



Development and Semi-Automated Analysis of an *in vitro* Dissemination Model
for Myeloma Cells Interacting with Mesenchymal Stromal Cells

Entwicklung und semi-automatisierte Analyse eines *in vitro* Modells
für die Disseminierung von Myelomzellen in Interaktion mit mesenchymalen Stromazellen

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Summary

This thesis integrates biomedical research and data science, focusing on an *in vitro* model for studying myeloma cell dissemination and a Python-based tool, `plotastic`, for semi-automated analysis of multidimensional datasets. Two major challenges are addressed: (1) understanding the early steps of myeloma dissemination and (2) improving data analysis efficiency to address the complexity- and reproducibility bottlenecks currently present in biomedical research.

In the experimental component, primary human mesenchymal stromal cells (hMSCs) are co-cultured with INA-6 myeloma cells to study cell proliferation, attachment, and detachment via time-lapse microscopy. Key findings reveal that detachment often follows cell division, predominantly driven by daughter cells. Novel separation techniques were developed to isolate myeloma subpopulations for further characterization by RNAseq, cell viability, and apoptosis assays. Differential expression of adhesion and retention factors upregulated by INA-6 cells correlates with patient survival. Overall, this work provides insights into myeloma dissemination mechanisms and identifies genes that potentially counteract dissemination through adhesion, which could be relevant for the design of new therapeutics.

To manage complex data, a Python-based software named `plotastic` was developed that streamlines analysis and visualization of multidimensional datasets. `plotastic` is built on the idea that statistical analyses are performed based on how the data is visualized. This approach not only simplifies data analysis, but semi-automates analysis in a standardized statistical protocol. The thesis becomes a case study as it reflects on the application of `plotastic` to the *in vitro* model, demonstrating how the software facilitates rapid adjustments and refinements in data analysis and presentation. Such efficiency is crucial for handling semi-big data transparently, which—despite being manageable—is complex enough to complicate analysis and reproducibility.

Together, this thesis illustrates the synergy between experimental methodologies and advanced data analysis tools. The *in vitro* model provides a robust platform for studying myeloma dissemination, while `plotastic` addresses the need for efficient data analysis. Combined, they offer an approach to handling complex experiments, advancing both cancer biology and research practices, in support of exploratory and transparent analysis of challenging phenomena.

Zusammenfassung

Diese Doktorarbeit integriert biomedizinische Forschung und Datenwissenschaften und konzentriert sich auf ein *in vitro*-Modell zur Untersuchung der Dissemination von Myelomzellen sowie ein Python-basiertes Werkzeug, `plotastic`, zur semi-automatisierten Analyse multidimensionaler Datensätze. Zwei Hauptprobleme werden bearbeitet: (1) das Verständnis der frühen Schritte der Myelomdissemination und (2) die Verbesserung der Effizienz der Datenanalyse, um die derzeit in der biomedizinischen Forschung vorhandenen Engpässe bezüglich Komplexität und Reproduzierbarkeit zu adressieren.

Im experimentellen Teil werden primäre menschliche mesenchymale Stromazellen (hMSCs) mit INA-6-Myelomzellen kokultiviert, um Zellproliferation, Anhaftung und Ablösung mittels Zeitraffer-Mikroskopie zu untersuchen. Zentrale Erkenntnisse zeigen, dass die Ablösung oft auf die Zellteilung folgt und vorwiegend von Tochterzellen angetrieben wird. Neue Trennungstechniken wurden entwickelt, um Myelom-Subpopulationen für weitere Charakterisierungen durch RNAseq, Zellviabilität und Apoptose-Assays zu isolieren. Die differentielle Expression von Adhäsions- und Retentionsfaktoren, die durch INA-6 Zellen hochreguliert werden, korreliert mit dem Überleben der Patienten. Insgesamt liefert diese Arbeit Einblicke in die Mechanismen der Myelomdissemination und identifiziert Gene, die potenziell die Dissemination durch Adhäsion konterkarieren könnten, was für die Entwicklung neuer Therapeutika relevant sein könnte.

Zur Verwaltung komplexer Daten wurde eine Python-basierte Software namens `plotastic` entwickelt, welche die Analyse und Visualisierung multidimensionaler Datensätze optimiert. `plotastic` basiert auf der Idee, dass statistische Analysen basierend darauf durchgeführt werden, wie die Daten visualisiert werden. Dieser Ansatz vereinfacht nicht nur die Datenanalyse, sondern automatisiert sie auch teilweise in einem standardisierten statistischen Protokoll. Die Arbeit wird zu einer Fallstudie, da sie die Anwendung von `plotastic` auf das *in vitro*-Modell reflektiert und zeigt, wie die Software schnelle Anpassungen und Verfeinerungen in der Datenanalyse und -präsentation erleichtert. Eine solche Effizienz ist entscheidend für den transparenten Umgang mit Semi-Big-Data, die trotz ihrer Handhabbarkeit komplex genug ist, um die Analyse und Reproduzierbarkeit zu erschweren.

Zusammengefasst veranschaulicht diese Dissertation die Synergie zwischen experimentellen Methoden und fortgeschrittenen Werkzeugen der Datenanalyse. Das *in vitro*-Modell bietet eine robuste Plattform für die Untersuchung der Myelomdissemination, während `plotastic` den Bedarf an effizienter Datenanalyse adressiert. Zusammen bieten sie einen Ansatz für die Bearbeitung komplexer Experimente, fördern sowohl die Krebsbiologie als auch die Forschungspraktiken und unterstützen die explorative und transparente Analyse herausfordernder Phänomene.

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Abbreviations

APRIL A Proliferation-Inducing Ligand	6
aMM asymptomatic Multiple Myeloma	3
BAFF B-cell Activating Factor	6
BM Bone Marrow	3
BMME Bone Marrow Microenvironment	4
BMPC Bone Marrow Plasma Cell	6
BMSC Bone Marrow Stromal Cell	6
CAD Cell Adhesion Dynamics	96
CM hMSC-conditioned medium	35
CM-INA6 MSC-Conditioned-Medium-treated INA-6	47
CAM Cell Adhesion Molecule	6
CLI Command Line Interface	70
CXCL12 C-X-C Motif Chemokine Ligand 12	6
DKK1 Dickkopf-1	12
ECM Extracellular Matrix	6
EMT Epithelial-Mesenchymal Transition	5
FACS Fluorescence-Activated Cell Sorting	85
GFI1 Growth Factor Independence 1	13
GUI Graphical User Interface	70
HDAC1 Histone Deacetylase 1	13
hMSC human Mesenchymal Stromal Cell	9
HSC Hematopoietic Stem Cell	9
IL-6 Interleukin-6	6
IGF-1 Insulin-like Growth Factor 1	6
JNK c-Jun N-terminal Kinase	12
LLM Large Language Model	70
MA MSC-adhering	44
MACS Magnetic-Activated Cell Sorting	37
MIP-1α Macrophage Inflammatory Protein-1 α	12
MSC Mesenchymal Stromal Cell	8
MGUS Monoclonal Gammopathy of Undetermined Significance	3
MM Multiple Myeloma	3
MMR Multiple Myeloma Relapse	4
MBD Multiple Myeloma related Bone Disease	11
NDMM Newly Diagnosed Multiple Myeloma	4
NF-κB Nuclear Factor- κ B	12
nMA non-MSC-adhering	44
OS Overall Survival	50
PCL Plasma Cell Leukemia	4

PFS	Progression-Free Survival	50
PPAR-γ	Peroxisome Proliferator-Activated Receptor- γ	13
RUNX2	Runt-related Transcription Factor 2	12
RANKL	Receptor Activator of Nuclear Factor- κ B Ligand	12
SASP	Senescence-Associated Secretory Phenotype	12
SDF-1	Stromal Cell-Derived Factor-1	6
sMM	smouldering Multiple Myeloma	3
SOST	Sclerostin	12
SP	Solitary Plasmacytoma	3
TAD	Topologically Associated Domain	9
TGF-β	Transforming Growth Factor- β	8
TNF-α	Tumor Necrosis Factor- α	13
TNFSF13	Tumor Necrosis Factor Superfamily, Member 13	6
VCAM-1	Vascular Cell Adhesion Molecule-1	6
VEGF	Vascular Endothelial Growth Factor	6
VLA-4	Very Late Antigen-4—also known as CD49d or ITGA4 Subunit α —	6
Wnt	Wingless-related Integration Site	12
WPSC	Well Plate Sandwich Centrifugation	36

Glossary

Introduction

Aims

This PhD thesis is designed to bridge significant gaps in the understanding and analysis of myeloma cell behavior and the handling of complex biomedical datasets. The specific aims are as follows:

- Develop an *in vitro* model to elucidate the mechanisms of myeloma cell dissemination in interaction with mesenchymal stromal cells (hMSCs), focusing particularly on:
 - Observing and quantifying cell proliferation, attachment, and detachment dynamics using time-lapse microscopy.
 - Isolating and characterizing distinct myeloma subpopulations interacting with hMSCs to understand differential gene expression related to cell adhesion and patient survival.
- Design and implement a Python-based software tool, `plotastic`, to facilitate the analysis of multidimensional datasets generated in biomedical research. This tool will aim to:
 - Streamline the data analysis process, making it more efficient and reproducible.
 - Integrate visualization and statistical analysis capabilities to ensure that data analysis protocols are aligned with the ways in which data is visualized.
 - Provide a case study demonstrating the application of `plotastic` in the analysis of *in vitro* dissemination experiments, emphasizing the tool's ability to handle semi-big data and enhance reproducibility.
- Synthesize the findings from the experimental and software development components to advance the understanding of myeloma dissemination and improve research practices in biomedical data analysis.

These aims are crafted to address both the biological and technical challenges in current cancer research methodologies and data science applications in biomedicine, fostering advancements that could lead to novel therapeutic strategies and more robust scientific inquiries.

Chapter 1: Modelling Myeloma Dissemination *in vitro*

The following chapter—including referenced appendices—has been taken with slight adjustments from Kuric et al. (2024), published in *Cancer Research Communications* under the CC BY 4.0 license.

Abstract

Multiple myeloma involves early dissemination of malignant plasma cells across the bone marrow; however, the initial steps of dissemination remain unclear. Human bone marrow-derived mesenchymal stromal cells (hMSCs) stimulate myeloma cell expansion (e.g., IL-6) and simultaneously retain myeloma cells via chemokines (e.g., CXCL12) and adhesion factors. Hence, we hypothesized that the imbalance between cell division and retention drives dissemination. We present an *in vitro* model using primary hMSCs co-cultured with INA-6 myeloma cells. Time-lapse microscopy revealed proliferation and attachment/detachment dynamics. Separation techniques (V-well adhesion assay and well plate sandwich centrifugation) were established to isolate MSC-interacting myeloma subpopulations that were characterized by RNAseq, cell viability and apoptosis. Results were correlated with gene expression data ($n = 837$) and survival of myeloma patients ($n = 536$). On dispersed hMSCs, INA-6 saturate hMSC-surface before proliferating into large homotypic aggregates, from which single cells detached completely. On confluent hMSCs, aggregates were replaced by strong heterotypic hMSC-INA-6 interactions, which modulated apoptosis time-dependently. Only INA-6 daughter cells (nMA-INA6) detached from hMSCs by cell division but sustained adherence to hMSC-adhering mother cells (MA-INA6). Isolated nMA-INA6 indicated hMSC-autonomy through superior viability after IL-6 withdrawal and upregulation of proliferation-related genes. MA-INA6 upregulated adhesion and retention factors (CXCL12), that, intriguingly, were highly expressed in myeloma samples from patients with longer overall and progression-free survival, but their expression decreased in relapsed myeloma samples. Altogether, *in vitro* dissemination of INA-6 is driven by detaching daughter cells after a cycle of hMSC-(re)attachment and proliferation, involving adhesion factors that represent a bone marrow-retentive phenotype with potential clinical relevance.

Statement of Significance

Novel methods describe *in vitro* dissemination of myeloma cells as detachment of daughter cells after cell division. Myeloma adhesion genes were identified that counteract *in vitro* detachment with potential clinical relevance.

Introduction

Multiple myeloma arises from clonal expansion of malignant plasma cells in the bone marrow (BM). At diagnosis, myeloma cells have disseminated to multiple sites in the skeleton and, in some cases, to “virtually any tissue” (Bladé et al., 2022; Rajkumar et al., 2014). However, the mechanism through which myeloma cells initially disseminate remains unclear. Dissemination is a multistep process involving invasion, intravasation, intravascular arrest, extravasation, and colonization (Zeissig et al., 2020). To initiate dissemination, myeloma cells overcome adhesion, retention, and dependency on the BM microenvironment, which could involve the loss of adhesion factors such as CD138 (Akhmetzyanova et al., 2020; García-Ortiz et al., 2021). BM retention is mediated by multiple factors: First, chemokines (CXCL12 and CXCL8) produced by mesenchymal stromal cells (MSCs), which attract plasma cells and prime their cytoskeleton and integrins for adhesion (Aggarwal et al., 2006; Alsayed et al., 2007). Second, myeloma cells must overcome the anchorage and physical boundaries of the extracellular matrix (ECM), consisting of e.g. fibronectin, collagens, and proteoglycans such as decorin (X. Hu et al., 2021; Huang et al., 2015; Katz, 2010; Kibler et al., 1998). Simultaneously, ECM provides signals inducing myeloma cell cycle arrest or progression the cell cycle (X. Hu et al., 2021; Katz, 2010). ECM is also prone to degradation, which is common in several osteotropic cancers, and is the cause of osteolytic bone disease. This is driven by a ‘vicious cycle’ that maximizes bone destruction by extracting growth factors (EGF and TGF- β) that are stored in calcified tissues (Glavey et al., 2017). Third, direct contact with MSCs physically anchors myeloma cells to the BM (Zeissig et al., 2020; Sanz-Rodríguez et al., 1999). Fourth, to disseminate to distant sites, myeloma cells require, at least partially, independence from essential growth and survival signals provided by MSCs in the form of soluble factors or cell adhesion signaling (García-Ortiz et al., 2021; Chatterjee et al., 2002; Hideshima et al., 2007). For example, the VLA-4 (Myeloma)-VCAM-1 (MSC)-interface activates NF- κ B in both myeloma and MSCs, inducing IL-6 expression in MSCs. The independence from MSCs is then acquired through autocrine survival signaling (Frassanito et al., 2001; Urashima et al., 1995). In short, anchorage of myeloma cells to MSCs or ECM is a ‘double-edged sword’: adhesion counteracts dissemination, but also presents signaling cues for growth, survival, and drug resistance (Solimando et al., 2022).

To address this ambiguity, we developed an *in vitro* co-culture system modeling diverse adhesion modalities to study dissemination, growth, and survival of myeloma cells and hMSCs. Co-cultures of hMSCs and the myeloma cell line INA-6 replicated tight interactions and aggregate growth, akin to “micrometastases” in Ghobrial’s metastasis concept (Ghobrial, 2012). We characterized the growth conformations of hMSCs and INA-6 as ho-

motypic aggregation *vs.* heterotypic hMSC adherence and their effects on myeloma cell survival. We tracked INA-6 detachments from aggregates and hMSCs, thereby identifying a potential “disseminated” subpopulation lacking strong adhesion. Furthermore, we developed innovative techniques (V-well adhesion assay and well plate sandwich centrifugation) to separate weakly and strongly adherent subpopulations for the subsequent analysis of differential gene expression and cell survival. Notably, our strategy resolves the differences in gene expression and growth behavior between cells of one cell population in “direct” contact with MSCs. In contrast, previous methods differentiated between “direct” and “indirect” cell-cell contact using transwell inserts (Dziadowicz et al., 2022). To evaluate whether genes mediating adhesion and growth characteristics of INA-6 were associated with patient survival, we analyzed publicly available datasets (Seckinger et al., 2017, 2018).

Materials and Methods

See Appendix A.3 for a complete method list and description.

Ethics Statement

Primary human MSCs were collected with the written informed consent of all patients. The procedure was conducted in accordance with recognized ethical guidelines (Helsinki Declaration) and approved by the local Ethics Committee of the University of Würzburg (186/18).

Cultivation and Co-Culturing of primary hMSCs and INA-6

Primary human MSCs were obtained from the femoral head of 34 non-myeloma patients (Appendix A: Tab. 1: 21 male and 13 female, mean age 68.9 ± 10.6) undergoing elective hip arthroplasty. The INA-6 cell line (*DSMZ Cat# ACC-862, RRID:CVCL_5209*, link) was initially isolated from a pleural effusion sample obtained from an 80-year-old male with multiple myeloma (Burger, Günther, et al., 2001; Gramatzki et al., 1994). hMSCs were not tested for mycoplasma, whereas stocks of INA-6 were tested in this study (Appendix A: Tab. 1) using the *Venor GEM OneStep* kit (Minerva Biolabs, Berlin, Germany). For each co-culture, hMSCs were seeded 24 h before INA-6 addition to generate the hMSC-conditioned medium (CM). INA-6 cells were washed with PBS, resuspended in MSC medium, and added to hMSCs so that the co-culture comprised 33 % (v/v) of CM gathered directly from the respective hMSC donor. The co-cultures were not substituted for IL-6 (Chatterjee et al., 2002).

Cell Viability and Apoptosis Assay

Cell viability and apoptosis rates were measured using *CellTiter-Glo Luminescent Cell Viability Assay* and *Caspase-Glo 3/7 Assay*, respectively (Promega GmbH, Mannheim, Germany).

Automated Fluorescence Microscopy

Microscopic images were acquired using an Axio Observer 7 (Zeiss) with a COLIBRI LED light source and motorized stage top using 5x and 10x magnification. The tiled images had an automatic 8–10 % overlap and were not stitched.

Live Cell Imaging

hMSCs (stained with PKH26) were placed into an ibidi Stage Top Incubation System and equilibrated to 80 % humidity and 5 % CO₂. INA-6 (2×10^3 cells/cm²) were added directly before the start of acquisition. Brightfield and fluorescence images of up to 13 mm² of the co-culture area were acquired every 15 min for 63 h. Each event of interest was manually analyzed and categorized into defined event parameters.

V-well Adhesion Assay

INA-6 cells were arrested during mitosis by two treatments with thymidine, followed by nocodazole. Arrested INA-6 were released and added to 96 V-well plates (10⁴ cells/cm²) on top of confluent hMSCs and adhered for 1–3 h. The co-culture was stained with calcein-AM (Thermo Fisher Scientific, Darmstadt, Germany) before non-adherent INA-6 were pelleted into the tip of the V-well (555 g, 5–10 min). MSC-adhering INA-6 cells were manually detached by rapid pipetting. The pellet brightness was measured microscopically and the pellet was isolated by pipetting.

Cell Cycle Profiling by Image Cytometry

Isolated INA-6 cells were fixed in 70 % ice-cold ethanol, washed, resuspended in PBS, distributed in 96-well plates, and stained with Hoechst 33342. The plates were scanned at 5x magnification. A pre-trained convolutional neural network (Intellesis, Zeiss) was fine-tuned to segment the scans into single nuclei and exclude fragmented nuclei. Nuclei were filtered to exclude extremes of size and roundness. The G0/G1 frequency was determined by Gaussian curve fitting.

Well Plate Sandwich Centrifugation (WPSC)

hMSCs were grown to confluence in 96-well plates coated with collagen I (rat tail; Corning, NY, USA). INA-6 cells were added and the cells were allowed to adhere for 24 h. A second plate (“catching plate”) was attached upside down to the top of the co-culture plate. That “well plate sandwich” was turned around and the content of the co-culture plate was centrifuged into the catching plate three times (40 s at 110 g) while gently adding 30 µL of medium in between centrifugation steps. Non-MSC-adhering INA-6 cells were collected from the catching plate, whereas MSC-adhering INA-6 cells were isolated by digesting the co-culture with accutase. For RNA sequencing (RNAseq), all samples were purified using

anti-CD45 Magnetic-Activated Cell Sorting (MACS) (Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach).

RNA Isolation

RNA was isolated using the *NucleoSpin RNA II Purification Kit* (Macherey-Nagel) according to the manufacturer's instructions. RNA was isolated from INA-6 cells co-cultured with a unique hMSC donor ($n = 5$ for RNA sequencing, $n = 11$ for qPCR).

RNA sequencing, Differential Expression, and Functional Enrichment Analysis

RNA sequencing (RNAseq) was performed at the Core Unit Systems Medicine, University of Würzburg. mRNA was enriched with polyA beads. Fastq files were aligned to the GRCh38 reference genome using STAR (*RRID:SCR_004463*, link) and raw read counts were generated using HTseq (*RRID:SCR_005514*, link) (Anders et al., 2015; Dobin et al., 2013; Zerbino et al., 2018). Differential gene expression was analyzed using edgeR in R (version 3.6.3) (*RRID:SCR_012802*, link). Functional enrichment analysis was performed using Metascape (*RRID:SCR_016620*, link) (Y. Zhou et al., 2019).

RT-qPCR

RNA (1 µg) was reverse transcribed using *SuperScript IV reverse transcriptase* (Thermo Fisher Scientific). qPCR was performed using 10 µL *GoTaq qPCR Master Mix* (Promega), 1:10 diluted cDNA, and 5 pmol of primers obtained from Biomers.net or Qiagen (Appendix A: Tab. 3).

Statistical Analysis

Inferential statistics were performed using Python (IPython, *RRID:SCR_001658*, link) (3.10) packages *pingouin* (0.5.1) and *statsmodels* (0.14.0) (Vallat, 2018; Seabold & Perktold, 2010). The figures were plotted using *plotastic* (0.0.1) (Kuric & Ebert, 2024). Normality (for $n \geq 4$) and sphericity were ensured using Mauchly's and Shapiro-Wilk tests, respectively. Data points were Log_{10} transformed to convert the scale from multiplicative to additive or to fulfill sphericity requirements. $p = 0.05 > * > 0.01 > ** > 10^{-3} > *** > 10^{-4} > ****$. p -values were either adjusted (*p*-adj) or not adjusted (*p*-unc) for family wise error rate. Power calculations were not performed to determine the sample size.

Patient Cohort, Analysis of Survival and Expression

Survival and gene expression data were obtained as previously described (Seckinger et al., 2017, 2018) and are available at the European Nucleotide Archive (ENA) under accession numbers PRJEB36223 and PRJEB37100. The expression level was categorized into “high” and “low” using `maxstat` (Maximally selected Rank Statistics) thresholds (Hothorn & Lausen, 2017).

Data Availability Statement

A detailed description of the methods is provided in the Supplementary Material section. Raw tabular data and examples of analyses and videos are available in the github repository, [link](#). Raw RNAseq data are available from the NCBI Gene Expression Omnibus (GEO) (*RRID:SCR_005012*, [link](#)) (GSE261423). Microscopy data are available at BioStudies (EMBL-EBI) (*RRID:SCR_004727*, [link](#)) (S-BIAD1092).

Results

INA-6 Cells Saturate hMSC-Interaction to Proliferate into Aggregates

hMSCs are isolated as a heterogeneous cell population. To analyze whether INA-6 cells could adhere to every hMSC, we saturated hMSCs with INA-6. A seeding ratio of 1:4 (hMSC:INA-6) resulted in the occupation of $93 \pm 6\%$ of single hMSCs by one or more INA-6 cells within 24 hours after INA-6 addition, escalating to 6% after 48 hours (Fig. 1A, B). Therefore, most hMSCs provide an interaction surface for INA-6 cells.

INA-6 exhibits homotypic aggregation when cultured alone, a phenomenon observed in some freshly isolated myeloma samples (up to 100 cells after 6 hours) (Kawano et al., 1991; Okuno et al., 1991). Adding hMSCs at a 1:1 ratio led to smaller aggregates after 24 hours (size 1–5 cells), all of which were distributed over $52 \pm 2\%$ of all hMSCs (Fig. 1A, B). Intriguingly, INA-6 aggregation was notably absent when grown on confluent hMSCs, and occurred only when heterotypic interactions were limited to 0.2 hMSCs per INA-6 cell (Fig. 1C). We concluded that INA-6 cells prioritize heterotypic over homotypic interactions.

To monitor the formation of such aggregates, we conducted live-cell imaging of hMSC/INA-6 co-cultures for 63 hours. We observed that INA-6 cells adhered long after cytokinesis, constituting $55 \pm 12\%$ of all homotypic interactions between 13 hours and 26 hours, increasing to more than 75% for the remainder of the co-culture (Fig. 1D). Therefore, homotypic INA-6 aggregates were mostly formed by cell division.

Apoptosis of INA-6 Depends on Ratio Between Heterotypic and Homotypic Interaction

Although direct interaction with hMSCs has been shown to enhance myeloma cell survival through NF- κ B signaling (Hideshima et al., 2007), the impact of aggregation on myeloma cell viability during hMSC interaction remains unclear. To address this, we measured the cell viability (ATP) and apoptosis rates of INA-6 cells growing as homotypic aggregates compared to those in heterotypic interactions with hMSCs by modulating hMSC density (Fig. 1E). To equalize the background signaling caused by soluble MSC-derived factors, all cultures were incubated in hMSC-conditioned medium and the results were normalized to INA-6 cells cultured without direct hMSC contact (Fig. 1E, left).

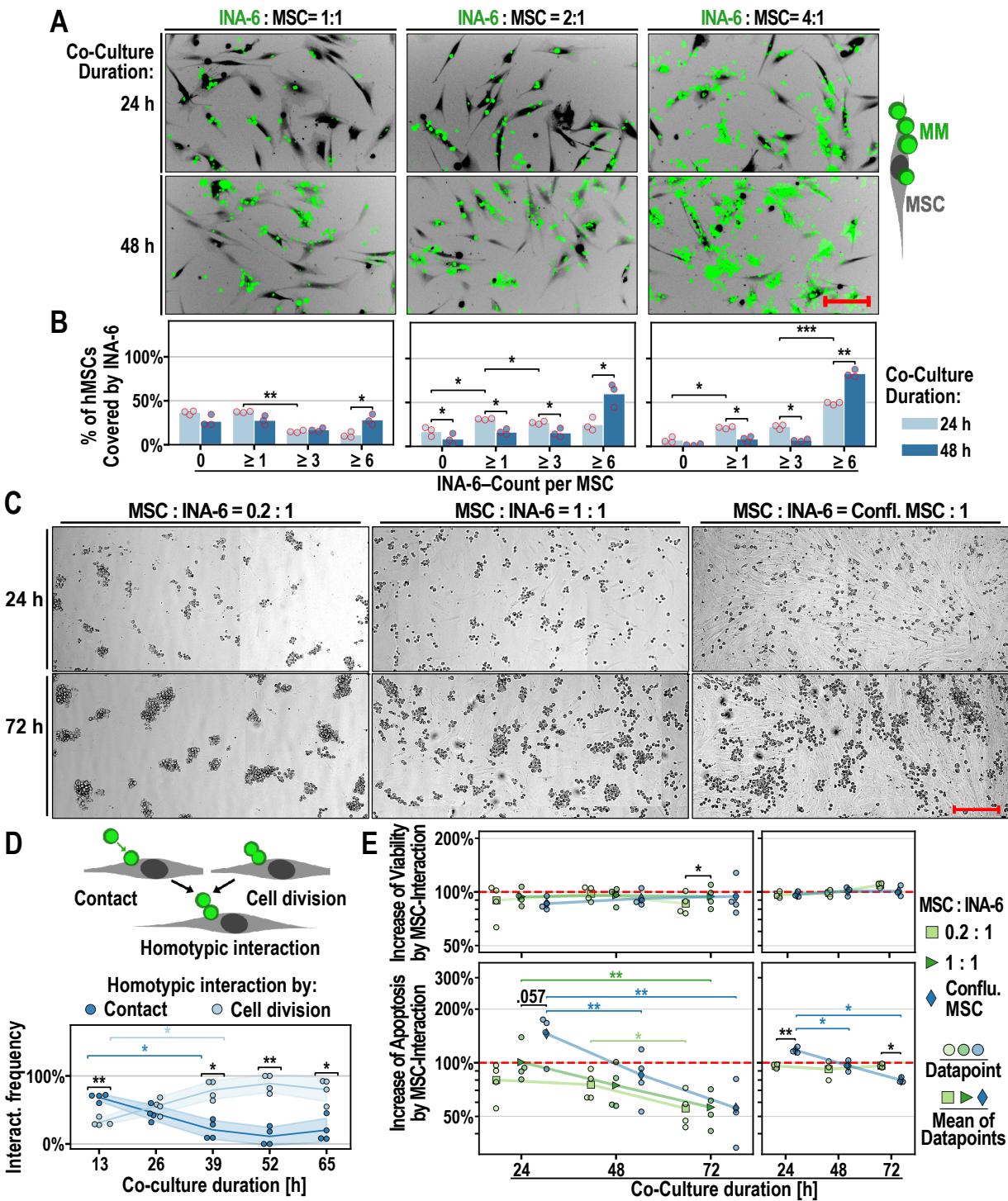


Figure 1: INA-6 growth conformations and survival on hMSCs. **A:** Interaction of INA-6 (green) with hMSCs (black, negative staining) at different INA-6 densities (constant hMSC densities). Scale bar = 200 μ m. **B:** Frequency of single hMSCs (same as A) that are covered by INA-6 of varying group sizes. Technical replicates = three per datapoint; 100 single hMSCs were evaluated per technical replicate. **C:** Interaction of INA-6 with hMSCs at different hMSC densities (constant INA-6 densities). Scale bar = 300 μ m. **D:** Two types of homotypic interaction: Attachment after cell contact and sustained attachment of daughter cells after cell division. Datapoints represent one of four independent time-lapse recordings, each evaluating 116 interaction events.

– continued on next page

Figure 1: continued from previous page – **E:** Effects of hMSC-density on the viability (ATP, top) and apoptosis (Caspase3/7 activity, bottom). INA-6:MSC ratio = 4:1; Technical replicates = four per datapoint; **E left:** Signals were measured in INA-6 washed off from hMSCs and normalized by INA-6 cultured in MSC-conditioned medium (= red line) ($n = 4$). **E right:** Signals were measured in co-cultures and normalized by the sum of the signals measured in hMSC and INA-6 cultured separately (= red line) ($n = 3$). **Statistics:** Paired t-test, two-factor RM-ANOVA. Datapoints represent independent co-cultures with hMSCs from three (A, B, D, E right), four (E left) unique donors. Confl. = Confluent. This content has been taken as is from Kuric et al. (2024), published in *Cancer Research Communications* under the CC BY 4.0 license.

INA-6 viability (ATP) was not affected by the direct adhesion of hMSCs at any density. However, apoptosis rates decreased over time [$F(2, 6) = 23.29$, $p\text{-unc} = 1.49 \times 10^{-3}$] (Two-factor RM-ANOVA), interacting significantly with MSC density [$F(4, 12) = 6.98$, $p\text{-unc} = 3.83 \times 10^{-3}$] For example, 24 hours of adhesion to confluent MSCs increased apoptosis rates by 1.46 ± 0.37 fold, while culturing INA-6 cells on dispersed hMSCs (ratio 1:1) did not change the apoptosis rate (1.01 ± 0.26 fold).

We presumed that sensitive apoptotic cells might have been lost when harvesting INA-6 cells from hMSCs. Hence, we measured survival parameters in the co-culture and in hMSC and INA-6 cells cultured separately (Fig. 1E, right). We defined MSC interaction effects when the survival measured in the co-culture differed from the sum of the signals measured from INA-6 and hMSCs alone. RM-ANOVA confirmed that adherence to confluent MSCs increased apoptosis rates of INA-6 cells 24 hours after adhesion and decreased after 72 hours [$F(2, 4) = 26.86$, $p\text{-unc} = 4.80 \times 10^{-3}$] (interaction between MSC density and time, Two-factor RM-ANOVA), whereas INA-6 cells were unaffected when grown on dispersed hMSCs. In summary, the growth conformation of INA-6 cells, measured as the ratio between homotypic aggregation and heterotypic MSC interactions, affected apoptosis rates of INA-6 cells.

Single INA-6 Cells Detach Spontaneously from Aggregates of Critical Size

Using time-lapse microscopy, we observed that $26 \pm 8\%$ of INA-6 aggregates growing on single hMSCs spontaneously shed INA-6 cells (Fig. 2A, B; Supplementary Video 1). Notably, all detached cells exhibited similar directional movements, suggesting entrainment in convective streams generated by temperature gradients within the incubation chamber. INA-6 predominantly detached from other INA-6 cells or aggregates (Fig. 2C), indicating weaker adhesive forces in homotypic interactions than in heterotypic interactions. The detachment frequency increased after 52 hours, when most aggregates that shed INA-6 cells were categorized as large (greater than 30 cells) (Fig. 2D). Since approximately 10-20 INA-6 cells already fully covered a single hMSC, we suggest that myeloma cell detachment depended not only on hMSC saturation but also required a minimum aggregate size.

Interestingly, INA-6 detached mostly as single cells, independent of aggregate size categories [$F(2, 6) = 4.68, p\text{-unc} = 0.059$] (Two-factor RM-ANOVA) (Fig. 2E), showing that aggregates remained mostly stable despite losing cells.

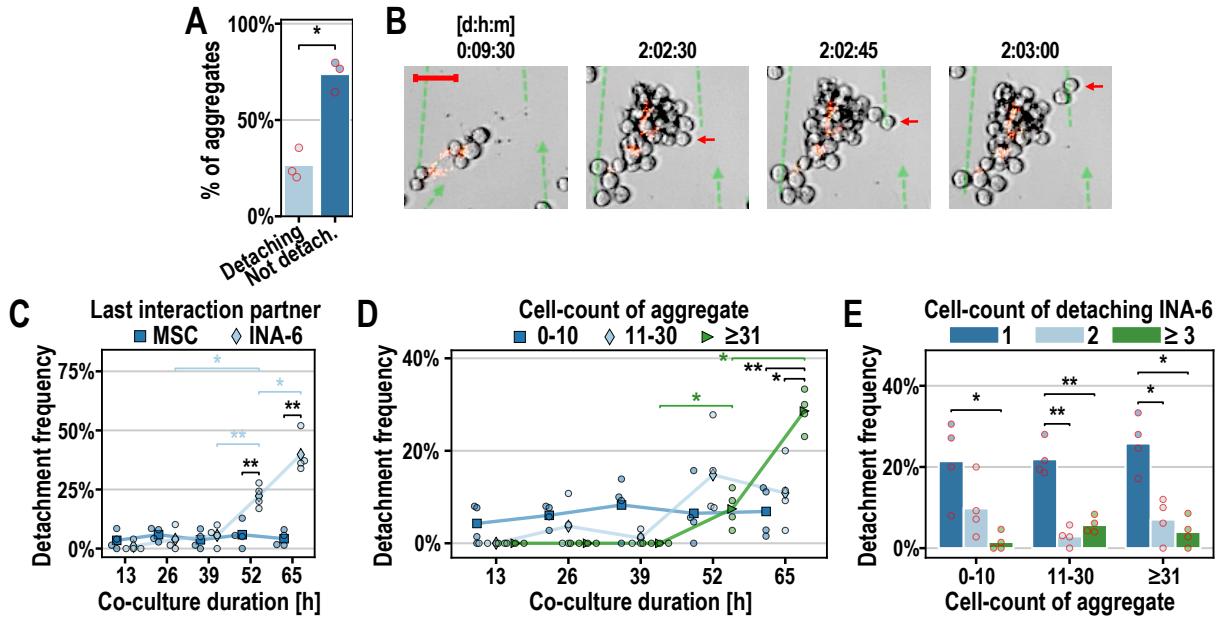


Figure 2: Time-lapse analysis of INA-6 detachment from INA-6 aggregates and hMSCs. **A:** Frequency of observed INA-6 aggregates that did or did not lose INA-6 cell(s). 87 aggregates were evaluated per datapoint. **B:** Example of a “disseminating” INA-6 aggregate growing on fluorescently (PKH26) stained hMSC (from A-D). Dashed green lines are trajectories of detached INA-6 cells. Scale bar = 50 μ m. **C-E:** Quantitative assessment of INA-6 detachments. 45 detachment events were evaluated per datapoint. Seeding ratio INA-6:MSC = 4:1. **C:** Most INA-6 cells dissociated from another INA-6 cell and not from an hMSC [$F(1, 3) = 298, p\text{-unc} = 4.2 \times 10^{-4}$]. **D:** Detachment frequency of aggregate size categories. **E:** Detachment frequency of INA-6 cells detaching as single, pairs or more than three cells. **Statistics:** (A): Paired-t-test; (C-E): Paired-t-test, Two-factor RM-ANOVA; Datapoints represent three (A) or four (C-E) independent time-lapse recordings of co-cultures with hMSCs from two (A) or three (C-E) unique donors. This content has been taken as is from Kuric et al. (2024), published in *Cancer Research Communications* under the CC BY 4.0 license.

Cell Division Generates a Daughter Cell Detached from hMSC

We suspected that cell division drives detachment because we observed that MSC-adhering INA-6 cells could generate daughter cells that “roll over” the mother cell (Fig. 3A; Supplementary Video 2). We recorded and categorized the movement of INA-6 daughter cells in confluent hMSCs after cell division. Half of all INA-6 divisions yielded two daughter cells that remained stationary, indicating hMSC adherence (Fig. 3B, C; Supplementary Video 3). The other half of division events generated one hMSC-adhering cell and one non-hMSC-adhering cell, which rolled around the MA-INA6 cell for a median time of 2.5 hours post division ($Q_1=1.00$ hour, $Q_3=6.25$ hours) until it stopped and re-adhered

to the hMSC monolayer (Fig. 3D; Supplementary Video 2, Supplementary Video 4). Thus, cell division establishes a time window in which one daughter cell can detach.

To validate that cell division reduced adhesion, we measured both the size and cell cycle profile of the nMA-INA6 and MA-INA6 populations using an enhanced V-well assay (method described in Fig. 3E, Appendix A: Fig. 1, 2). For comparison, we fully synchronized and arrested INA-6 cells at mitosis and released their cell cycle immediately before addition to the hMSC monolayer, rendering them more likely to divide while adhering. Mitotic arrest significantly increased the number of nMA-INA6 cells and decreased the number of MA-INA6 cells (Fig. 3F). Furthermore, the nMA-INA6 population contained significantly more cells cycling in the G0/G1 phase than the MA-INA6 population, both in synchronously and asynchronously cycling INA-6 (Fig. 3G, Appendix A: Fig. 3, 4). The number of nMA-INA6 INA-6 cells increased due to a higher cell division frequency. Taken together, we showed that INA-6 detach from aggregates by generating one temporarily detached daughter cell after cell division, a process that potentially contributes to the initiation of dissemination.

RNAseq of Non-MSC-Adhering and MSC-Adhering Subpopulations

To characterize the subpopulations separated by WPSC, we conducted RNAseq, revealing 1291 differentially expressed genes between nMA-INA6 *vs.* CM-INA6, 484 between MA-INA6 *vs.* CM-INA6, and 195 between MA-INA6 *vs.* nMA-INA6. We validated RNAseq and found that the differential expression of 18 genes correlated with those measured with qPCR for each pairwise comparison (Fig. 4C–E, Appendix A: Fig. 5): nMA-INA6 *vs.* CM-INA6 [$\rho(16) = .803, p = 6.09 \times 10^{-5}$], MA-INA6 *vs.* CM-INA6 [$\rho(16) = .827, p = 2.30 \times 10^{-5}$], and MA-INA6 *vs.* nMA-INA6 [$\rho(16) = .746, p = 3.74 \times 10^{-4}$] (Spearman’s rank correlation). One of the 18 genes (*MUC1*) measured by qPCR showed a mean expression opposite to that obtained by RNAseq (nMA-INA6 *vs.* CM-INA6), although the difference was insignificant (Fig. 4C). For nMA-INA6 *vs.* CM-INA6, the difference in expression measured by qPCR was significant for only two of the 11 genes (*DKK1, OPG*), whereas the other genes (*DKK1, OPG, BCL6, BMP4, BTG2, IL10RB, IL24, NOTCH2, TNFRSF1A, TRAF5*) only confirmed the tendency measured by RNAseq (Fig. 4C–E). For MA-INA6 *vs.* CM-INA6, qPCR validated the significant upregulation of seven genes (*DKK1, OPG, BCL6, BMP4, BTG2, IL10RB, IL24, NOTCH2, TNFRSF1A, TRAF5, TGM2, DCN, LOX, MMP14, MMP2, CXCL12, CXCL8*), whereas the downregulation of *BMP4* was insignificant.

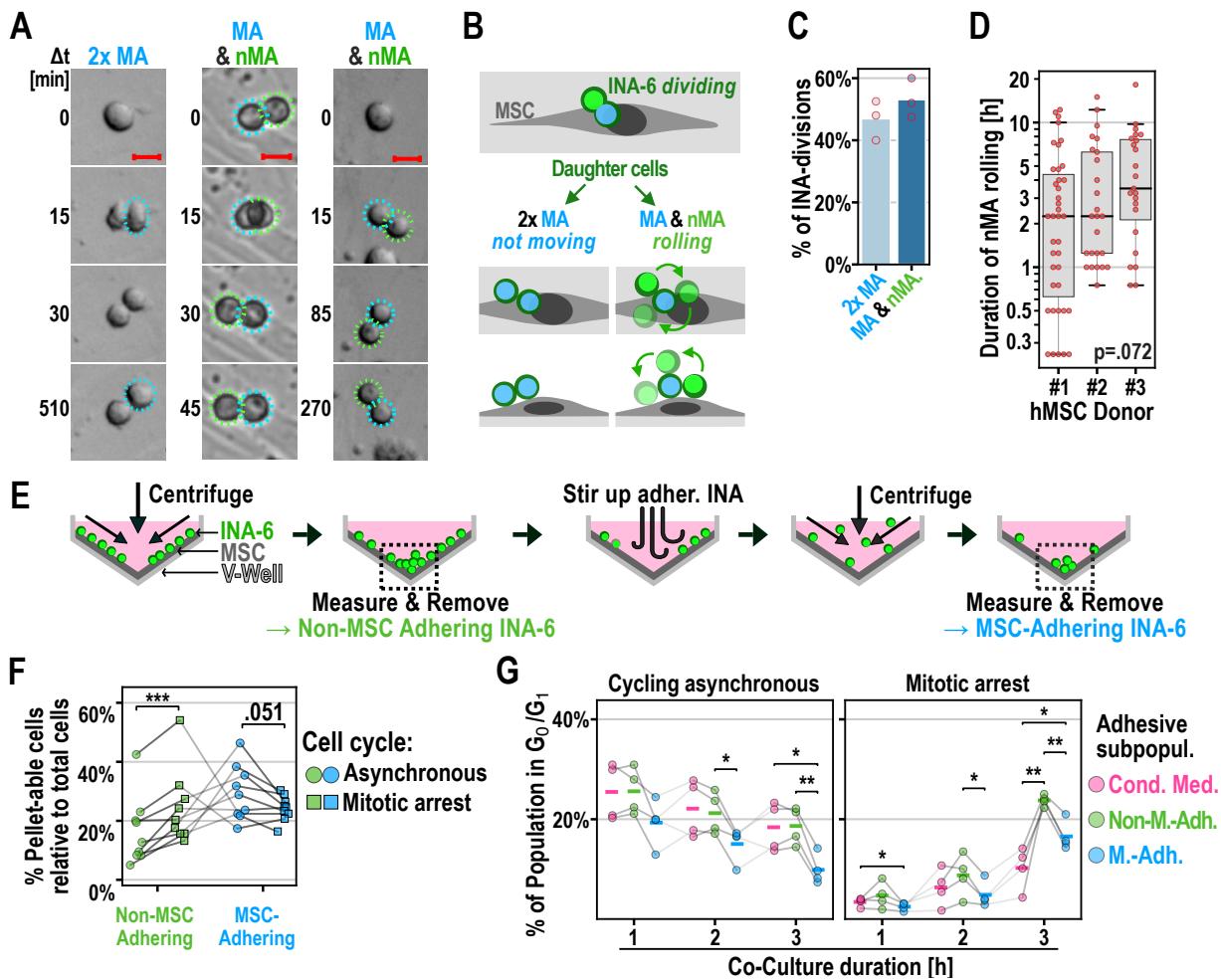


Figure 3: Detachment of INA-6 daughter cells after Cell Division. **A-D:** INA-6 divisions in interaction with confluent hMSCs. Seeding ratio INA-6:MSC = 4:20. **A:** Three examples of dividing INA-6 cells generating either two MA, or one MA and one nMA daughter cells as described in (G). Dashed circles mark mother cells (white), MA cell (blue), and first position of nMA cell (green). Scale bar: 20 μ m. **B:** Cell division of MSC-adhering (MA) mother cell can yield one mobile non-MSC-adhering (nMA) daughter cell. **C:** Frequencies of INA-6 pairs defined in (A, B) per observed cell division. 65 divisions were evaluated for each of three independent time-lapse recordings. **D:** Rolling duration of nMA cells after division did not depend on hMSC donor [$H(2) = 5.250$, $p\text{-unc} = .072$]. Datapoints represent single nMA-cells after division. **E-G:** Adhesive and cell cycle assessment of MSC-interacting INA-6 subpopulations using the V-Well assay. **E:** Schematic of V-Well Assay (see Appendix A: Fig. 1 for detailed analysis). MSC-interacting subpopulations were separated by subsequent centrifugation and removal of the pellet. The pellet size was quantified by its total fluorescence brightness. Adhering subpopulations were resuspended by rough pipetting. **F:** Relative cell pellet sizes of adhesive INA-6 subpopulations that cycle either asynchronously or were synchronized at mitosis. Gray lines in-between points connect dependent measurements of co-cultures ($n = 9$) that shared the same hMSC-donor and INA-6 culture. Co-cultures were incubated for three different durations (1 h, 2 h and 3 h after INA-6 addition). Time points were pooled, since time did not show an effect on cell adhesion [$F(2, 4) = 1.414$, $p\text{-unc} = 0.343$]. Factorial RM-ANOVA shows an interaction between cell cycle and the kind of adhesive subpopulation [$F(1, 8) = 42.67$, $p\text{-unc} = 1.82 \times 10^{-4}$]. Technical replicates = 4 per datapoint. **G:** Cell cycles were profiled in cells gathered from the pellets of four independent co-cultures ($n = 4$) and the frequency of G₀/G₁ cells are displayed depending on co-culture duration (see Appendix A: Fig. 3 for cell cycle profiles). Four technical replicates were pooled after pelleting. **Statistics:** (D): Kruskal-Wallis H-test. (F): Paired t-test, (G): Paired t-test, two-factor RM-ANOVA. Datapoints represent INA-6 from independent co-cultures with hMSCs from three unique donors. This content has been taken as is from Kuric et al. (2024), published in *Cancer Research Communications* under the CC BY 4.0 license.

Non-MSC-Adhering INA-6 and MSC-Adhering INA-6 Have Distinct Expression Patterns of Proliferation or Adhesion, Respectively

To functionally characterize the unique transcriptional patterns in nMA-INA6 and MA-INA6, we generated lists of genes that were differentially expressed *vs.* the other two subpopulations [termed nMA *vs.* (MA & CM) and MA *vs.* (nMA & CM)]. Functional enrichment analysis was performed, and the enriched terms were displayed as ontology clusters (Fig. 5A). nMA-INA6 upregulated genes enriched with loosely connected term clusters associated with proliferation (e.g., “positive regulation of cell cycle”).

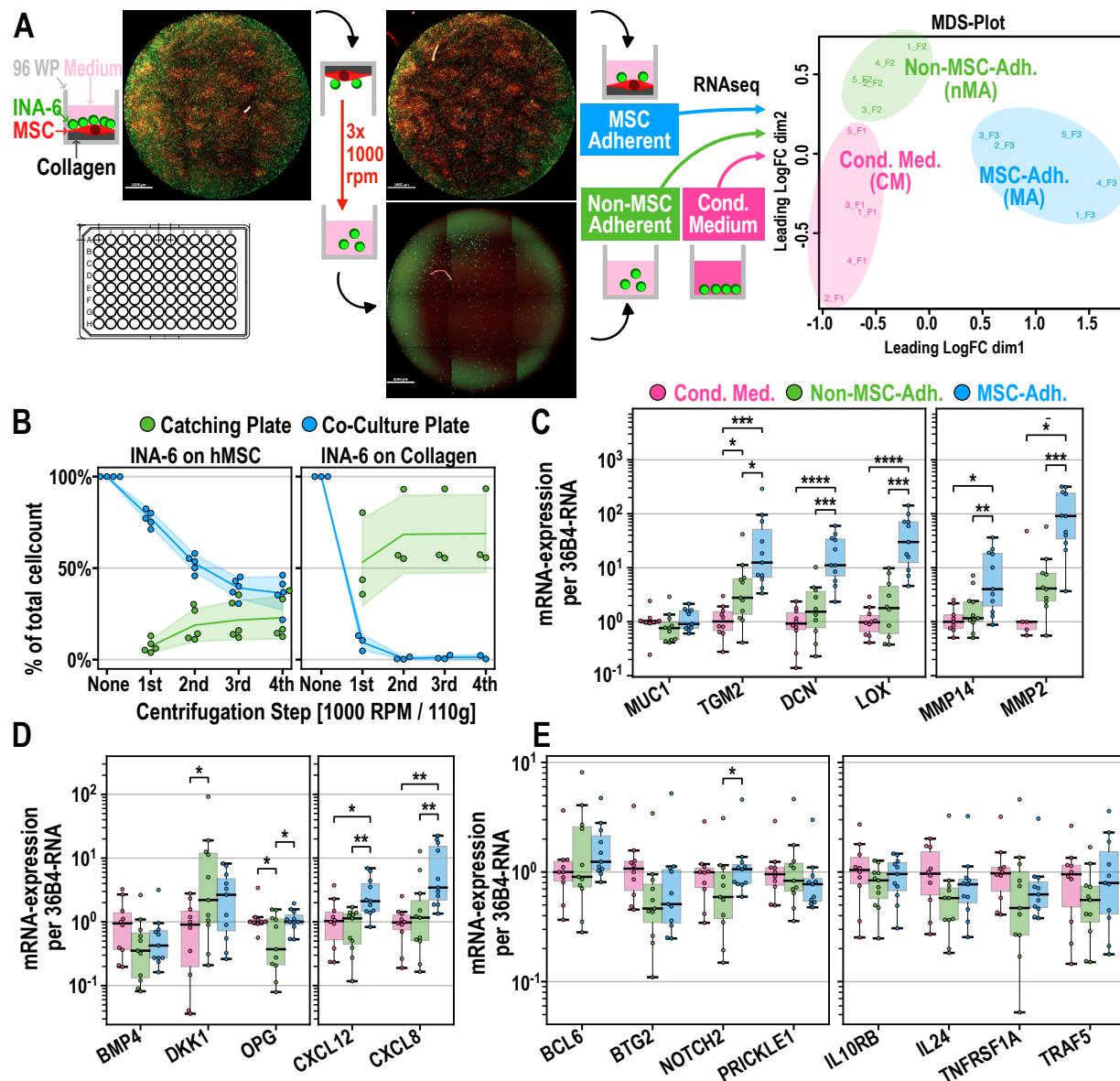


Figure 4: Separation and gene expression of INA-6 subpopulations. **A:** Schematic of “Well-Plate Sandwich Centrifugation” (WPSC) separating nMA- from MA-INA6. A co-culture 96-well plate is turned upside down and attached on top of a “catching plate”, forming a “well-plate sandwich”. nMA-INA6 cells are collected in the catching plate by subsequent rounds of centrifugation and gentle washing. MA-INA6 are enzymatically dissociated from hMSCs or by rough pipetting. Subsequent RNAseq of MSC-interacting subpopulations reveals distinct expression clusters [right, multidimensional scaling plot (MDS) ($n = 5$)]. **B:** Separation was microscopically tracked after each centrifugation step. **C-E:** RT-qPCR of genes derived from RNAseq results. Expression was normalized to the median of CM-INA6. Samples include those used for RNAseq and six further co-cultures ($n = 11$; non-detects were discarded). **C:** Adhesion factors, ECM proteins, and matrix metalloproteinases. **D:** Factors involved in bone remodeling and bone homing chemokines. **E:** Factors involved in (immune) signaling. **Statistics:** (C-E): Paired t-test. Datapoints represent the mean of three (B-E) technical replicates. INA-6 were isolated from independent co-cultures with hMSCs from five (A, B), nine (C-E) unique donors. This content has been taken as is from Kuric et al. (2024), published in *Cancer Research Communications* under the CC BY 4.0 license.

MA-INA6 upregulated genes enriched with tightly connected term clusters related to

cell adhesion and the production of ECM factors (e.g., “cell-substrate adhesion”). Similar ontology terms were enriched in the gene lists obtained from pairwise comparisons nMA *vs.* CM, MA *vs.* CM, and MA *vs.* nMA (Fig. 5B). In particular, nMA *vs.* CM (but not MA *vs.* CM) upregulated genes that were enriched with “G1/S transition”, showing that WPSC isolated nMA-INA6 daughter cells after cell division.

To check for similarities between lists of differentially expressed genes from hMSC-interacting subpopulations, we performed enrichment analysis on gene lists from the overlaps (“ \cap ”) between all pairwise comparisons (Fig. 5B, Appendix A: Fig. 6), and showed the extent of these overlaps in circos plots (Fig. 5C). The overlap between MA *vs.* CM and nMA *vs.* CM showed neither enrichment with proliferation- nor adhesion-related terms but with apoptosis-related terms. A direct comparison of MSC-interacting subpopulations (MA *vs.* nMA) showed a major overlap with MA *vs.* CM (Fig. 5C, middle). This overlap was enriched with terms related to adhesion but not proliferation. Hence, MA-INA6 and nMA-INA6 mostly differed in their expression of adhesion genes.

To assess whether nMA-INA6 and MA-INA6 were regulated by separate transcription factors, we examined the enrichment of curated regulatory networks from the TRRUST database (Fig. 5B, bottom). All the lists were enriched for p53 regulation. E2F1 regulation was observed only in genes upregulated in nMA *vs.* CM and downregulated in MA *vs.* nMA. Genelists involving MA-INA6 were enriched in regulation by subunits of NF- κ B (NFKB1/p105 and RELA/p65) and factors of immediate early response (SRF, JUN). Correspondingly, NF- κ B and JUN are known to regulate the expression of adhesion factors in multiple myeloma and B-cell lymphoma, respectively (Blonska et al., 2015; Tai et al., 2006).

Taken together, MSC-interacting subpopulations showed unique regulatory patterns, focusing on either proliferation or adhesion.

nMA-INA6 and MA-INA6 Show Increased Apoptosis Signaling Mediated by ER-Stress, p53 and Death Domain Receptors

As previously stated, apoptosis rates increased in INA-6 cells grown on confluent hMSCs compared with MSC-Conditioned-Medium-treated INA-6 (CM-INA6) cells after 24 hours of co-culture (Fig. 1D). Since this setup was similar to that used to separate hMSC-interacting subpopulations using WPSC, we looked for enrichment of apoptosis-related terms (Fig. 5B). “Regulation of cellular response to stress” and “intrinsic apoptotic signaling pathway (in response to ER-stress)” are terms that were enriched in nMA *vs.* CM, MA *vs.* CM and their overlap.

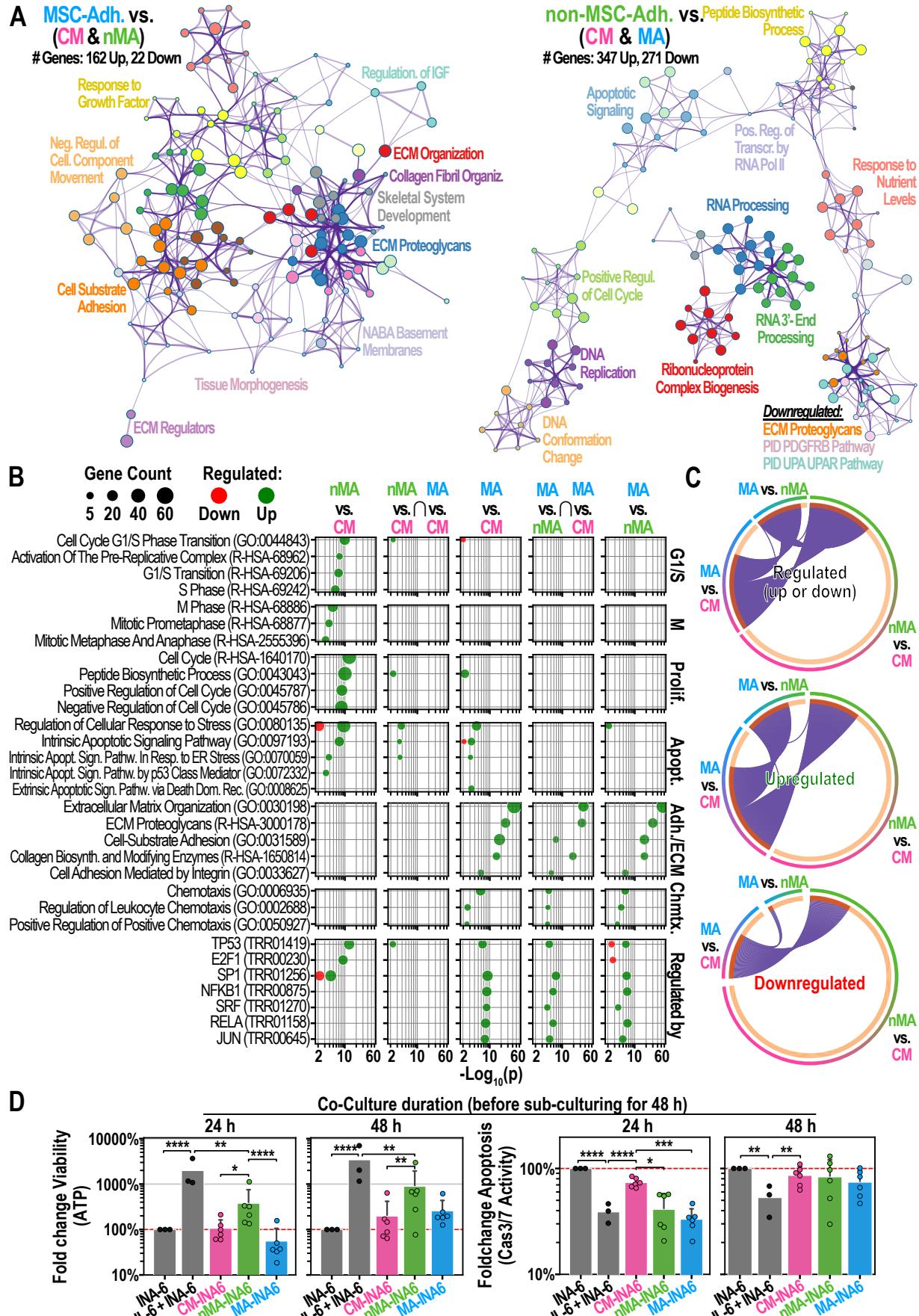


Figure 5: Functional analysis of MSC-interacting subpopulations (A-C): Functional enrichment analysis of differentially expressed genes (from RNAseq) using Metascape. **A:** Gene ontology (GO) cluster analysis of gene lists that are unique for MA (left) or nMA (right) INA-6. Circle nodes represent subsets of input genes falling into similar GO-term. Node size grows with the number of input genes. Node color defines a shared parent GO-term. Two nodes with a similarity score > 0.3 are linked. **B:** Enrichment analysis of pairwise comparisons between MA subpopulations and their combinations (nMA + MA) using GSEA. **C:** Circular diagrams showing the distribution of regulated genes (up or down) between MA vs. nMA comparisons. The inner ring represents 'Regulated (up or down)', the middle ring represents 'Upregulated', and the outer ring represents 'Downregulated'. The diagrams are color-coded by comparison: MA vs. CM (blue), MA vs. nMA (green), and nMA vs. CM (orange). **D:** Bar charts showing fold change viability (ATP) and fold change apoptosis (Cas3/7 activity) for different co-culture durations (24 h and 48 h) and conditions (IL-6 + INA-6, CM-INA6, nMA-INA6, MA-INA6).

We also found specific stressors for either nMA-INA6 (“intrinsic apoptotic signaling pathway by p53 class mediator”) or MA-INA6 (“extrinsic apoptotic signaling pathway via death domain receptor”). Therefore, apoptosis may be driven by ER stress in both nMA-INA6 and MA-INA6, but also by individual pathways such as p53 and death domain receptors, respectively.

nMA-INA6 and MA-INA6 Regulate Genes Associated with Bone Loss

Myeloma cells cause bone loss by degradation and dysregulation of bone turnover via *DKK1* and *OPG* (Standal et al., 2002; Van Valckenborgh et al., 2004; F. Zhou et al., 2013). RNAseq of hMSC-interacting subpopulations showed enrichment with functional terms “skeletal system development” and “ossification” (Fig. 5A, Appendix A: Fig. 6), as well as the regulation of *MMP2*, *MMP14*, *DKK1*, and *OPG*. Validation by qPCR (Fig. 4C, D) showed that MA-INA6 significantly upregulated both *MMP14* and *MMP2* compared with either nMA-INA6 or CM-INA6. The expression of *DKK1*, however, was upregulated significantly in nMA-INA6 (and not significantly upregulated in MA-INA6), while *OPG* was significantly downregulated only in nMA-INA6.

Together, hMSC-interacting subpopulations might contribute to bone loss through different mechanisms: MA-INA6 expression of matrix metalloproteinases and nMA-INA6 cells via paracrine signaling.

MA-INA6 Upregulate Collagen and Chemokines Associated with Bone Marrow Retention

Retention of myeloma cells within the bone marrow is mediated by adhesion to the ECM (e.g., collagen VI) and the secretion of chemokines (*CXCL8* and *CXCL12*), potentially counteracting dissemination (Alsayed et al., 2007; Katz, 2010). RNAseq of hMSC-interacting subpopulations showed that genes upregulated in MA-INA6 were enriched with collagen biosynthesis and modifying enzymes, as well as chemotaxis and chemotaxis-related terms (Fig. 5B). Using qPCR, we validated the upregulation of collagen crosslinkers (*LOX* and *TGM2*), collagen-binding *DCN*, and chemokines (*CXCL8* and *CXCL12*) in MA-INA6 compared with both nMA-INA6 and CM-INA6 (Fig. 4D). Therefore, MA-INA6 can provide both an adhesive surface and soluble signals for the retention of malignant plasma cells in the bone marrow.

nMA-INA6 Show Highest Viability During IL-6 Withdrawal

Although RNAseq did not reveal IL-6 induction in any WPSC-isolated subpopulation, nMA-INA6 upregulated *IGF-1* 1.35-fold [RNAseq, nMA *vs.* (MA & CM)], which was shown to stimulate growth in CD45+ and IL-6 dependent myeloma cell lines such as INA-6, implying increased autonomy for nMA-INA6 (40). To test the autonomy of hMSC-interacting INA-6 subpopulations, we isolated them using WPSC after 24 hours and 48 hours of co-culture, sub-cultured them for 48 hours under IL-6 withdrawal, and measured both viability and apoptosis (Fig. 5D). Among the subpopulations, nMA-INA6 was the most viable. Compared to MA-INA6, nMA-INA6 increased cell viability by 8 or 4 fold when co-cultured for 24 hours or 48 hours, respectively [Hedges *g* of $\text{Log}_{10}(\text{Fold Change}) = 2.31$ or 0.82]. However, the difference was no longer significant after 48 hours of co-culture, probably because nMA-INA6 adhered to the hMSC layer (turning into MA-INA6) during prolonged co-culture, which could also explain why the viability of MA-INA6 cell subcultures increased with prolonged co-culture. Nevertheless, nMA-INA6 did not achieve the same viability as that of INA-6 cells cultured with IL-6. Despite the differences in viability, subcultures of hMSC-interacting subpopulations did not show any differences in caspase 3/7 activity when co-cultured for 48 hours (Fig. 5D, right).

Overall, among the hMSC-interacting subpopulations, nMA-INA6 had the highest chance of surviving IL-6 withdrawal.

Genes Upregulated by MA-INA6 are Associated with an Improved Disease Prognosis

To relate the adhesion of MA-INA6 observed *in vitro* to the progression of multiple myeloma, we assessed patient survival [$n = 535$, Seckinger et al. (2017, 2018)] depending on the expression level of 101 genes, which were upregulated in MA *vs.* (nMA & CM) and are part of the ontology terms “Extracellular matrix organization,” “ECM proteoglycans,” “cell-substrate adhesion,” and “negative regulation of cell-substrate adhesion” (Fig. 6A, Appendix A: Tab. 2). As a reference, we generated a list of 173 cell cycle-related genes that were upregulated by nMA *vs.* (MA & CM).

As expected, longer patient survival was associated with low expression of the majority of cell cycle genes [71 or 68 genes for Progression-Free Survival (PFS) or Overall Survival (OS)]. Only a few cell cycle genes (two for PFS and seven for OS) were associated with survival when highly expressed. Intriguingly, adhesion genes showed an inverse pattern: a large group of adhesion genes (24 for PFS and 26 for OS) was significantly associated with improved survival when highly expressed, whereas only a few genes (two for PFS and

four for OS) improved survival when expressed at low levels (Tab. 1). We concluded that the myeloma-dependent expression of adhesion factors determined in our *in vitro* study correlates with improved patient survival.

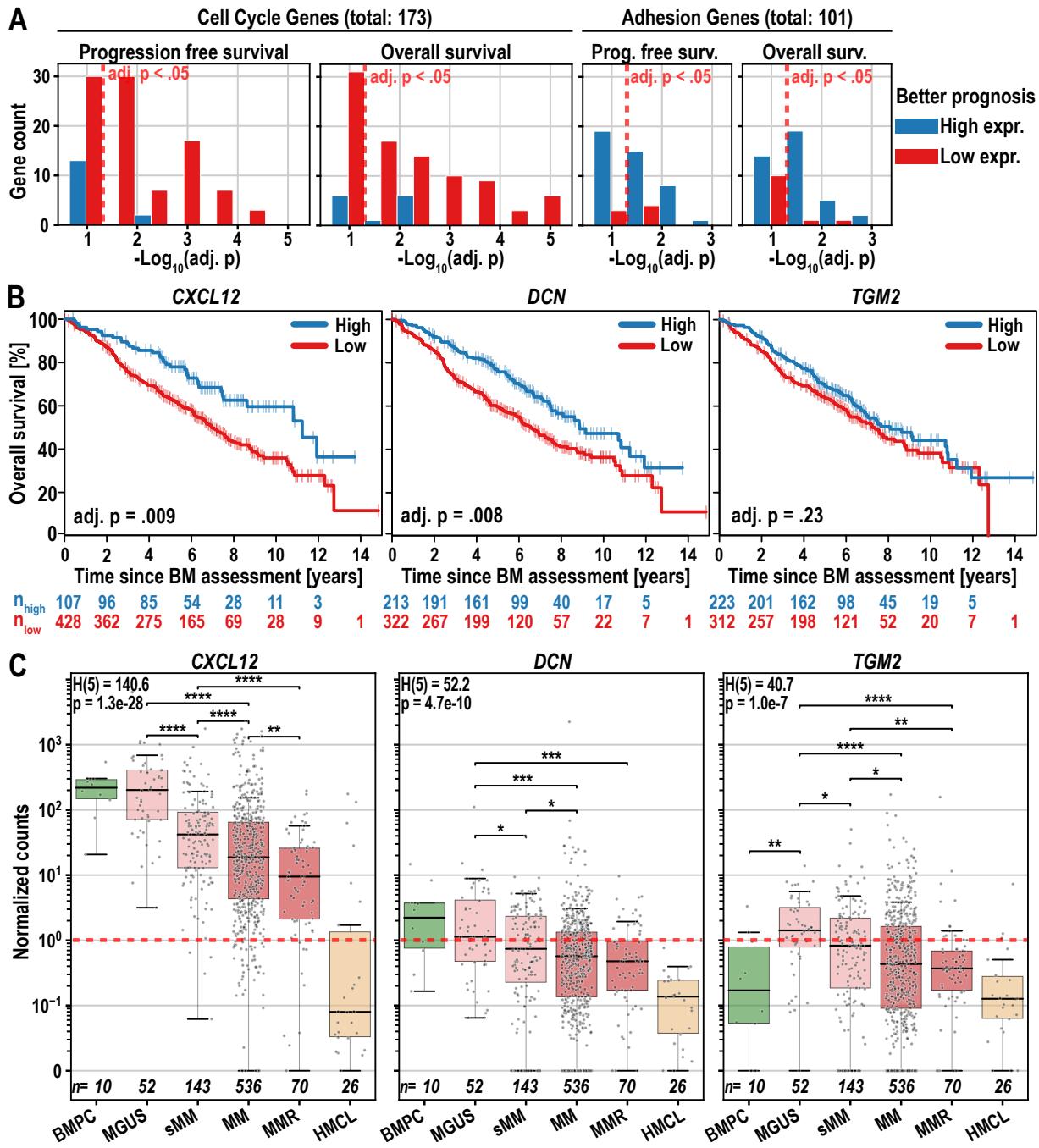


Figure 6: Survival of patients with multiple myeloma regarding the expression levels of adhesion and bone retention genes. **A:** p-value distribution of genes associated with patient survival ($n = 535$) depending on high or low expression levels. Red dashed line marks the significance threshold of $p\text{-adj} = 0.05$. Histogram of p -values was plotted using a bin width of $-\log_{10}(0.05)/2$. Patients with high and low gene expression were delineated using maximally selected rank statistics (maxstat). **B:** Survival curves for three genes taken from the list of adhesion genes shown in (A), maxstat thresholds defining high and low expression were: *CXCL12*: 81.08; *DCN*: 0.75; *TGM2*: 0.66 normalized counts. – *continued on next page*

Figure 6: continued from previous page – **C:** Gene expression (RNAseq, $n = 873$) measured in normalized counts (edgeR) of *CXCL12*, *DCN* in Bone Marrow Plasma Cell (BMPC), Monoclonal Gammopathy of Undetermined Significance (MGUS), smoldering Multiple Myeloma (sMM), Multiple Myeloma (MM), Multiple Myeloma Relapse (MMR), Human Myeloma Cell Lines (HMCL). The red dashed line marks one normalized read count. **Statistics:** (A, B): Log-rank test; (C): Kruskal-Wallis, Mann–Whitney U Test. All p -values were corrected using the Benjamini-Hochberg procedure. This content has been taken as is from Kuric et al. (2024), published in *Cancer Research Communications* under the CC BY 4.0 license.

Expression of Adhesion- or Retention-related Genes (*CXCL12*, *DCN* and *TGM2*) is Decreased During Progression of Multiple Myeloma

To examine how the disease stage affects the adhesion and bone marrow retention of myeloma cells *in vitro*, we analyzed the expression of *CXCL12* in healthy plasma cell (BMPC) cohorts of patients at different disease stages and in myeloma cell lines (HMCL) [described in Seckinger et al. (2018)] (Fig. 6C). We also included *DCN* and *TGM2* since both are suggested to inhibit metastasis in different cancers by promoting cell-matrix interactions (X. Hu et al., 2021; Tabolacci et al., 2019). In accordance with independent reports (Huang et al., 2015; Bao et al., 2013), high expression of *CXCL12* and *DCN* by myeloma cells was associated with improved overall survival (adj. $p = .009$ and $.008$, respectively) (Fig. 6B).

Table 1: Adhesion and ECM genes (shown in Fig. 6A) were filtered by their association with patient survival (p -adj. < 0.01) and were categorized as continuously downregulated during disease progression. The complete list is presented in Appendix A: Tab. 2. Bone Marrow Plasma Cells (BMPC), Monoclonal Gammopathy of Undetermined Significance (MGUS), smoldering Multiple Myeloma (sMM), Multiple Myeloma (MM), and Multiple Myeloma Relapse (MMR). p-unc: unadjusted p -values; p-adj: p -values adjusted using the Benjamini-Hochberg method with 101 genes. This content has been taken as is from Kuric et al. (2024), published in *Cancer Research Communications* under the CC BY 4.0 license.

Regulation during disease progression	Gene	Ensemble ID	Progression Free / Overall Survival	Better Prognosis with high/low expression	Association of expression with survival	
					[p-unc]	[p-adj]
Not Downregulated (or overall low expression)	CCNE2	ENSG00000175305	Overall	low	5.34E-04	8.64E-03
	MMP2	ENSG00000087245	Prog. Free	high	2.29E-05	2.32E-03
	OSMR	ENSG00000145623	Prog. Free	high	5.67E-04	7.15E-03
Continuously Downregulated (BMPC)	AXL	ENSG00000167601	Overall	high	3.64E-05	1.84E-03
	COL1A1	ENSG00000108821	Prog. Free	high	3.03E-04	4.37E-03
			Overall	high	5.93E-04	8.64E-03
MGUS continued on next page sMM > MM > MMR)	CXCL12	ENSG00000107562	Prog. Free	high	1.16E-04	2.93E-03

continued on next page
sMM > MM >

Table 1 – continued from previous page

		Overall	high	6.48E-04	8.64E-03
CYP1B1	ENSG00000138061	Overall	high	6.84E-04	8.64E-03
DCN	ENSG0000011465	Overall	high	2.47E-04	8.33E-03
LRP1	ENSG00000123384	Overall	high	4.34E-04	8.64E-03
LTBP2	ENSG00000119681	Prog. Free	high	9.03E-05	2.93E-03
CYP1B1	ENSG00000138061	Overall	high	6.84E-04	8.64E-03
DCN	ENSG0000011465	Overall	high	2.47E-04	8.33E-03
LRP1	ENSG00000123384	Overall	high	4.34E-04	8.64E-03
LTBP2	ENSG00000119681	Prog. Free	high	9.03E-05	2.93E-03
MFAP5	ENSG00000197614	Prog. Free	high	2.43E-04	4.09E-03
MMP14	ENSG00000157227	Prog. Free	high	6.93E-05	2.93E-03
MYL9	ENSG00000101335	Prog. Free	high	1.46E-04	2.95E-03
		Overall	high	1.56E-05	1.57E-03

CXCL12 is expressed by BMPCs (median = 219 normalized counts), but its expression levels are significantly lower from MGUS to relapsed multiple myeloma (MMR) (median = 9 normalized counts in MMR and absent expression in most HMCL). *DCN* (but not *TGM2*) was weakly expressed in BMPCs ($Q_1 = 0.7$, $Q_3 = 3.7$, normalized counts), whereas *TGM2* was weakly expressed only in patients with monoclonal gammopathy of undetermined significance (MGUS) ($Q_1 = 0.4$, $Q_3 = 4.1$ normalized counts). The median and upper quartiles of both *DCN*- and *TGM2* decreased continuously after each stage, ending at $Q_3 = 0.9$ and $Q_3 = 0.6$, respectively, in MMR. 49 of the 101 adhesion genes (Fig. 6A) followed a similar pattern of continuous downregulation in the advanced stages of multiple myeloma (Appendix A: Fig. 7 and 8), of which 19 genes were associated with longer PFS when they were highly expressed. The other 52 (out of 101) adhesion genes that were not downregulated across disease progression (or were expressed at a level too low to make that categorization) contained only five genes that were associated with longer PFS at high expression (Tab. 1, Appendix A: Tab. 2).

Together, the expression of adhesion or bone marrow retention-related markers (*CXCL12*, *DCN*, and *TGM2*) is reduced or lost at advanced stages of multiple myeloma, which could enhance dissemination and reduce retention in the BM microenvironment.

Discussion

In this study, we developed an *in vitro* model to investigate the attachment/detachment dynamics of INA-6 cells to/from hMSCs and established methods to isolate the attached and detached intermediates nMA-INA6 and MA-INA6. Secondly, we characterized a cycle of (re)attachment, division, and detachment, linking cell division to the switch that causes myeloma cells to detach from hMSC adhesion (Fig. 7). Thirdly, we identified clinically relevant genes associated with patient survival, where better or worse survival was based on the adherence status of INA-6 to hMSCs.

INA-6 cells emerged as a robust choice for studying myeloma dissemination *in vitro*, showing rapid and strong adherence, as well as aggregation exceeding MSC saturation. The IL-6 dependency of INA-6 enhanced the resemblance of myeloma cell lines to patient samples, with INA-6 ranking 13th among 66 cell lines (Sarin et al., 2020). Despite variations in bone marrow MSCs between multiple myeloma and healthy states, we anticipated the robustness of our results, given the persistent strong adherence and growth signaling from MSCs to INA-6 during co-cultures (Dotterweich et al., 2016).

We acknowledge that INA-6 cells alone cannot fully represent the complexity of myeloma aggregation and detachment dynamics. However, the diverse adhesive properties of myeloma cell lines pose a challenge. We reasoned that attempting to capture this complexity within a single publication would not be possible. Our focus on INA-6 interactions with hMSCs allowed for a detailed exploration of the observed phenomena, such as the unique aggregation capabilities that facilitate the easy detection of detaching cells *in vitro*. The validity of our data was demonstrated by matching the *in vitro* findings with the gene expression and survival data of the patients (e.g., *CXCL12*, *DCN*, and *TGM2* expression, $n = 873$), ensuring biological consistency and generalizability regardless of the cell line used. The protocols presented in this study offer a cost-efficient and convenient solution, making them potentially valuable for a broader study of cell interactions. We encourage optimizations to meet the varied adhesive properties of the samples, such as decreasing the number of washing steps if the adhesive strength is low. We caution against strategies that average over multiple cell lines without prior understanding their diverse attachment/detachment dynamics, such as homotypic aggregation. Such detailed insights may prove instrumental when considering the diversity of myeloma patient samples across different disease stages (Kawano et al., 1991; Okuno et al., 1991).

The intermediates, nMA-INA6 and MA-INA6, were distinct but shared similarities in response to cell stress, intrinsic apoptosis, and regulation by p53. Unique regulatory patterns were related to central transcription factors: E2F1 for nMA-INA6; and NF-

κ B, SRF, and JUN for MA-INA6. This distinction may have been established through antagonism between p53 and the NF- κ B subunit RELA/p65 (Wadgaonkar et al., 1999; Webster & Perkins, 1999). Similar regulatory patterns were found in transwell experiments with RPMI1-8226 myeloma cells, where direct contact with the MSC cell line HS5 led to NF- κ B signaling and soluble factors to E2F signaling (Dziadowicz et al., 2022).

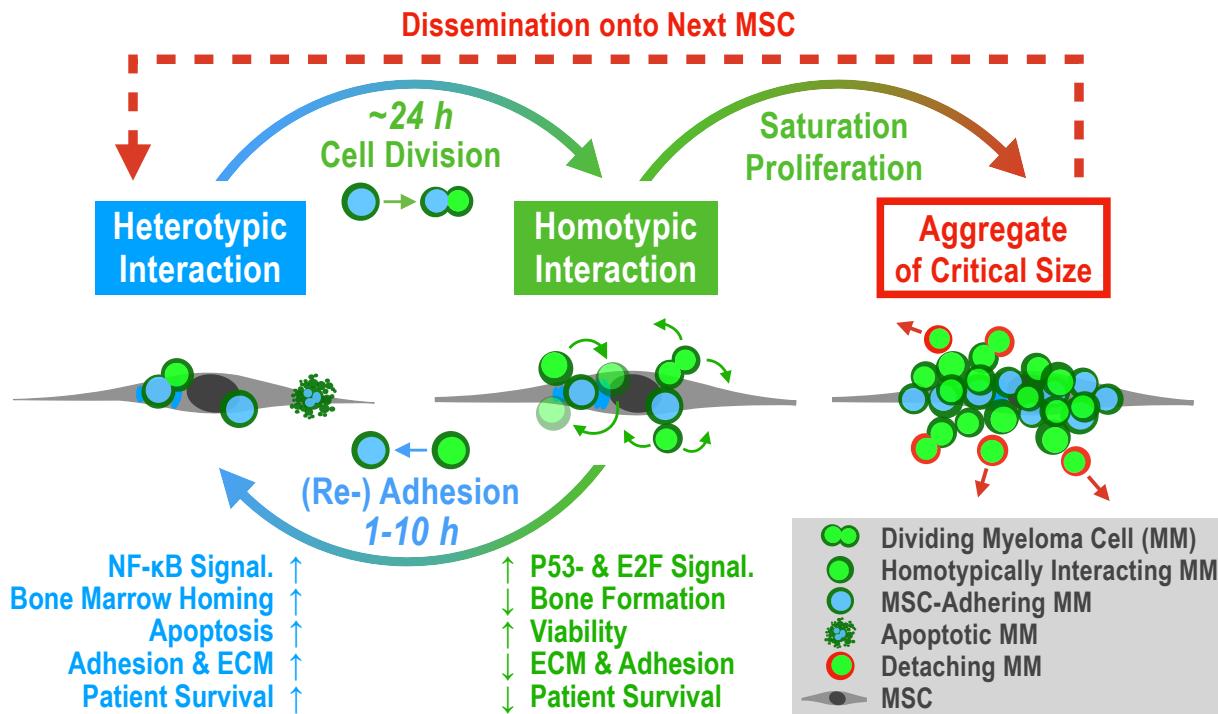


Figure 7: Proposed model of “Detached Daughter Driven Dissemination” (DDDD) in aggregating multiple myeloma. **Heterotypic Interaction:** Malignant plasma cells colonize the bone marrow microenvironment by adhering to an MSC (or osteoblast, ECM, etc.) to maximize growth and survival through paracrine and adhesion mediated signaling, even if contact may trigger initial apoptosis. Gene expression will focus on establishing a strong anchor within the bone marrow, but also on attracting other myeloma cells (via secretion of ECM factors and CXCL12/CXCL8, respectively). **Cell Division:** Cell fission can generate one daughter cell that no longer adheres to the MSC (nMA). **Homotypic Interaction:** If myeloma cells have the capacity to grow as aggregates, the daughter cell stays attached to their MSC-adhering mother cell (MA). **Re-Adhesion:** The daughter cell “rolls around” the mother cell until it re-adheres to the MSC. Our model estimates the rolling duration to be 1–10 h long. **Proliferation & Saturation:** We estimate that a single myeloma cell covers one MSC completely after roughly four population doublings. When heterotypic adhesion is saturated, subsequent daughter cells benefit from a homotypic interaction, since they stay close to growth-factor secreting MSCs and focus gene expression on proliferation (e.g. driven by E2F) and not adhesion (driven by NF- κ B). **Critical Size:** Homotypic interaction is weaker than heterotypic interaction, and each cell fission destabilizes the aggregate. Hence, detachment of myeloma cells may depend mostly on aggregate size. **Dissemination:** After myeloma cells have detached, they gained a viability advantage through IL-6-independence (with unknown duration), which enhances their survival outside of the bone marrow and allows them to spread throughout the body. This content has been taken as is from Kuric et al. (2024), published in *Cancer Research Communications* under the CC BY 4.0 license.

The first subpopulation, nMA-INA6, represented proliferative and disseminative cells;

They drove detachment through cell division, which was regulated by E2F, p53, and likely their crosstalk (Polager & Ginsberg, 2009). nMA-INA6 upregulate cell cycle progression genes associated with worse prognosis, because proliferation is a general risk factor for an aggressive disease course (Hose et al., 2011). Additionally, nMA-INA6 survived IL-6 withdrawal better than CM-INA6 and MA-INA6, implying their ability to proliferate independently of the bone marrow (Bladé et al., 2022). Indeed, xenografted INA-6 cells developed autocrine IL-6 signaling but remained IL-6-dependent after explantation (Burger, Günther, et al., 2001). The increased autonomy of nMA-INA6 cells can be explained by the upregulation of *IGF-1*, being the major growth factor for myeloma cell lines (Sprynski et al., 2009). Other reports characterized disseminating cells differently: Unlike nMA-INA6, circulating myeloma tumor cells were reported to be non-proliferative and bone marrow retentive (Garcés et al., 2020). In contrast to circulating myeloma tumor cells, nMA-INA6 were isolated shortly after detachment and therefore these cells are not representative of further steps of dissemination, such as intravasation, circulation or intravascular arrest (Zeissig et al., 2020). Furthermore, Brandl et al. described proliferative and disseminative myeloma cells as separate entities, depending on the surface expression of CD138 or JAM-C (Akhmetzyanova et al., 2020; A. Brandl et al., 2022). Although CD138 was not differentially regulated in nMA-INA6 or MA-INA6, both subpopulations upregulated JAM-C, indicating disease progression (A. Brandl et al., 2022).

Furthermore, nMA-INA6 showed that cell division directly contributed to dissemination. This was because INA-6 daughter cells emerged from the mother cell with distance to the hMSC plane in the 2D setup. A similar mechanism was described in an intravasation model in which tumor cells disrupt the vessel endothelium through cell division and detach into blood circulation (Wong & Searson, 2017). Overall, cell division offers key mechanistic insights into dissemination and metastasis.

The other subpopulation, MA-INA6, represented cells retained in the bone marrow; MA-INA6 strongly adhered to MSCs, showed NF- κ B signaling, and upregulated several retention, adhesion, and ECM factors. The production of ECM-associated factors has recently been described in MM.1S and RPMI-8226 myeloma cells (Maichl et al., 2023). Another report did not identify the upregulation of such factors after direct contact with the MSC cell line HS5; hence, primary hMSCs may be crucial for studying myeloma-MSC interactions (Dziadowicz et al., 2022). Moreover, MA-INA6 upregulated adhesion genes associated with prolonged patient survival and showed decreased expression in relapsed myeloma. As myeloma progression implies the independence of myeloma cells from the bone marrow (Bladé et al., 2022; Sarin et al., 2020), we interpreted these adhesion genes as mediators of bone marrow retention, decreasing the risk for dissemination and thereby potentially prolonging patient survival. However, the overall impact of cell adhesion and

ECM on patient survival remains unclear. Several adhesion factors have been proposed as potential therapeutic targets (A. Brandl et al., 2022; Bou Zerdan et al., 2022). Recent studies have described the prognostic value of multiple ECM genes, such as those driven by NOTCH (Maichl et al., 2023). Another study focused on ECM gene families, of which only six of the 26 genes overlapped with our gene set (Appendix A: Tab. 2) (Evers et al., 2023). The expression of only one gene (*COL4A1*) showed a different association with overall survival than that in our cohort. The lack of overlap and differences can be explained by dissimilar definitions of gene sets (homology *vs.* gene ontology), methodological discrepancies, and cohort composition.

In summary, our *in vitro* model provides a starting point for understanding the initiation of dissemination and its implications for patient survival, providing innovative methods, mechanistic insights into attachment/detachment, and a set of clinically relevant genes that play a role in bone marrow retention. These results and methods might prove useful when facing the heterogeneity of disseminative behaviors among myeloma cell lines and primary materials.

Summarizing Discussion

The subsequent sections will discuss the chapters presented earlier, focusing on how they fit within current scientific fields and the technical and academic challenges encountered during this project. Given the extensive scope of the topics covered, this discussion is divided into three main sections: Data analysis, microscopy and cancer cell biology. Each section will detail key experiments that led to shifts in understanding and present intermediary conclusions to ensure clarity on broad topics.

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Appendices

A Supplementary Data & Methods

A.1 Figures

A.2 Tables

A.3 Materials & Methods

B Documentation of `plotastic`