a built-in RefSeq (O’Leary et al., 2016) gene annotation. We quantified the gene expression levels using RPKM (reads per kilobase of the exon model per million mapped) (Mortazavi et al., 2008). The Bioconductor package, edgeR (Robinson et al., 2010) was used to examine the differential expression (DE), accounting for biological and technical variability, with the statistical criteria: FDR < 0.05, fold change >1.5, and CPM (count per million; log₂) > 0. To determine the molecular mechanisms of complex disorders and the expression changes of transwell to monoculture, we used Gene Set Enrichment Analysis through GSEA v4.0.2 (Subramanian et al., 2005). The files (rnk) used to run GSEA with a fold change of expression were generated for four comparisons: transwell versus monoculture in wildtype, knockout (KO) versus wildtype (WT) in monoculture, KO versus WT in transwell, and transwell KO versus WT to monoculture KO versus WT. The rnk files contain the gene symbol and expression fold-change. The rnk files served as an input in GSEA that helped us examine the significant preferential expression for various genesets such as MSigDB (Subramanian et al., 2005) hallmark gene sets (Liberzon et al., 2015), BM stroma-induced transcriptional signatures to early plasma cells (NB2PC atlas, <http://www.genomicscape.com/>), Dormant Cells (Lawson et al., 2015), Pre-Plasma Cells (Chaidos et al., 2013), and Pre-Plasma Cells versus Plasma Cells (Chaidos et al., 2013).

Chapter 1

Bone marrow stroma-induced transcriptional signatures of MM cells

We have previously characterized the transcriptome signature of MM cells induced by a BMSC cell line HS5, derived from a healthy donor. We found that transcription changes induced by BMSCs are mainly driven by soluble factors secreted during the interaction between MM cells and BMSCs (Dziadowicz et al., 2022). In this work, we replaced the HS-5 with MSP-1, a BMSC cell line derived from MM patients, and characterized the induced transcriptome signatures.

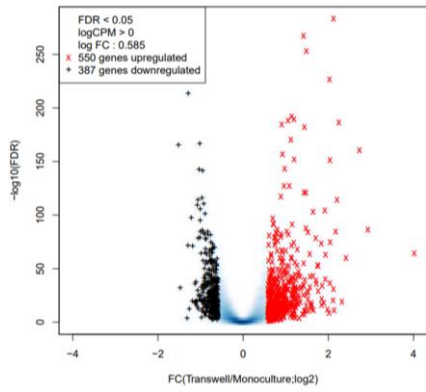
Following our previous work (Dziadowicz et al., 2022), we use transwell systems to characterize the role of JunB in transcription regulation. We placed MM cells in both the upper transwell and the lower chamber, where MSP-1 co-exists. The physical interaction between MM and MSP-1 is expected to stimulate the secretion of various soluble factors from both cell lines. The soluble factors traveling through the membrane of the transwell affected the MM cells presenting in the transwell.

We first characterized the transcriptome signatures of MM cells as induced by MSP-1 as compared to monoculture. Figure 1 visualizes the transcriptional signatures in MM in response to the soluble factors secreted from a co-culture with MSP-1 (Fig 1). We have identified 550 genes upregulated and 387 genes downregulated. GSEA against MSigDB (Subramanian et al., 2005) hallmark gene sets (Liberzon et al., 2015) revealed that soluble factors from the coculture activated several signaling pathways including $\text{TNF}\alpha$, KRAS, IL6/JAK/STAT3, IL2/STAT5, and $\text{TGF}\beta$ (Fig 1B). Consistent with the previous observation (Dziadowicz et al., 2022), gene sets related to cell proliferation such as MYC target, E2F1 target, and G2M checkpoint are downregulated by MSP-1.

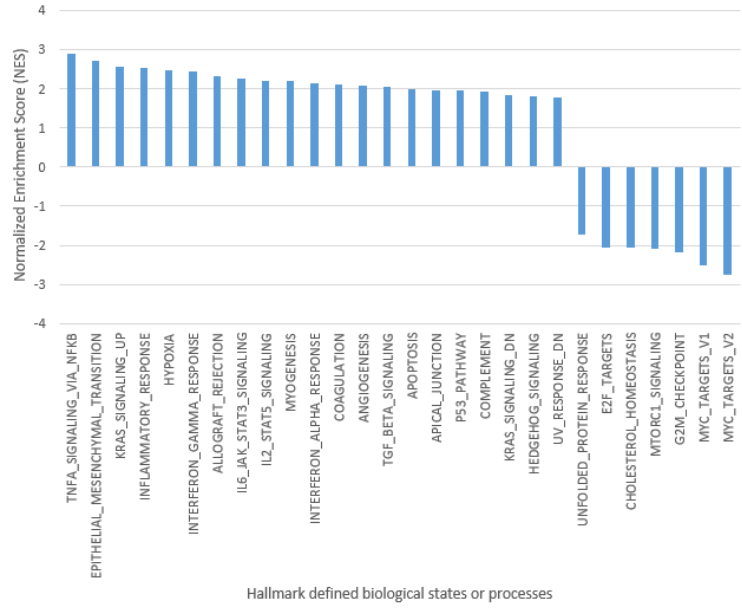
Gene Ontology (GO) enrichment analysis provides a framework for understanding the function of genes with three major ontologies: cellular components, biological processes, and molecular functions. For the current study, we focused on the biological process in upregulated (upper) and downregulated (lower) genes using Metascape (Zhou et al., 2019) (Fig 1C). GO terms such as innate immune response and the inflammatory response were with the Hallmark data analysis (Fig 1B) as well as previous works (Dziadowicz et al., 2022).

Our early work (Dziadowicz et al., 2022) aligned the BM stroma-induced transcriptional signatures to early plasma cells (NB2PC atlas, <http://www.genomicscape.com/>), and Dormant Cells (Lawson et al., 2015). The same gene sets were reanalyzed using the current system (Fig 1D and 1E) with the addition of unfavorable prognostic markers compiled in the previous work (fig 1F). We found that the signature gene of dormant cells and early plasmas cells were upregulated in MM cells by MSP-1, while unfavorable prognostic markers were generally downregulated.

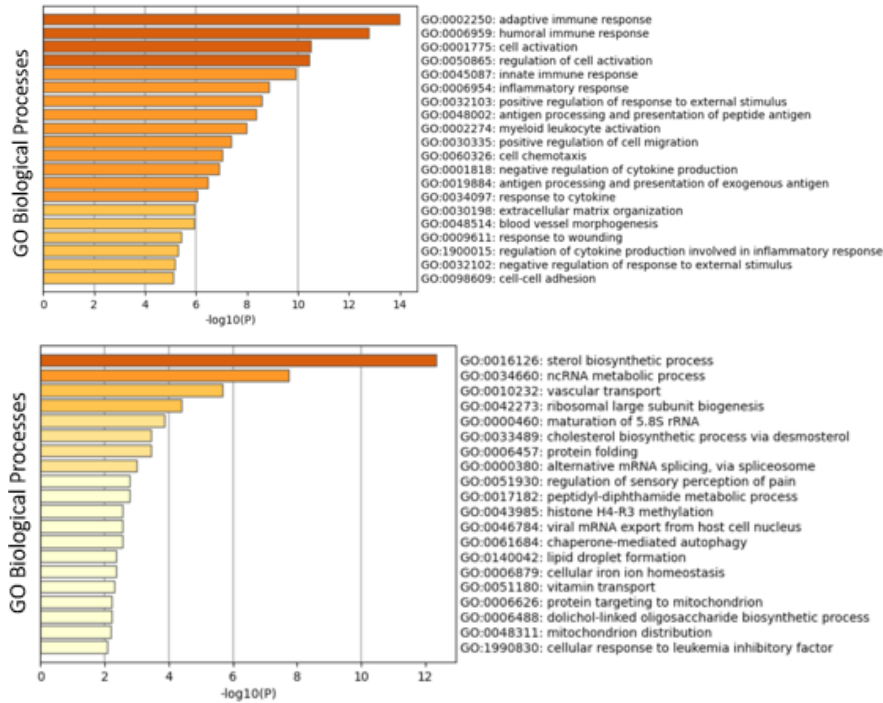
A.



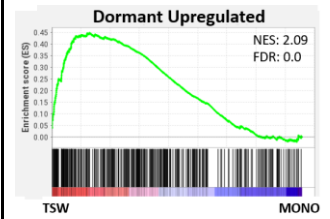
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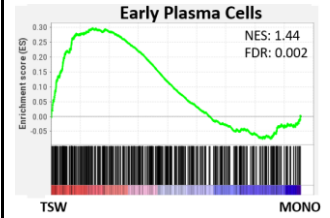
C.



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E.



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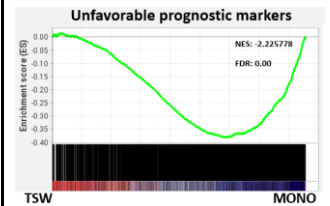


Figure 1: Transcriptional Signatures in Transwell versus Monoculture using Wild Type RPMI8226 cells. A. Volcano plot, demonstrating 550 upregulated genes in red and 387 downregulated genes in black, constructed using an in-house script that plots the negative logarithm of false discovery rate (FDR) on the y axis against the logarithm of fold change (FC) in transwell versus monoculture (FDR<0.05; Fold Change> 1.5). B. Gene-set enrichment analysis (GSEA) against hallmark gene sets (FDR < 0.05). The GSEA consisted of expressed genes that are sorted in order of the expression fold change (Transwell versus mono) from the high (red gradient) to low (blue gradient) against the leading genes that are represented by vertical bars. C. Gene ontology analysis on upregulated genes (upper panel) and downregulated genes (lower panel). D. GSEA analysis against upregulated genes in dormant geneset. E. GSEA analysis against early plasma cells geneset. F. GSEA analysis against unfavorable prognostic markers.

Chapter 2

Transcriptome changes by JunB knockout with and without MSP-1 stimulation

The second chapter focuses on the knockout model compared to wildtype in the absence of MSP-1 (monoculture) or in the presence of MSP-1 (coculture). We first characterized the changes in gene expression in response to loss of JUNB in monoculture without MSP-1 stimulation. Figure 2 visualizes the transcriptional signatures in knockout versus wildtype in monoculture. The volcano plot visualizes 39 upregulated genes in red and 12 downregulated genes in black (fig 2A). The MSigDB (Subramanian et al., 2005) hallmark genesets (Liberzon et al., 2015) has three statistically significant biological processes: xenobiotic metabolism (up-regulated), MYC targets (up-regulated), and cholesterol homeostasis (downregulated) (Fig 2B). The Metascape (Zhou et al., 2019) GO analysis represents upregulated genes (upper panel) and downregulated genes (lower panel) with interesting GO terms such as epithelial cell differentiation (up-regulated), humoral immune response (down-regulated), and regulation of cell growth (down-regulated) (Fig 2C).

In the presence of MSP-1, JUNB deletion upregulates the expression of 32 genes and down-regulates 28 (fig 3A). The MSigDB (Subramanian et al., 2005) hallmark genesets (Liberzon et al., 2015) has various statistically significant biological processes represented as downregulated by JUNB depletion, including hypoxia, IL2/STAT5 signaling, inflammatory response, and TNF α signaling via NF κ B (Fig 3B). The Metascape (Zhou et al., 2019) GO analysis represents upregulated genes (upper panel) and downregulated genes (lower panel) with interesting GO terms such as epithelial cell differentiation, humoral immune response, and regulation of angiogenesis that can be explored further (Fig 3C).

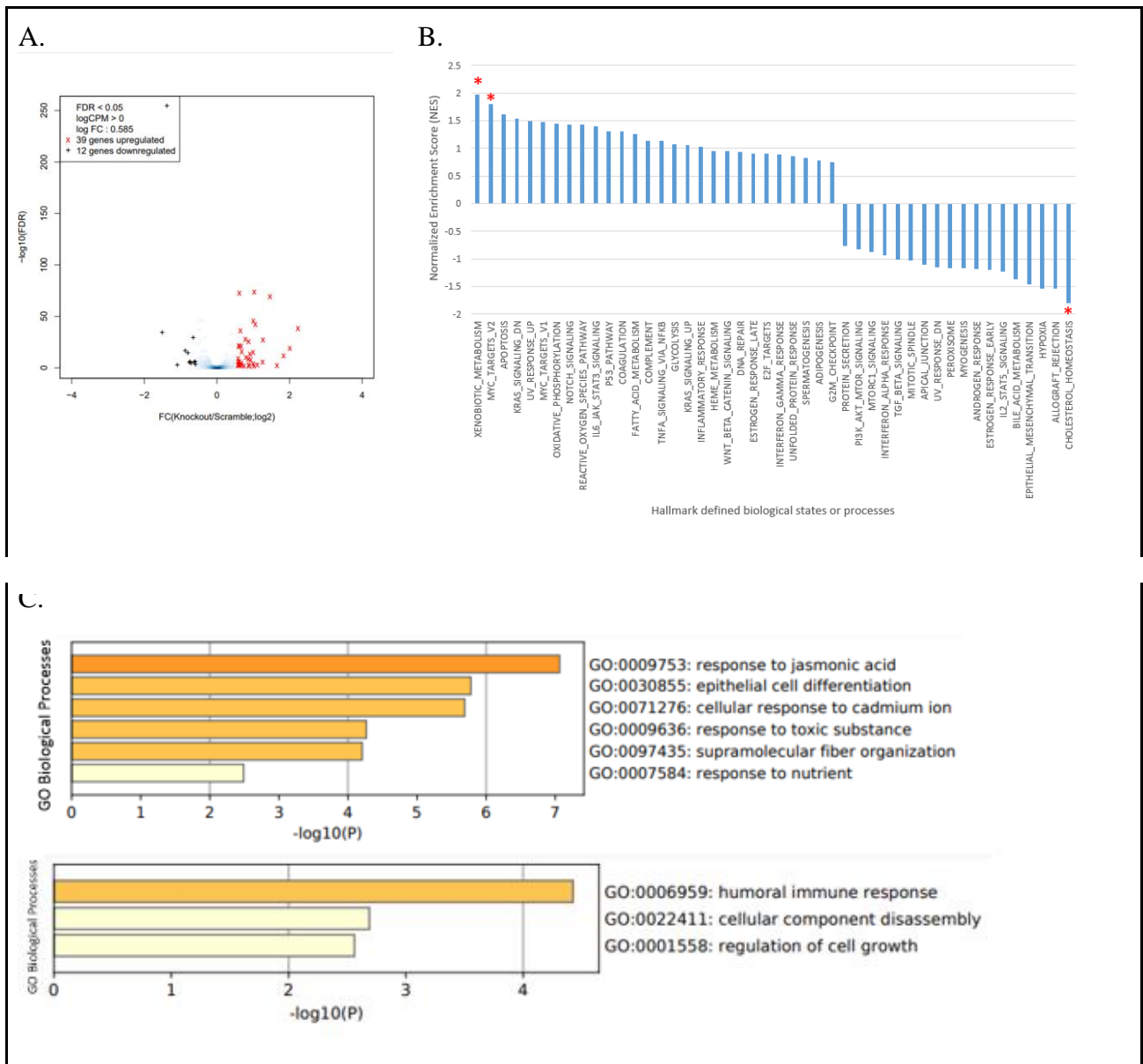


Figure 2: Transcriptional Signatures in Knockout versus Wildtype RPMI8226 in Monoculture. A. Volcano plot demonstrating 39 upregulated genes in red and 12 downregulated genes in black ($FDR < 0.05$; Fold Change > 1.5). B. Gene-set enrichment analysis (GSEA) against hallmark geneset ($FDR < 0.05$ is *). C. Gene ontology analysis on upregulated genes (upper panel) and downregulated genes (lower panel).

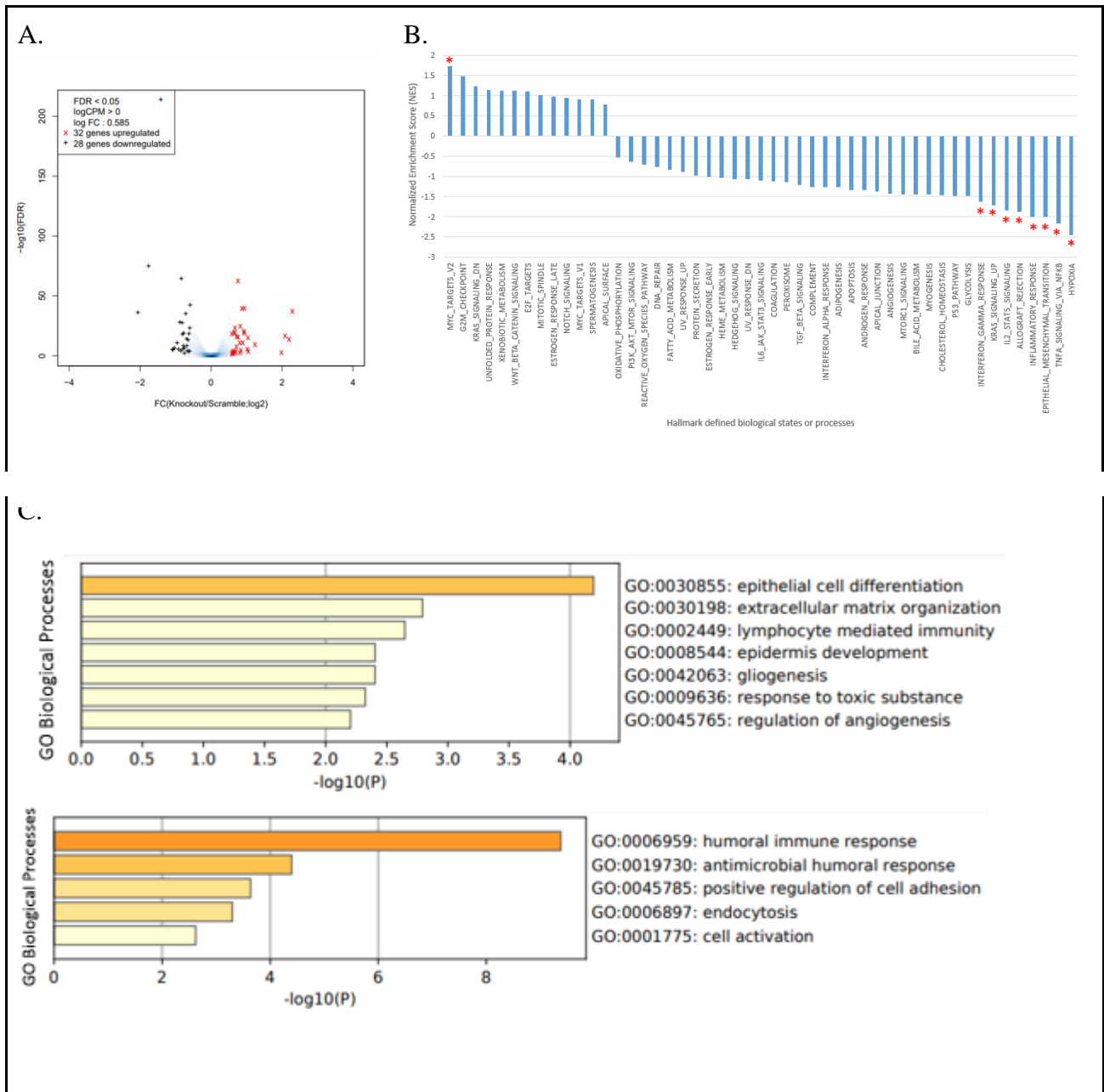


Figure 3: Transcriptional Signatures in Knockout versus Wildtype RPMI8226 in Transwell Co Culture. A. Volcano plot demonstrating 32 upregulated genes in red and 28 downregulated genes in black ($\text{FDR} < 0.05$; Fold Change > 1.5). B. Gene-set enrichment analysis (GSEA) against hallmark geneset ($\text{FDR} < 0.05$ is *). C. Gene ontology analysis on upregulated genes (upper panel) and downregulated genes (lower panel).

Chapter 3

MSP-1 induced and JUNB dependent transcription signatures

We have found a limited number of genes are responsive to the loss of JUNB at baseline level without MSP-1 stimulation. MSP-1 induces an expression upregulation of JUNB by two fold in MM. Consistently loss of JUNB induced more number of differentially expressed genes in the presence of MSP-1 than the absence. We next proceeded to define transcription signatures induced by MSP-1 in a JUNB dependent way.

There are four data groups of focus in this project: transwell knockout, transwell wild type, monoculture knockout, and monoculture wild type. To understand the contribution of all the groups, a flexible statistical framework of two factor analysis was implemented. The analysis compares transwell knockout versus wildtype to monoculture knockout versus wildtype essentially summarizing the project. The gene expression matrix was obtained from the DEGs with statistical filtering of FDR < 0.05 (Fig 4A). Based on the gene expression heatmap generated via MeV, we defined three major groups with distinctive gene expression patterns: namely A, B, and C. In Group A, JunB has an integral in the upregulation of these genes (activator role)(Fig 4B). In Group B, JunB demonstrates a repressive function (Fig 4C). In Group C, JunB also demonstrates a repressive function (Fig 4D). The MSigDB (Subramanian et al., 2005) hallmark genesets (Liberzon et al., 2015) has various statistically significant biological processes represented such as hypoxia, xenobiotic metabolism, inflammatory response, IL6/JAK/STAT3 signaling, and TNF α signaling via NF κ B (Fig 4E).

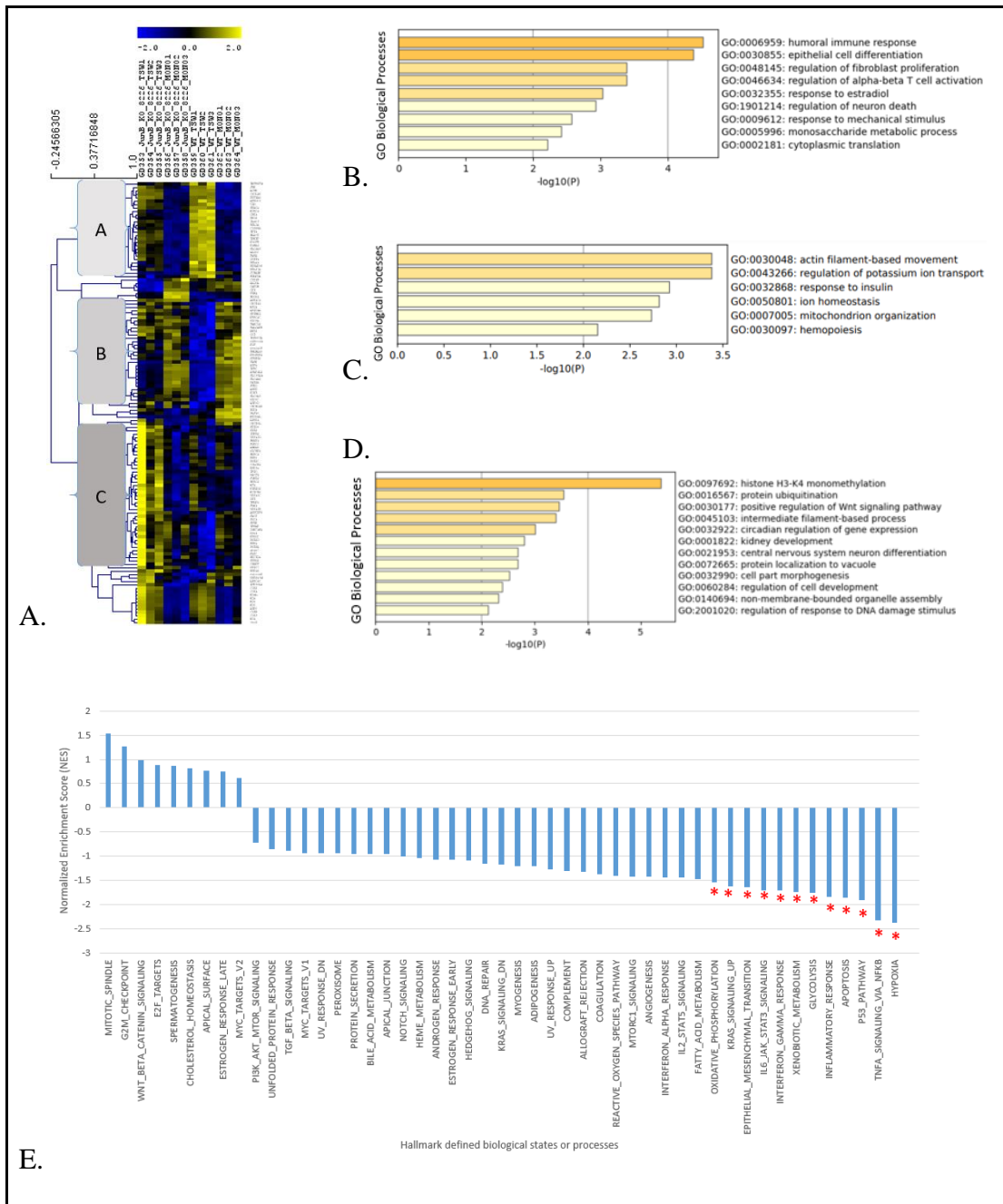


Figure 4: Statistical framework of two factor analysis to compare transwell knockout versus wildtype against monoculture knockout versus wildtype. **A.** Heatmap visualizing all differentially expressed genes (FDR<0.05) and categorized into three groups A-C. **B.** Gene ontology analysis on differentially regulated genes in Group A. **C.** Gene ontology analysis on differentially regulated genes in Group B. **D.** Gene ontology analysis on differentially regulated genes in Group C. **E.** Gene-set enrichment analysis (GSEA) against hallmark geneset (FDR < 0.05 is *).

Discussion

Multiple myeloma (MM) is a bone marrow (BM) disease of malignant plasma cells. MM in its early stages is represented by a significant increase in BM angiogenesis, which is also microenvironment-dependent. There have been connections in the development of drug resistance and the BMSCs interaction with MM cells. Even with phenomenal therapeutic advances in drug discovery and patient survival improvements, MM has remained terminal with recurring need for new therapies. The acquisition of resistance has been one of the leading causes of the failure of cancer therapy, and the aspects of the tumor microenvironment and its impact on targeted cancer therapies can be potential research interests (Fu et al., 2015).

Clonal evolution of MM cells and the BM contributes to drug resistance. Two drug resistances introduced in the background are de novo cancer drug resistance and acquired cancer drug resistance. Epigenetic events, and genetic changes such as histone modifications, DNA methylation, and acetylation have been studied to understand the chemoresistance and the resulting relapse in malignancies (Aziz and Ahmad, 2020). Novel epigenetic therapies are important in understanding the regulation of several physiological processes and how their expression is controlled through epigenetic regulation (Issa et al., 2017) (Aziz and Ahmad, 2020). In the BM, the SM-DR and CAM-DR occur rapidly, but are reversible once the microenvironment stimulations are relieved, suggesting the role of epigenetic regulations as an attribute to gain drug resistance.

The exploratory research characterizes the transcriptional signature in MM induced by soluble factors and adhesion to a MM patient derived BMSC cell line (namely, MSP-1). The number of differentially expressed genes was substantially less than what we observed previously using a BMSC cell line from health donor (namely HS5) (Dziadowicz et al., 2022). We found that the MSP-1 of the current batch is not effectively secreting IL-6 (data not shown), which may explain the mild expression changes.

The in-vitro tranwell and coculture system was integral in examining the BMSC induced chromatin reorganization in MM (Dziadowicz et al., 2022) and we further expanded on the connection between transcription reprogramming and the transcriptome signatures that might be induced by BMSCs. While the results lay consistent with previous studies (Dziadowicz et al., 2022; Lam et al., 2018; McMillin et al., 2010), there has been a less-differentiated MM cell phase when compared to the pre-PC cells and PC cell signatures, observed when interacting with BMSCs. This attribute helps us explore the stem-like nature of the MM cells in the BM microenvironment and can be beneficial for studying drug resistance.

BMSCs induce upregulation of JunB in MM (Fan et al., 2021). JunB has been characterized as a regulator associated with angiogenesis, along with hepatocyte growth factor (HGF), angiopoietins, and VEGF (Soliman et al., 2021). JunB is essential in development, cell proliferation, cell invasion, migration, and metastasis (da Costa Reis Monte-Mór et al., 2009; Hyakusoku et al., 2016; Kenner et al., 2004). As we continue to explore the function of JunB in MM, one of the key questions we would like to explore is if there is JunB functional redundancy with other members of the AP-1 family that may compensate for the JunB knockout.

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