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

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

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Doctoral Thesis for a Doctoral Degree

*at the*

GRADUATE SCHOOL OF LIFE SCIENCES,  
JULIUS-MAXIMILIANS-UNIVERSITÄT WÜRZBURG,  
SECTION BIOMEDICINE

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## **Summary**

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## **Zusammenfassung**

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## Acknowledgements

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# Introduction

To provide a comprehensive background for the following chapters that focus on the interaction of human mesenchymal stromal cells (hMSCs) with multiple myeloma (MM) cells, this

## Human Mesenchymal Stem/Stromal Cells

Explaining what a mesenchymal stromal cell (MSC) is, is not such an easy task as one might expect. MSCs are derived from multiple MSCs different sources, serve a wide array of functions and are always isolated as a heterogenous group of cells. This makes it particularly challenging to find a consensus on their exact definition, nomenclature, exact function and *in vivo* differentiation potential. Therefore, the most effective approach to describe hMSCs is to present their historical context.

hMSCs first gained popularity as a stem cell. Stem cells lay the foundation of multicellular organisms. Embryonic stem cells orchestrate the growth and patterning during embryonic development, while adult stem cells are responsible for regeneration during adulthood. The classical definition of a stem cell is that of a relatively undifferentiated cell that divides asymmetrically, producing another stem cell and a differentiated cell (Cooper, 2000; Shenghui et al., 2009). Because of their significance in biology and regenerative medicine, stem cells have become a prominent subject in modern research. Especially human mesenchymal stromal cells (hMSCs) have proven to be a promising candidate in this context (Ullah et al., 2015).

Mesenchyme first appears in embryonic development during gastrulation. There, cells that are committed to a mesodermal fate, lose their cell junctions and exit the epithelial layer in order to migrate freely. This process is called epithelial-mesenchymal transition (Tam & Beddington, 1987; Nowotschin & Hadjantonakis, 2010). Hence, the term mesenchyme describes non-epithelial embryonic tissue differentiating into mesodermal lineages such as bone, muscles and blood. Interestingly, it was shown nearly twenty years earlier that cells within adult bone marrow seemed to have mesenchymal properties as they were able to differentiate into bone tissue (A. J. Friedenstein et al., 1966; A. Friedenstein & Kuralesova, 1971; Bianco, 2014). This was the origin of the “mesengenic process”-hypothesis: This concept states that mesenchymal stem cells serve as progenitors for multiple mesodermal tissues (bone, cartilage, muscle, marrow stroma, tendon, fat, dermis and connective tissue) during both adulthood and embryonic development (A. Caplan, 1991; A. I. Caplan, 1994). The mesenchymal nature of these cells (termed bone marrow stromal cells: BMSCs) was confirmed later when they were shown to differentiate into adipocytic (fat) and chondrocytic (cartilage) lineages (Pittenger et al., 1999). Since then,

the term “mesenchymal stem cell” (MSC) has grown popular as an adult multipotent precursor to a couple of mesodermal tissues. hMSCs derived from bone marrow (hMSCs) were shown to differentiate into osteocytes, chondrocytes, adipocytes and cardiomyocytes (Gronthos et al., 1994; Muruganandan et al., 2009; Xu et al., 2004). Most impressively, these cells also exhibited ectodermal and endodermal differentiation potential, as they produced neuronal cells, pancreatic cells and hepatocytes (Barzilay et al., 2009; Wilkins et al., 2009; Gabr et al., 2013; Stock et al., 2014).

Furthermore, cultures with MSC properties can be established from “virtually every post-natal organs and tissues”, and not just bone marrow (da Silva Meirelles et al., 2006). However, it has to be noted that hMSCs can differ greatly in their transcription profile and *in vivo* differentiation potential depending on which tissue they originated from (Jansen et al., 2010; Sacchetti et al., 2016).

Since “hMSCs” are a heterogenous group of cells, they were defined by their *in vitro* characteristics. A minimal set of criteria are the following (Dominici et al., 2006) : First, hMSCs must be plastic adherent. Second, they must express or lack a set of specific surface antigens (positive for CD73, CD90, CD105; negative for CD45, CD34, CD11b, CD19). Third, hMSCs must differentiate to osteoblasts, adipocytes and chondroblasts *in vitro*. Together, hMSCs exhibit diverse differentiation potentials and can be isolated from multiple sources of the body. This offers great opportunity for regenerative medicine, if the particular hMSC-subtype is properly characterized.

## Multiple Myeloma

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 (Rajkumar & Kumar, 2020; Bladé et al., 2022).

## Myeloma-hMSC Interactions

Since plasma cells can not survive outside the bone marrow, MM cells also require survival signals for growth and disease progression. These signals are produced by the bone marrow microenvironment, including ECM, MSCs and ACs (Kibler et al., 1998; García-Ortiz et al., 2021).

## Myeloma Bone Disease

Bone is a two-phase system in which the mineral phase provides the stiffness and the collagen fibers provide the ductility and ability to absorb energy (Viguet-Carrin et al., 2006). On a molecular level, bone tissue is composed of extracellular matrix (ECM) proteins that are calcified by hydroxyapatite crystals. This ECM consists mostly of collagen type I, but also components with major regulatory activity, such as fibronectin and proteoglycans that are essential for healthy bone physiology (Alcorta-Sevillano et al., 2020). Bone tissue is actively remodeled by bone-forming osteoblasts and bone-degrading osteoclasts. Osteoblasts are derived from mesenchymal stromal cells (MSCs) that reside in the bone marrow (A. J. Friedenstein et al., 1966; Pittenger et al., 1999). MSCs also give rise to adipocytes (ACs) to form Bone Marrow Adipose Tissue (BMAT), which can account for up to 70% of bone marrow volume (Fazeli et al., 2013).

MM indirectly degrades bone tissue by stimulating osteoclasts and inhibiting osteoblast differentiation, which leads to MM-related bone disease (MBD) (Glavey et al., 2017). MBD is present in 80% of patients at diagnosis and is characterized by osteolytic lesions, osteopenia and pathological fractures. (Terpos et al., 2018)

## Dissemination of Myeloma Cells

dissemination is still widely unclear - multistep process - invasion, intravasation, intravascular arrest, extravasation, colonization - overcome adhesion, retention, and dependency on the BM microenvironment - loss of adhesion factors such as CD138

## The Increasing Role of Software in Biomedicine

The increasing importance of software in biomedicine is a direct consequence of the increasing complexity of biological data. The biological community has been producing more data in shorter time, posing new challenges (Yang et al., 2017). - RNAseq - single cell rnaseq - sequence

Artificial intelligence (AI) has been a game changer in the field of biomedicine. The early development of AI itself was driven by radiology, where it was designed to detect pathologies in medical images.

## Code Quality Ensures Scientific Reproducibility

A main reason to write software is to define re-usable instructions for task automation (Narzt et al., 1998). However, the complexity of the code makes it prone to errors and can prevent usage by persons other than the author himself. This is a problem for the general scientific community, as the software is often the only way to reproduce the results of a study (Sandve et al., 2013). Hence, modern journals aim to enforce standards to software development, including software written and used by biological researchers (Smith et al., 2018). Here, we provide a brief overview of the standards utilized by **plotastic** that to ensure its reliability and reproducibility by the scientific community.

Modern software development is a long-term commitment of maintaining and improving code after initial release (Boswell & Foucher, 2011). Hence, it is good practice to write the software such that it is scalable, maintainable and usable. Scalability or, to be precise, structural scalability means that the software can easily be expanded with new features without major modifications to its architecture (Bondi, 2000). This is achieved by writing the software in a modular fashion, where each module is responsible for a single function. Maintainability means that the software can easily be fixed from bugs and adapted to new requirements (Kazman et al., 2020). This is achieved by writing the code in a clear and readable manner, and by writing tests that ensure that the code works as expected (Boswell & Foucher, 2011). Usability is hard to define (Brooke, 1996), yet one can consider a software as usable if the commands have intuitive names and if the software's manual, termed “documentation”, is up-to-date and easy to understand for new users with minimal coding experience. A software package that has not received an update for a long time (approx. one year) could be considered abandoned. Abandoned software is unlikely to be fully functional, since it relies on other software (dependencies) that has changed in functionality or introduce bugs that were not expected by the developers of all dependencies. Together, software that's scalable, maintainable and usable requires continuous changes to its codebase. There are best practices that standardize the continuous change of the codebase, including version control, continuous integration (often referred to as CI), and

software testing.

Version control is a system that records changes to the codebase line by line, allowing the documentation of the history of the codebase, including who made which changes and when. This is required to isolate new and experimental features into newer versions and away from the stable version that's known to work. The most popular version control system is Git, which is considered the industry standard for software development (Chacon & Straub, 2024). Git can use GitHub.com as a platform to store and host codebases in the form of software repositories. GitHub's most famous feature is called "pull request". A pull request is a request from anyone registered on GitHub to include their changes to the codebase (as in "please pull this into your main code"). One could see pull requests as the identifying feature of the open source community, since it exposes the codebase to potentially thousands of independent developers, reaching a workforce that is impossible to achieve with closed source models used by paid software companies.

Continuous integration (CI) is a software development practice in which developers integrate code changes into a shared repository several times a day (Duvall et al., 2007). Each integration triggers the test suite, aiming to detect errors as soon as possible. The test suite includes building the software, setting up an environment for the software to run and then executing the programmed tests, ensuring that the software runs as a whole. Continuous integration is often used together with software branches. Branches are independent copies of the codebase that are meant to be merged back into the original code once the changes are finished. Since branches accumulate multiple changes over time, this can lead to minor incompatibilities between the branches of all developers (integration conflicts), which is something that CI helps to prevent.

Continuous integration especially relies on a thorough software testing suite. Software testing is the practice of writing code that checks if the codebase works as expected (Myers et al., 2011). The main type of software testing is unit testing, which tests the smallest units of the codebase (functions and classes) in isolation (Listing 1). The quality of the software testing suite is measured by the code coverage, the precision of the tests, and the number of test-cases that are checked. The code coverage is the percentage of the codebase that is called by the testing functions, which should be as close to 100% as possible, although it does not measure how well the code is tested. The precision of the test is not a measurable quantity, but it represents if the tests truly checks if the code works as expected. The number of test-cases is the number of different scenarios that are checked by the testing functions, for example testing every possible option or combinations of options for functions that have multiple options. The most popular software testing framework for python is `pytest`, which is utilized by `plotastic` (Krekel et al., 2004).

**Listing 1:** Example of an arbitrary python function and its respective unit test function. The first function simply returns the number 5. The second function tests if the first function indeed returns the number 5. The test function is named with the prefix “`test_`” and is placed in a file that ends with the suffix “`_test.py`”. The test function is executed by the testing framework `pytest`.

```
1 # Define a function called "give_me_five" that returns the number 5
2 def give_me_five():
3     return 5
4 # Define a test function asserting that "give_me_five" returns 5
5 def test_give_me_five():
6     assert give_me_five() == 5
```

## Python as a Programming Language

Here, we provide a general overview of the python programming language, explaining terms like “type”, “method”, etc., in order to prepare readers without prior programming experience for the following chapters. We also describe the design principles of python to lay out the key concepts that differentiate python compared to other programming languages.

Languages such as python are considered “high-level”, which means that it is designed to be easy to read and write, but also independent of hardware ([The Python Language Reference](#), n.d.). A key principle of python is the emphasis on implementing a syntax that is concise and close to human language (Listing 2, Listing 3).

**Listing 2:** Example of readable python code. This one-line code returns the words (string) “Hello, World!” when executed. The command is straightforward and easy to understand.

```
1 print("Hello, World!")
2 # Output: Hello, World!
```

**Listing 3:** Example of less readable code written in the low-level programming language C. This code is doing exactly the same as the python code in Listing 2. The command is harder to understand because more steps are needed to access the same functionality, including the definition of a function

```
1 #include <stdio.h>
2 int main() {
3     printf("Hello, World!");
4     return 0;
5 }
```

Furthermore, python is an interpreted language, which means that the code is executed line by line. This makes coding easier because the programmer can see the results of the code immediately after writing it. This is in contrast to compiled languages, where the code has to be compiled into machine code before it can be executed. The advantage of compiled languages is that the code runs faster, because the machine code is optimized for the hardware.

Python automates tasks that would otherwise require an advanced understanding of computer hardware, like the need for manual allocation of memory space. This is achieved by using a technique called “garbage collection”, which automatically frees memory space that is no longer needed by the program. This is a feature that is not present in low-level programming languages like C or C++, that were designed to maximize control over hardware.

Another hallmark of python is its dynamic typing system. In python the type is inferred automatically during code execution (Listing 4). This is in contrast to statically typed languages like C, where the type of a variable has to be declared explicitly and cannot be changed during code execution (Listing 5) ([The Python Language Reference](#), n.d.). This makes python a very beginner-friendly language, since one does not have to keep track of the type of each variable. However, this also makes python a slower language, because the interpreter has to check the type of each variable during code execution. Also, dynamic typing systems are more prone to bugs, because they allow for implicit type conversions, making mistakes, and require disciplined programming conventions. One such convention are type hints, which are a way to declare the type of a variable in python, and are used to make the code more readable and understandable for other developers (Listing 6) (van Rossum et al., 2014).

**Listing 4:** Example of dynamic typing in python. The variable “a” is assigned the value 5, which is an integer. The variable “a” is then assigned the value “Hello, World!”, which is a string. Note that code after “#” is considered a comment and not executed.

```
1  a = 5 # Type integer
2  a = "Hello, World!" # Type string
3  a = 5.0 # Type float
4  a = True # Type boolean
5  a = False # Type boolean
6  a = [1, 2, 3] # Type list of integers
7  a = {"name": "Regina"} # Type dictionary
```

**Listing 5:** Example of static typing in C. The variable “a” is declared as an integer, and can only store integers. The variable “a” is then assigned the value 5, which is an integer. The variable “a” is then assigned the value “Hello, World!”, which is a string. This results in a compilation error, because the variable “a” can only store integers.

```
1  int a; // Declare type as integer
2  a = 5;
3  a = "Hello, World!"; // Compilation error!
```

**Listing 6:** Example of type hints used in python. Explicitly stating the type of the variable is optional and does not change the behavior of the code.

```
1  a: int = 5
2  a: str = "Hello, World!"
```

Python supports both functional and object-oriented programming paradigms. In functional programming, the code is written in a way that the program is a sequence of function calls,

where each function call returns a value that is used for the next function call (Listing 7). This approach is useful when multiple actions have to be performed on the same data and the structure of the data is relatively simple, for example a string of a gene.

**Listing 7:** Example of functional programming in Python. The code defines three functions: “create\_person”, “say\_hello”, and “introduce”. “create\_person” creates a dictionary representing a person, “say\_hello” prints a greeting, and “introduce” uses the other two functions to introduce a person.

```
1
2     gene1 = "TGAGCTGAGCTGATGCGCTATTTAGGCG"
3
4     def find_restriction_site(gene: str):
5         return gene.find("GCGC")
6
7     def cut(gene: str):
8         position = find_restriction_site(gene)
9         return gene[position:]
10
11    gene1_cut = cut(gene1)
12    print(gene1_cut)
13    # Output: GCGCTATTTAGGCG
```

However, when the data itself gains in complexity, an object-oriented approach is more suitable (Listing 8). Object-oriented programming is a programming paradigm that uses objects and classes. An object is a collection of both data and functions, and a class is a blueprint for creating objects. Functions that are associated with a class are called methods.

The main benefit of using an object oriented versus a functional approach is that every method has access to the data of the object

**Listing 8:** Example of a class in python. The class is called Person and has two functions, “\_\_init\_\_” and “say\_hello”. The function “\_\_init\_\_” is called when creating (“initializing”) an object and fills the object with data. The parameter “self” is used to reference the object itself internally. The function “say\_hello” is a method that prints the string “Hello!” when called. The method “introduce” is a method that calls the method “say\_hello” and combines the string “I am” with the name of the object.

```
1     class Gene:
2         def __init__(self, sequence: str, organism: str, exons: list):
3             self.sequence: str = sequence
4             self.organism: str = organism
5             self.exons: list = exons
6             self.promotor: str = self.find_promotor()
7         def find_promotor(self):
8             return self.sequence.find("TATA")
9         def find_restriction_site(self):
10            return self.sequence.find("GCGC")
```

```
11     def cut(self):
12         position = self.find_restriction_site()
13         return self.sequence[position:]
14
15 gene1 = Gene(
16     sequence="TGAGCTGAGCTGATGCGCTATATTAGGCG",
17     organism="Human"
18 )
19 gene1_cut = gene1.cut()
20 print(gene1_cut)
21 # Output: GCGCTATATTAGGCG
```

Initially, object-oriented programming was designed to be used when the functions rely on the state of the data and modifications of such state.

This state could be for example a Table containing data, and the functions could be the operations that modify the table. When designing software, both paradigms can be used together, where object oriented programming is often used to design the overall architecture of the software, and functional programming is used to write the code that implements the functionalities.

## Data Science with Python

the ease of use has made python a very popular language (Rayhan & Gross, 2023)

Like any other programming language, python alone does not provide specialized tools like those used for data analysis ([The Python Language Reference](#), n.d.). However, python was designed to be extended by packages developed by its users. A python package consists of multiple python modules, where each module is a text-file with a .py ending containing python code. Famous examples of such packages are **pytorch** and **tensorflow**, that are used to build models of artificial intelligence, including ChatGPT (Paszke et al., 2019; Abadi et al., 2016; Radford et al., 2019). Here, we outlay the most important packages used for **plotastic**.

Interactive Python - Jupyter

Python overcame the issues of interpreted language by utilizing Code written in C numpy:

- Acceleration,
- SIMD instructions

Tabular operations - pandas

Data visualization - matplotlib - seaborn

Inferential Statistics - pingouin

AI: - pytorch and tensorflow - example: VGG19 is just a few lines of code (??) asdfdf

## Convolutional Neural Networks

This work greatly benefited from the use of convolutional neural networks provided by the Zeiss ZEN software. Here, we provide a brief

# Chapter 1: Modelling Myeloma Dissemination *in vitro*

1 Keep it Together: Modelling Myeloma Dissemination *in vitro* with hMSC-  
2 Interacting Subpopulations of INA-6 Cells and their Aggregation/Detachment  
3 Dynamics

4

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23

24 **Running Title**

25 Keep it Together: Modelling Myeloma Dissemination *in vitro*

26

27 **Keywords**

28 Multiple Myeloma, Human Mesenchymal Stromal Cells (hMSC), Cell Adhesion,  
29 Dissemination, Survival

30

31 **Additional Information**

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40 **Conflict of Interest:**

41 The authors declare no potential conflicts of interest.

42

## 43 Abstract

44 Multiple myeloma involves early dissemination of malignant plasma cells across the bone  
45 marrow; however, the initial steps of dissemination remain unclear. Human bone marrow-  
46 derived mesenchymal stromal cells (hMSCs) stimulate myeloma cell expansion (e.g., IL-6)  
47 and simultaneously retain myeloma cells via chemokines (e.g., CXCL12) and adhesion  
48 factors. Hence, we hypothesized that the imbalance between cell division and retention  
49 drives dissemination.

50 We present an *in vitro* model using primary hMSCs co-cultured with INA-6 myeloma cells.  
51 Time-lapse microscopy revealed proliferation and attachment/detachment dynamics.  
52 Separation techniques (V-well adhesion assay and well plate sandwich centrifugation) were  
53 established to isolate MSC-interacting myeloma subpopulations that were characterized by  
54 RNAseq, cell viability and apoptosis. Results were correlated with gene expression data  
55 (n=837) and survival of myeloma patients (n=536).

56 On dispersed hMSCs, INA-6 saturate hMSC-surface before proliferating into large homotypic  
57 aggregates, from which single cells detached completely. On confluent hMSCs, aggregates  
58 were replaced by strong heterotypic hMSC-INA-6 interactions, which modulated apoptosis  
59 time-dependently. Only INA-6 daughter cells (nMA-INA6) detached from hMSCs by cell  
60 division but sustained adherence to hMSC-adhering mother cells (MA-INA6).

61 Isolated nMA-INA6 indicated hMSC-autonomy through superior viability after IL-6 withdrawal  
62 and upregulation of proliferation-related genes. MA-INA6 upregulated adhesion and retention  
63 factors (CXCL12), that, intriguingly, were highly expressed in myeloma samples from patients  
64 with longer overall and progression-free survival, but their expression decreased in relapsed  
65 myeloma samples.

66 Altogether, *in vitro* dissemination of INA-6 is driven by detaching daughter cells after a cycle  
67 of hMSC-(re)attachment and proliferation, involving adhesion factors that represent a bone  
68 marrow-retentive phenotype with potential clinical relevance.

69    **Statement of Significance**

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

73

## 74 Introduction

75 Multiple myeloma arises from clonal expansion of malignant plasma cells in the bone marrow  
76 (BM). At diagnosis, myeloma cells have disseminated to multiple sites in the skeleton and, in  
77 some cases, to “virtually any tissue” (1, 2). However, the mechanism through which myeloma  
78 cells initially disseminate remains unclear.

79 Dissemination is a multistep process involving invasion, intravasation, intravascular arrest,  
80 extravasation, and colonization (3). To initiate dissemination, myeloma cells overcome  
81 adhesion, retention, and dependency on the BM microenvironment, which could involve the  
82 loss of adhesion factors such as CD138 (4, 5). BM retention is mediated by multiple factors:  
83 First, chemokines (CXCL12 and CXCL8) produced by mesenchymal stromal cells (MSCs),  
84 which attract plasma cells and prime their cytoskeleton and integrins for adhesion (6, 7).  
85 Second, myeloma cells must overcome the anchorage and physical boundaries of the  
86 extracellular matrix (ECM), consisting of e.g. fibronectin, collagens, and proteoglycans such  
87 as decorin (8-11). Simultaneously, ECM provides signals inducing myeloma cell cycle arrest  
88 or progression the cell cycle (8, 10). ECM is also prone to degradation, which is common in  
89 several osteotropic cancers, and is the cause of osteolytic bone disease. This is driven by a  
90 ‘vicious cycle’ that maximizes bone destruction by extracting growth factors (EGF and TGF-  
91 β) that are stored in calcified tissues (12). Third, direct contact with MSCs physically anchors  
92 myeloma cells to the BM (3, 13). Fourth, to disseminate to distant sites, myeloma cells  
93 require, at least partially, independence from essential growth and survival signals provided  
94 by MSCs in the form of soluble factors or cell adhesion signaling (5, 14, 15). For example,  
95 the VLA4 (Myeloma)-VCAM1 (MSC)-interface activates NF-κB in both myeloma and MSCs,  
96 inducing IL-6 expression in MSCs. The independence from MSCs is then acquired through  
97 autocrine survival signaling (16, 17). In short, anchorage of myeloma cells to MSCs or ECM  
98 is a ‘double-edged sword’: adhesion counteracts dissemination, but also presents signaling  
99 cues for growth, survival, and drug resistance (18).

100 To address this ambiguity, we developed an *in vitro* co-culture system modeling diverse  
101 adhesion modalities to study dissemination, growth, and survival of myeloma cells and

102 hMSCs. Co-cultures of hMSCs and the myeloma cell line INA-6 replicated tight interactions  
103 and aggregate growth, akin to "micrometastases" in Ghobrial's metastasis concept (19). We  
104 characterized the growth conformations of hMSCs and INA-6 as homotypic aggregation vs.  
105 heterotypic hMSC adherence and their effects on myeloma cell survival. We tracked INA-6  
106 detachments from aggregates and hMSCs, thereby identifying a potential "disseminated"  
107 subpopulation lacking strong adhesion. We developed innovative techniques (V-well  
108 adhesion assay and well plate sandwich centrifugation) to separate weakly and strongly  
109 adherent subpopulations for the subsequent analysis of differential gene expression and cell  
110 survival. Notably, our strategy resolves the differences in gene expression and growth  
111 behavior between cells of one cell population in "direct" contact with MSCs. In contrast,  
112 previous methods differentiated between "direct" and "indirect" cell-cell contact using  
113 transwell inserts (20). To evaluate whether genes mediating adhesion and growth  
114 characteristics of INA-6 were associated with patient survival, we analyzed publicly available  
115 datasets (21, 22).

## 116 Materials and Methods

117 See supplemental for a complete method list and description.

### 118 Ethics Statement

119 Primary human MSCs were collected with informed consent from all patients and the  
120 procedure was approved by the local Ethics Committee of the University of Würzburg  
121 (186/18).

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

123 Cell isolation, cultivation, and medium composition are provided in the supplemental  
124 materials and methods section. Primary human MSCs (hMSC) were obtained from 34 non-  
125 myeloma patients undergoing elective hip arthroplasty (Tab. S1: 21 male and 13 female,  
126 mean age  $68.9 \pm 10.6$ ). The INA-6 cell line (DSMZ Cat# ACC-862, RRID:CVCL\_5209, [link](#))  
127 was initially isolated from a pleural effusion sample obtained from an 80-year-old male with  
128 multiple myeloma (23, 24). hMSCs were not tested for mycoplasma, whereas stocks of INA-6

129 were tested in this study (Tab. S1) using the Venor GeM OneStep kit (Minerva Biolabs,  
130 Berlin, Germany).

131 For each co-culture, hMSCs were seeded 24 h before INA-6 addition to generate the MSC-  
132 conditioned medium (CM). INA-6 cells were washed with PBS, resuspended in MSC  
133 medium, and added to hMSCs so that the co-culture comprised 33% (v/v) of CM gathered  
134 directly from the respective hMSC donor. The co-cultures were not substituted for IL-6 ([14](#)).

### 135 **Cell Viability and Apoptosis Assay**

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

### 139 **Automated Fluorescence Microscopy**

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

### 143 **Live Cell Imaging**

144 hMSCs (stained with PKH26) were placed into an ibidi Stage Top Incubation System and  
145 equilibrated to 80% humidity and 5% CO<sub>2</sub>. INA-6 ( $2 \times 10^3$  cells/cm<sup>2</sup>) were added directly  
146 before the start of acquisition. Brightfield and fluorescence images of up to 13 mm<sup>2</sup> of the co-  
147 culture area were acquired every 15 min for 63 h. Each event of interest was manually  
148 analyzed and categorized into defined event parameters.

### 149 **V-well Adhesion Assay**

150 INA-6 cells were arrested during mitosis by two treatments with thymidine, followed by  
151 nocodazole. Arrested INA-6 were released and added to 96 V-well plates (10<sup>4</sup> cells/cm<sup>2</sup>) on  
152 top of confluent hMSCs and adhered for 1-3 h. The co-culture was stained with calcein-AM  
153 (Thermo Fisher Scientific, Darmstadt, Germany) before non-adherent INA-6 were pelleted  
154 into the tip of the V-well (2000 rpm, 5-10 min). MSC-adhering INA-6 cells were manually  
155 detached by rapid pipetting. The pellet brightness was measured microscopically and the  
156 pellet was isolated by pipetting.

157 **Cell Cycle Profiling by Image Cytometry**

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

164 **Well Plate Sandwich Centrifugation (WPSC)**

165 hMSCs were grown to confluence in 96-well plates coated with collagen I (rat tail; Corning,  
166 NY, USA). INA-6 were added and the cells were allowed to adhere for 24 h. A second plate  
167 ("catching plate") was attached upside down to the top of the co-culture plate. That "well  
168 plate sandwich" was turned around and the content of the co-culture plate was centrifuged  
169 into the catching plate three times (40 seconds at 110 g) while gently adding 30 µL of  
170 medium in between centrifugation steps. Non-MSC-adhering INA-6 cells were collected from  
171 the catching plate, whereas MSC-adhering INA-6 cells were isolated by digesting the co-  
172 culture with Accutase. For RNA sequencing (RNAseq), all samples were purified using anti-  
173 CD45 magnetic-assisted cell sorting (Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach).

174 **RNA Isolation**

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

178 **RNA sequencing, Differential Expression, and Functional Enrichment Analysis**

179 RNAseq was performed at the Core Unit Systems Medicine, University of Würzburg. mRNA  
180 was enriched with polyA beads. Fastq files were aligned to the GRCh38 reference genome  
181 using STAR (RRID:SCR\_004463, [link](#)) and raw read counts were generated using HTseq  
182 ([25-27](#)). Differential gene expression was analyzed using edgeR in R (version 3.6.3).  
183 Functional enrichment analysis was performed using Metascape ([28](#)).

184 **RT-qPCR**

185 RNA (1 µg) was reverse transcribed using SuperScript IV reverse transcriptase (Thermo  
186 Fisher Scientific, Darmstadt, Germany). qPCR was performed using 10 µL GoTaq qPCR  
187 Master Mix (Promega), 1:10 diluted cDNA, and 5 pmol of primers obtained from Biomers.net  
188 or Qiagen (Tab. S3).

189 **Statistics**

190 Inferential statistics were performed using Python (IPython, RRID:SCR\_001658, [link](#)) (3.10)  
191 packages pingouin (0.5.1) and statsmodels (0.14.0) ([29](#), [30](#)). The figures were plotted using  
192 plotastic (0.0.1) ([31](#)). Normality (for  $n \geq 4$ ) and sphericity were ensured using Mauchly's and  
193 Shapiro-Wilk tests, respectively. Data points were log10 transformed to convert the scale  
194 from multiplicative to additive or to fulfill sphericity requirements. p-value = 0.05 > \* > 0.01 >  
195 \*\* >  $10^{-3}$  > \*\*\*  $10^{-4}$  > \*\*\*\*. P-values were either adjusted (p-adj) or not adjusted (p-unc) for  
196 family wise error rate. Power calculations were not performed to determine the sample size.

197 **Patient Cohort, Analysis of Survival and Expression**

198 Survival and gene expression data were obtained as previously described ([21](#), [22](#)) and are  
199 available at the European Nucleotide Archive (ENA) under accession numbers PRJEB36223  
200 and PRJEB37100. The expression level was categorized into "high" and "low" using maxstat  
201 (Maximally selected Rank Statistics) thresholds ([32](#)).

202 **Data Availability Statement**

203 A detailed description of the methods is provided in the Supplementary Material section. Raw  
204 tabular data and examples of analyses and videos are available in the github repository ([33](#)).  
205 Raw RNAseq data are available from the NCBI Gene Expression Omnibus (GEO)  
206 (RRID:SCR\_005012, link) (GSExxxx). Microscopy data are available at the Image Data  
207 Resource (IDR) (RRID:SCR\_017421, link) (idrxxxx).

208 **Results**

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

210 hMSCs are isolated as a heterogeneous cell population. To analyze whether INA-6 cells  
211 could adhere to every hMSC, we saturated hMSCs with INA-6. A seeding ratio of 1:4

212 (hMSC:INA-6) resulted in the occupation of  $93\% \pm 6\%$  of single hMSCs by one or more INA-  
213 6 cells within 24 h after INA-6 addition, escalating to 98% after 48 h (Fig. 1A, B). Therefore,  
214 most hMSCs provide an interacting surface for INA-6 cells.

215 INA-6 exhibits homotypic aggregation when cultured alone, a phenomenon observed in  
216 some freshly isolated myeloma samples (up to 100 cells after 6 h) (34, 35). Adding hMSCs at  
217 a 1:1 ratio led to smaller aggregates after 24 h (size 1-5 cells), all of which were distributed  
218 over  $52 \pm 2\%$  of all hMSCs (Fig. 1A, B). Intriguingly, INA-6 aggregation was notably absent  
219 when grown on confluent hMSCs, and occurred only when heterotypic interactions were  
220 limited to 0.2 hMSCs per INA-6 cell (Fig. 1C). We concluded that INA-6 cells prioritize  
221 heterotypic over homotypic interactions.

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

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

228 Although direct interaction with hMSCs has been shown to enhance myeloma cell survival  
229 through NF- $\kappa$ B signaling (15), the impact of aggregation on myeloma cell viability during  
230 hMSC interaction remains unclear. To address this, we measured the cell viability (ATP) and  
231 apoptosis rates of INA-6 cells growing as homotypic aggregates compared to those in  
232 heterotypic interactions with hMSCs by modulating hMSC density (Fig. 1E). To equalize the  
233 background signaling caused by soluble MSC-derived factors, all cultures were incubated in  
234 hMSC-conditioned medium and the results were normalized to INA-6 cells cultured without  
235 direct hMSC contact (Fig. 1E, left).

236 INA-6 viability (ATP) was not affected by the direct adhesion of hMSCs at any density.  
237 However, apoptosis rates decreased over time [ $F(2, 6) = 23.29$ ,  $p\text{-unc} = 1.49e-03$ , Two-factor  
238 RM-ANOVA], interacting significantly with MSC density [ $F(4, 12) = 6.98$ ,  $p\text{-unc} = 3.83e-3$ ].  
239 For example, 24 h of adhesion to confluent MSCs increased apoptosis rates by  $1.46 \pm 0.37$

240 fold, while culturing INA-6 cells on dispersed hMSCs (ratio 1:1) did not change the apoptosis  
241 rate ( $1.01 \pm 0.26$ ).

242 We presumed that sensitive apoptotic cells might have been lost when harvesting INA-6 cells  
243 from hMSCs. Hence, we measured survival parameters in the co-culture and in hMSC and  
244 INA-6 cells cultured separately (Fig. 1E, right). We defined MSC interaction effects when the  
245 survival measured in the co-culture differed from the sum of the signals measured from INA-6  
246 and hMSCs alone. RM-ANOVA confirmed that adherence to confluent MSCs increased  
247 apoptosis rates of INA-6 cells 24 h after adhesion and decreased after 72 h [interaction  
248 between MSC density and time:  $F(2, 4) = 26.86$ ,  $p\text{-unc} = 4.80\text{e-}03$ , Two-factor RM-ANOVA],  
249 whereas INA-6 cells were unaffected when grown on dispersed hMSCs.

250 In summary, the growth conformation of INA-6 cells, measured as the ratio between  
251 homotypic aggregation and heterotypic MSC interactions, affected apoptosis rates of INA-6  
252 cells.

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

254 Using time-lapse microscopy, we observed that  $26 \pm 8\%$  of INA-6 aggregates growing on  
255 single hMSCs spontaneously shed INA-6 cells (Fig. 2A, B; Vid. S1). Notably, all detached  
256 cells exhibited similar directional movements, suggesting entrainment in convective streams  
257 generated by temperature gradients within the incubation chamber. INA-6 predominantly  
258 detached from other INA-6 cells or aggregates (Fig. 2C), indicating weaker adhesive forces  
259 in homotypic interactions than in heterotypic interactions. The detachment frequency  
260 increased after 52 h, when most aggregates that shed INA-6 cells were categorized as large  
261 ( $> 30$  cells) (Fig. 2D). Since  $\sim 1020$  INA6 cells already fully covered a single hMSC, we  
262 suggest that myeloma cell detachment depended not only on hMSC saturation, but also  
263 required a minimum aggregate size. Interestingly, INA-6 detached mostly as single cells,  
264 independent of aggregate size categories [ $F(2, 6)=4.68$ ,  $p\text{-unc}=.059$ , Two-factor RM-  
265 ANOVA] (Fig. 2D), showing that aggregates remained mostly stable despite losing cells.

**266 Cell Division Generates a Daughter Cell Detached from hMSC**

267 We suspected that cell division drives detachment because we observed that MSC-adhering  
268 INA-6 cells could generate daughter cells that “roll over” the mother cell (Fig. 3A; Vid. S2).  
269 We recorded and categorized the movement of INA-6 daughter cells in confluent hMSCs  
270 after cell division. Half of all INA-6 divisions yielded two daughter cells that remained  
271 stationary, indicating hMSC adherence (Fig. 3B, C; Vid. S3). The other half of division  
272 events generated one hMSC-adhering (MA) cell and one non-hMSC-adhering (nMA) cell,  
273 which rolled around the MA cell for a median time of 2.5 h post division ( $Q_1=1.00$  h,  $Q_3=6.25$   
274 h) until it stopped and re-adhered to the hMSC monolayer (Fig. 3D; Vid. S2, S4). Thus, cell  
275 division establishes a time window in which one daughter cell can detach.

276 To validate that cell division reduced adhesion, we measured both the size and cell cycle  
277 profile of the nMA and MA populations using an enhanced V-well assay (method described in  
278 Fig. 3E, S1, S2). For comparison, we fully synchronized and arrested INA-6 cells at mitosis  
279 and released their cell cycle immediately before addition to the hMSC monolayer, rendering  
280 them more likely to divide while adhering. Mitotic arrest significantly increased the number of  
281 nMA cells and decreased the number of MA cells (Fig. 3F). Furthermore, the nMA population  
282 contained significantly more cells cycling in the G<sub>0</sub>/G<sub>1</sub> phase than the MA population, both in  
283 synchronously and asynchronously cycling INA-6 (Fig. 3G, S3B). The number of nMA INA-6  
284 cells increased due to a higher cell division frequency. Taken together, we showed that INA-  
285 6 detach from aggregates by generating one temporarily detached daughter cell after cell  
286 division, a process that potentially contributes to the initiation of dissemination.

**287 Well Plate Sandwich Centrifugation (WPSC) Separates hMSC-Interacting INA-6  
288 Subpopulations**

289 To separate nMA and MA cells for further analysis, we developed the method “Well Plate  
290 Sandwich Centrifugation” (WPSC), outlined in Fig. 4A. To equalize the background signaling  
291 caused by MSC-derived factors and to focus on differences within directly MSC-interacting  
292 INA-6 subpopulations, all cultures were incubated in hMSC-conditioned medium (CM) from  
293 the respective donors and compared with INA-6 incubation in CM without hMSCs.

294 Microscopic tracking of nMA and MA INA-6 cell numbers during each WPSC separation step  
295 revealed successful separation after the third centrifugation step, whereas CM-treated INA-6  
296 cells required only one centrifugation step (Fig. 4B). Thus, WPSC generated cell numbers  
297 that were suitable for subsequent analyses.

298 **RNAseq of non-MSC-Adhering and MSC-Adhering Subpopulations**

299 To characterize the subpopulations separated by WPSC, we conducted RNAseq, revealing  
300 1291 differentially expressed genes between nMA vs. CM, 484 between MA vs. CM, and 195  
301 between MA vs. nMA. We validated RNAseq and found that the differential expression of 18  
302 genes correlated with those measured with qPCR for each pairwise comparison (Fig. 4C-E,  
303 S5): nMA vs. CM [ $\rho(16) = .803, p = 6.09e-5$ ], MA vs. CM [ $\rho(16) = .827, p = 2.30e-5$ ], and MA  
304 vs. nMA cells [ $\rho(16) = .746, p = 3.74e-4$ ] (Spearman's rank correlation). One of the 18 genes  
305 (*MUC1*) measured by qPCR showed a mean expression opposite to that obtained by  
306 RNAseq (nMA vs. CM), although the difference was insignificant (Fig. 4C). For nMA vs. CM,  
307 the difference in expression measured by qPCR was significant for only two of the 11 genes  
308 (*DKK1* and *OPG*), whereas the other genes (*BCL6*, *BMP4*, *BTG2*, *IL10RB*, *IL24*, *NOTCH2*,  
309 *TNFRSF1A*, *TRAF5*) only confirmed the tendency measured by RNAseq (Fig. 4C-E). For MA  
310 vs. CM, qPCR validated the significant upregulation of seven genes (*TGM2*, *DCN*, *LOX*,  
311 *MMP14*, *MMP2*, *CXCL12*, *CXCL8*), whereas the downregulation of *BMP4* was insignificant.

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

314 To functionally characterize the unique transcriptional patterns in nMA-INA6 and MA-INA6,  
315 we generated lists of genes that were differentially expressed vs. the other two  
316 subpopulations [termed nMA vs. (MA & CM) and MA vs. (nMA & CM)]. Functional enrichment  
317 analysis was performed, and the enriched terms were displayed as ontology clusters (Fig.  
318 5A). nMA-INA6 upregulated genes enriched with loosely connected term clusters associated  
319 with proliferation (e.g., "positive regulation of cell cycle"). MA-INA6 upregulated genes  
320 enriched with tightly connected term clusters related to cell adhesion and the production of  
321 ECM factors (e.g. "cell-substrate adhesion"). Similar ontology terms were enriched in the

322 gene lists obtained from pairwise comparisons (nMA vs. CM, MA vs. CM, and MA vs. nMA)  
323 (Fig. 5B). In particular, nMA vs. CM (but not MA vs. CM) upregulated genes that were  
324 enriched with “G1/S transition”, showing that WPSC isolated nMA daughter cells after cell  
325 division.

326 To check for similarities between lists of differentially expressed genes from hMSC-  
327 interacting subpopulations, we performed enrichment analysis on gene lists from the  
328 overlaps (“ $\cap$ ”) between all pairwise comparisons (Fig. 5B, S6), and showed the extent of  
329 these overlaps in circos plots (Fig. 5C). The overlap between MA vs. CM and nMA vs. CM  
330 showed neither enrichment with proliferation- nor adhesion-related terms but with apoptosis-  
331 related terms. A direct comparison of MSC-interacting subpopulations (MA vs. nMA) showed  
332 a major overlap with MA vs. CM (Fig. 5C, middle). This overlap was enriched with terms  
333 related to adhesion but not proliferation. Hence, MA-INA6 and nMA-INA6 mostly differed in  
334 their expression of adhesion genes.

335 To assess whether nMA-INA6 and MA-INA6 were regulated by separate transcription factors,  
336 we examined the enrichment of curated regulatory networks from the TRRUST database  
337 (Fig. 5B bottom). All the lists were enriched for p53 regulation. E2F1 regulation was observed  
338 only in genes upregulated in nMA vs. CM and downregulated in MA vs. nMA. Genelists  
339 involving MA-INA6 were enriched in regulation by subunits of NF- $\kappa$ B (NFKB1/p105 and  
340 RELA/p65) and factors of immediate early response (SRF, JUN). Correspondingly, NF- $\kappa$ B  
341 and JUN are known to regulate the expression of adhesion factors in multiple myeloma and  
342 B-cell lymphoma, respectively (36, 37).

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

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

347 As previously stated, apoptosis rates increased in INA-6 cells grown on confluent hMSCs  
348 compared to CM-INA6 cells after 24 h of co-culture (Fig. 1D). Since this setup was similar to  
349 that used to separate hMSC-interacting subpopulations using WPSC, we looked for

350 enrichment of apoptosis-related terms (Fig. 5B). “Regulation of cellular response to stress”  
351 and “intrinsic apoptotic signaling pathway (in response to ER-stress)” are terms that were  
352 enriched in nMA vs. CM, MA vs. CM and their overlap. We also found specific stressors for  
353 either nMA-INA6 (“intrinsic apoptotic signaling pathway by p53 class mediator”) or MA-INA6  
354 (“extrinsic apoptotic signaling pathway via death domain receptor”). Therefore, apoptosis  
355 may be driven by ER stress in both nMA-INA6 and MA-INA6, but also by individual pathways  
356 such as p53 and death domain receptors, respectively.

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

358 Myeloma cells cause bone loss by degradation and dysregulation of bone turnover via DKK1  
359 and OPG ([38-40](#)). RNAseq of hMSC-interacting subpopulations showed enrichment with  
360 functional terms “skeletal system development” and “ossification” (Fig. 5A, S6), as well as  
361 the regulation of *MMP2*, *MMP14*, *DKK1*, and *OPG*. Validation by qPCR (Fig. 4C, D) showed  
362 that MA-INA6 significantly upregulated both *MMP14* and *MMP2* compared with either nMA-  
363 INA6 or CM-INA6. The expression of *DKK1*, however, was upregulated significantly in nMA-  
364 INA6 (and not significantly upregulated in MA-INA6), while *OPG* was significantly  
365 downregulated only in nMA-INA6.

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

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

371 Retention of myeloma cells within the bone marrow is mediated by adhesion to the ECM  
372 (e.g., collagen VI) and the secretion of chemokines (CXCL8 and CXCL12) ([7](#), [11](#)). This  
373 directly counteracts dissemination, which is a hallmark of MA-INA6. RNAseq of hMSC-  
374 interacting subpopulations showed that genes upregulated in MA-INA6 were enriched with  
375 collagen biosynthesis and modifying enzymes, as well as chemotaxis and chemotaxis-  
376 related terms (Fig. 5B). Using qPCR, we validated the upregulation of collagen crosslinkers  
377 (*LOX* and *TGM2*), collagen-binding *DCN* and chemokines (CXCL8 and CXCL12) in MA-INA6

378 compared with both nMA-INA6 and CM-INA6 (Fig. 4D). Therefore, MA-INA6 can provide  
379 both an adhesive surface and soluble signals for the retention of malignant plasma cells in  
380 the bone marrow.

381 **nMA-INA6 Show Highest Viability During IL-6 Withdrawal**

382 Although RNAseq did not reveal IL-6 induction in any WPSC-isolated subpopulation, nMA-  
383 INA6 upregulated IGF-1 135%-fold [RNAseq, nMA vs. (MA & CM)], which was shown to  
384 stimulate growth in CD45+ and IL-6 dependent myeloma cell lines such as INA-6, implying  
385 increased autonomy for nMA-INA6 (41).

386 To test the autonomy of hMSC-interacting INA-6 subpopulations, we isolated them using  
387 WPSC after 24 h and 48 h of co-culture, sub-cultured them for 48 h under IL-6 withdrawal,  
388 and measured both viability and apoptosis (Fig. 5D). Among the subpopulations, nMA-INA6  
389 was the most viable. Compared to MA-INA6, nMA-INA6 increased cell viability by 8 or 4 fold  
390 when co-cultured for 24 or 48 h, respectively [Hedges g of  $\text{Log}_{10}(\text{Fold Change})$  = 2.31 or  
391 0.82]. However, the difference was no longer significant after 48 h of co-culture, probably  
392 because nMA-INA6 adhered to the hMSC layer (turning into MA-INA6) during prolonged co-  
393 culture, which could also explain why the viability of MA-INA6 cell subcultures increased with  
394 prolonged co-culture. Nevertheless, nMA-INA6 did not achieve the same viability as that of  
395 INA-6 cells cultured with IL-6. Despite the differences in viability, subcultures of hMSC-  
396 interacting subpopulations did not show any differences in caspase 3/7 activity when co-  
397 cultured for 48 h (Fig. 5D, right).

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

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

401 To relate the adhesion of MA-INA6 observed *in vitro* to the progression of multiple myeloma,  
402 we assessed patient survival [ $n = 535$ , Seckinger et al. 2018 (21, 22) depending on the  
403 expression level of 101 genes, which were upregulated in MA vs. (nMA & CM) and are part  
404 of the ontology terms “Extracellular matrix organization,” “ECM proteoglycans,” “cell-  
405 substrate adhesion” and “negative regulation of cell-substrate adhesion” (Fig. 6A, Tab. S2).

406 As a reference, we generated a list of 173 cell cycle-related genes that were upregulated by  
407 nMA-INA6 vs. (MA-INA6 & CM-INA6).  
408 As expected, longer patient survival was associated with low expression of the majority of  
409 cell cycle genes [71 or 68 genes for progression-free survival (PFS) or overall survival (OS)].  
410 Only a few cell cycle genes (two for PFS and seven for OS) were associated with survival  
411 when highly expressed. Intriguingly, adhesion genes showed an inverse pattern: a large  
412 group of adhesion genes (24 for PFS and 26 for OS) was significantly associated with  
413 improved survival when highly expressed, whereas only a few genes (two for PFS and four  
414 for OS) improved survival when expressed at low levels (Tab. 1). We concluded that the  
415 myeloma-dependent expression of adhesion factors determined in our *in vitro* study  
416 correlates with improved patient survival.

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

419 To examine how the disease stage affects the adhesion and bone marrow retention of  
420 myeloma cells *in vitro*, we analyzed the expression of CXCL12 in healthy plasma cell  
421 (BMPC) cohorts of patients at different disease stages and in myeloma cell lines (HMCL)  
422 [described in Seckinger et al. 2018 (29)] (Fig. 6C). We also included DCN and TGM2 since  
423 both are suggested to inhibit metastasis in different cancers by promoting cell-matrix  
424 interactions (8, 42). In accordance with independent reports (9, 43), high expression of  
425 CXCL12 and DCN by myeloma cells was associated with improved overall survival (adj. p =  
426 .009 and .008, respectively) (Fig. 6B).

427 CXCL12 is expressed by BMPCs (median = 219 normalized counts), but its expression  
428 levels are significantly lower from MGUS to relapsed multiple myeloma (MMR) (median = 9  
429 normalized counts in MMR and absent expression in most HMCL). DCN (but not TGM2) was  
430 weakly expressed in BMPCs ( $Q_1=0.7$ ,  $Q_3=3.7$ , normalized counts), whereas TGM2 was  
431 weakly expressed only in patients with monoclonal gammopathy of undetermined  
432 significance (MGUS) ( $Q_1=0.4$ ,  $Q_3=4.1$  normalized counts). The median and upper quartiles of  
433 both DCN- and TGM2 decreased continuously after each stage, ending at  $Q_3=0.9$  and

434  $Q_3=0.6$ , respectively, in MMR. 49 of the 101 adhesion genes (Fig. 6A) followed a similar  
435 pattern of continuous downregulation in the advanced stages of multiple myeloma (Fig. S7  
436 and S8), of which 19 genes were associated with longer PFS when they were highly  
437 expressed. The other 52 (out of 101) adhesion genes that were not downregulated across  
438 disease progression (or were expressed at a level too low to make that categorization)  
439 contained only five genes that were associated with longer PFS at high expression (Tab. 1,  
440 Tab. S2).

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

## 444 Discussion

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

452 INA-6 cells emerged as a robust choice for studying myeloma dissemination *in vitro*, showing  
453 rapid and strong adherence, as well as aggregation exceeding MSC saturation. The IL-6  
454 dependency of INA-6 enhanced the resemblance of myeloma cell lines to patient samples,  
455 with INA-6 ranking 13th among 66 cell lines (44). Despite variations in bone marrow MSCs  
456 between multiple myeloma (MM) and healthy states, we anticipated the robustness of our  
457 results, given the persistent strong adherence and growth signaling from MSCs to INA-6  
458 during co-cultures (45).

459 We acknowledge that INA-6 cells alone cannot fully represent the complexity of myeloma  
460 aggregation and detachment dynamics. However, the diverse adhesive properties of  
461 myeloma cell lines pose a challenge. We reasoned that attempting to capture this complexity

462 within a single publication would not be possible. Our focus on INA-6 interactions with  
463 hMSCs allowed for a detailed exploration of the observed phenomena, such as the unique  
464 aggregation capabilities that facilitate the easy detection of detaching cells *in vitro*. The  
465 validity of our data was demonstrated by matching the *in vitro* findings with the gene  
466 expression and survival data of the patients (e.g. CXCL12, DCN, and TGM2 expression,  
467 n=873), ensuring biological consistency and generalizability regardless of the cell line used.  
468 The protocols presented in this study offer a cost-efficient and convenient solution, making  
469 them potentially valuable for a broader study of cell interactions. We encourage optimizations  
470 to meet the varied adhesive properties of the samples, such as decreasing the number of  
471 washing steps if the adhesive strength is low. We caution against strategies that average  
472 over multiple cell lines without prior understanding their diverse attachment/detachment  
473 dynamics, such as homotypic aggregation. Such detailed insights may prove instrumental  
474 when considering the diversity of myeloma patient samples across different disease stages  
475 ([34](#), [35](#)).

476 The intermediates, nMA-INA6 and MA-INA6, were distinct but shared similarities in response  
477 to cell stress, intrinsic apoptosis, and regulation by p53. Unique regulatory patterns were  
478 related to central transcription factors: E2F1 for nMA-INA6; and NF- $\kappa$ B, SRF, and JUN for  
479 MA-INA6. This distinction may have been established through antagonism between p53 and  
480 the NF- $\kappa$ B subunit RELA/p65 ([46](#), [47](#)). Similar regulatory patterns were found in transwell  
481 experiments with RPMI1-8226 myeloma cells, where direct contact with the MSC cell line  
482 HS5 led to NF- $\kappa$ B signaling and soluble factors to E2F signaling ([20](#)).

483 The first subpopulation, nMA-INA6, represented proliferative and disseminative cells; nMA-  
484 INA6 drove detachment through cell division, which was regulated by E2F, p53, and likely  
485 their crosstalk ([48](#)). They upregulate cell cycle progression genes associated with worse  
486 prognosis, because proliferation is a general risk factor for an aggressive disease course  
487 ([49](#)). Additionally, nMA-INA6 survived IL-6 withdrawal better than CM-INA6 and MA-INA6,  
488 implying their ability to proliferate independently of the bone marrow ([1](#)). Indeed, xenografted  
489 INA-6 cells developed autocrine IL-6 signaling but remained IL-6-dependent after

490 explantation (23). The increased autonomy of nMA-INA6 cells can be explained by the  
491 upregulation of IGF-1, being the major growth factor for myeloma cell lines (41). Other  
492 reports characterized disseminating cells differently: Unlike nMA-INA6, circulating myeloma  
493 tumor cells were reported to be non-proliferative and bone marrow retentive (50). In contrast  
494 to circulating myeloma tumor cells, nMA-INA6 were isolated shortly after detachment and  
495 therefore these cells are not representative of further steps of dissemination, such as  
496 intravasation, circulation or intravascular arrest (3). Furthermore, Brandl et al. described  
497 proliferative and disseminative myeloma cells as separate entities, depending on the surface  
498 expression of CD138 or JAM-C (4, 51). Although CD138 was not differentially regulated in  
499 nMA-INA6 or MA-INA6, both subpopulations upregulated JAM-C, indicating disease  
500 progression (51).

501 Furthermore, nMA-INA6 showed that cell division directly contributed to dissemination. This  
502 was because INA-6 daughter cells emerged from the mother cell with distance to the hMSC  
503 plane in the 2D setup. A similar mechanism was described in an intravasation model in which  
504 tumor cells disrupt the vessel endothelium through cell division and detach into blood  
505 circulation (52). Overall, cell division offers key mechanistic insights into dissemination and  
506 metastasis.

507 The other subpopulation, MA-INA6, represented cells retained in the bone marrow; MA-INA6  
508 strongly adhered to MSCs, showed NF- $\kappa$ B signaling, and upregulated several retention,  
509 adhesion, and ECM factors. The production of ECM-associated factors has recently been  
510 described in MM.1S and RPMI-8226 myeloma cells (53). Another report did not identify the  
511 upregulation of such factors after direct contact with the MSC cell line HS5; hence, primary  
512 hMSCs may be crucial for studying myeloma-MSC interactions (20). Moreover, MA-INA6  
513 upregulated adhesion genes associated with prolonged patient survival and showed  
514 decreased expression in relapsed myeloma. As myeloma progression implies the  
515 independence of myeloma cells from the bone marrow (1, 44), we interpreted these adhesion  
516 genes as mediators of bone marrow retention, decreasing the risk for dissemination and  
517 thereby potentially prolonging patient survival. However, the overall impact of cell adhesion

518 and ECM on patient survival remains unclear. Several adhesion factors have been proposed  
519 as potential therapeutic targets (51, 54). Recent studies have described the prognostic value  
520 of multiple ECM genes, such as those driven by NOTCH (53). Another study focused on  
521 ECM gene families, of which only six of the 26 genes overlapped with our gene set (Tab. S2)  
522 (55). The expression of only one gene (*COL4A1*) showed a different association with overall  
523 survival than that in our cohort. The lack of overlap and differences can be explained by  
524 dissimilar definitions of gene sets (homology vs. gene ontology), methodological  
525 discrepancies, and cohort composition.

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

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541

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- 697

698 **Figure Legends**

699 **Fig. 1:** INA-6 growth conformations and survival on hMSCs. **A:** Interaction of INA-6 (green)  
700 with hMSCs (black, negative staining) at different INA-6 densities (constant hMSC densities).  
701 Scale bar = 200  $\mu\text{m}$ . **B:** Frequency of single hMSCs (same as A) that are covered by INA-6  
702 of varying group sizes. Technical replicates = three per datapoint; Single hMSCs evaluated:  
703 100 per technical replicate. **C:** Interaction of INA-6 with hMSCs at different hMSC densities  
704 (constant INA-6 densities). Scale bar = 300  $\mu\text{m}$ . **D:** Two types of homotypic interaction:  
705 Attachment after cell contact and sustained attachment of daughter cells after cell division.  
706 Datapoints represent one of four independent time-lapse recordings, each evaluating 116  
707 interaction events. **E:** Effects of hMSC-density on the viability (ATP, top) and apoptosis  
708 (Caspase3/7 activity, bottom). INA-6:MSC ratio = 4:1; Technical replicates = four per  
709 datapoint; **E left:** Signals were measured in INA-6 washed off from hMSCs and normalized  
710 by INA-6 cultured in MSC-conditioned medium (= red line) (n=4). **E right:** Signals were  
711 measured in co-cultures and normalized by the sum of the signals measured in hMSC and  
712 INA-6 cultured separately (= red line) (n=3). **Statistics:** Paired t-test, two-factor RM-ANOVA.  
713 Datapoints represent independent co-cultures with hMSCs from three (A, B, D, E right), four  
714 (E left) unique donors. Confl. = Confluent.

715

716 **Fig. 2:** Time-lapse analysis of INA-6 detachment from INA-6 aggregates and hMSCs. **A:**  
717 Frequency of observed INA-6 aggregates that did or did not lose INA-6 cell(s). 87 aggregates  
718 were evaluated per datapoint. **B:** Example of an “disseminating” INA-6 aggregate growing on  
719 fluorescently (PKH26) stained hMSC (from A-D). Dashed green lines are trajectories of  
720 detached INA-6 cells. Scale bar = 50  $\mu\text{m}$ . **(C-E):** Quantitative assessment of INA-6  
721 detachments. 45 detachment events were evaluated per datapoint. Seeding ratio INA-6:MSC  
722 = 4:1. **C:** Most INA-6 cells dissociated from another INA-6 cell and not from an hMSC  
723 [F(1, 3) = 298, p-unc=4.2e-4]. **D:** Detachment frequency of aggregate size categories. **E:**  
724 Detachment frequency of INA-6 cells detaching as single, pairs or more than three cells.  
725 **Statistics:** (A): Paired-t-test; (C-E): Paired-t-test, Two-factor RM-ANOVA; Datapoints

726 represent three (A) or four (C-E) independent time-lapse recordings of co-cultures with  
727 hMSCs from two (A) or three (C-E) unique donors.

728

729 **Fig. 3:** Detachment of INA-6 daughter cells after Cell Division. **(A-D):** INA-6 divisions in  
730 interaction with confluent hMSCs. Seeding ratio INA-6:MSC = 4:20. **A:** Three examples of  
731 dividing INA-6 cells generating either two MA, or one MA and one nMA daughter cells as  
732 described in (G). Dashed circles mark mother cells (white), MA cell (blue), and first position  
733 of nMA cell (green). Scale bar: 20  $\mu$ m. **B:** Cell division of MSC-adhering (MA) mother cell can  
734 yield one mobile non-MSC-adhering (nMA) daughter cell. **C:** Frequencies of INA-6 pairs  
735 defined in (A, B) per observed cell division. 65 divisions were evaluated for each of three  
736 independent time-lapse recordings. **D:** Rolling duration of nMA cells after division did not  
737 depend on hMSC donor [ $H(2) = 5.250$ ,  $p\text{-unc} = .072$ ]. Datapoints represent single nMA-cells  
738 after division. **(E-G):** Adhesive and cell cycle assessment of MSC-interacting INA-6  
739 subpopulations using the V-Well assay. **E:** Schematic of V-Well Assay (See Fig. S1 for  
740 detailed analysis). MSC-interacting subpopulations were separated by subsequent  
741 centrifugation and removal of the pellet. The pellet size was quantified by its total  
742 fluorescence brightness. Adhering subpopulations were resuspended by rough pipetting. **F:**  
743 Relative cell pellet sizes of adhesive INA-6 subpopulations that cycle either asynchronously  
744 or were synchronized at mitosis. Gray lines in-between points connect dependent  
745 measurements of co-cultures ( $n=9$ ) that shared the same hMSC-donor and INA-6 culture.  
746 Co-cultures were incubated for three different durations (1 h, 2 h, and 3 h after INA-6  
747 addition). Time points were pooled, since time did not show an effect on cell adhesion  
748 [ $F(2,4) = 1.414$ ,  $p\text{-unc} = 0.343$ ]. Factorial RM-ANOVA shows an interaction between cell  
749 cycle and the kind of adhesive subpopulation [ $F(1, 8) = 42.67$ ,  $p\text{-unc} = 1.82e-04$ ]. Technical  
750 replicates = 4 per datapoint. **G:** Cell cycles were profiled in cells gathered from the pellets of  
751 four independent co-cultures ( $n=4$ ) and the frequency of G0/G1 cells are displayed  
752 depending on co-culture duration (see Fig. S3 for cell cycle profiles). Four Technical  
753 replicates were pooled after pelleting. **Statistics:** (D): Kruskal-Wallis H-test. (F): Paired t-test,

754 (G): Paired t-test, two-factor RM-ANOVA. Datapoints represent INA-6 from independent co-  
755 cultures with hMSCs from three unique donors.

756

757 **Fig. 4:** Separation and gene expression of INA-6 subpopulations. **A:** Schematic of “Well-  
758 Plate Sandwich Centrifugation” (WPSC) separating nMA- from MA-INA6. A co-culture 96 well  
759 plate is turned upside down and attached on top of a “catching plate”, forming a “well-plate  
760 sandwich”. nMA-INA6 cells are collected in the catching plate by subsequent rounds of  
761 centrifugation and gentle washing. MA-INA6 are enzymatically dissociated from hMSCs or by  
762 rough pipetting. Subsequent RNAseq of MSC-interacting subpopulations reveals distinct  
763 expression clusters [right, multidimensional scaling plot (MDS) (n=5)]. **B:** Separation was  
764 microscopically tracked after each centrifugation step. **(C-E):** RT-qPCR of genes derived  
765 from RNAseq results. Expression was normalized to the median of CM-INA6. Samples  
766 include those used for RNAseq and six further co-cultures (n=11; non-detects were  
767 discarded). **C:** Adhesion factors, ECM proteins and matrix metalloproteinases. **D:** Factors  
768 involved in bone remodeling and bone homing chemokines. **E:** Factors involved in (immune)  
769 signaling. **Statistics:** (C-E): Paired t-test. Datapoints represent the mean of three (B-E)  
770 technical replicates. INA-6 were isolated from independent co-cultures with hMSCs from five  
771 (A, B), nine (C-E) unique donors.

772

773 **Fig. 5:** Functional analysis of MSC-interacting subpopulations **(A-C):** Functional enrichment  
774 analysis of differentially expressed genes (from RNAseq) using Metascape. **A:** Gene  
775 ontology (GO) cluster analysis of gene lists that are unique for MA (left) or nMA (right) INA-6.  
776 Circle nodes represent subsets of input genes falling into similar GO-term. Node size grows  
777 with the number of input genes. Node color defines a shared parent GO-term. Two nodes  
778 with a similarity score > 0.3 are linked. **B:** Enrichment analysis of pairwise comparisons  
779 between MA subpopulations and their overlaps (arranged in columns). GO terms were  
780 manually picked and categorized (arranged in rows). Raw Metascape results are shown in  
781 Fig. S6. For each GO-term, the p-values (x-axis) and the counts of matching input genes

782 (circle size) were plotted. The lowest row shows enrichment of gene lists from the TRRUST-  
783 database. **C:** Circos plots by Metascape. Sections of a circle represent lists of differentially  
784 expressed genes. Purple lines connect same genes appearing in two gene lists.  $\cap$ :  
785 Overlapping groups, MA: MSC-adhering, nMA: non-MSC-adhering, CM: MSC-Conditioned  
786 Medium. **D:** INA-6 were co-cultured on confluent hMSC for 24 h or 48 h, separated by WPSC  
787 and sub-cultured for 48 h under IL-6 withdrawal (n=6), except the control (IL-6 + INA-6)  
788 (n=3). Signals were normalized (red line) to INA-6 cells grown without hMSCs and IL-6 (n=3).  
789 Statistics (D): Paired t-test, two-factor RM-ANOVA. Datapoints represent the mean of four  
790 technical replicates. INA-6 were isolated from independent co-cultures with hMSCs from six  
791 unique donors.

792

793 **Fig. 6:** Survival of patients with multiple myeloma regarding the expression levels of  
794 adhesion and bone retention genes. **A:** p-value distribution of genes associated with patient  
795 survival (n=535) depending on high or low expression levels. Red dashed line marks the  
796 significance threshold of  $p\text{-adj}=0.05$ . Histogram of p-values was plotted using a bin width of -  
797  $\log_{10}(0.05)/2$ . Patients with high and low gene expression were delineated using maximally  
798 selected rank statistics (maxstat). **B:** Survival curves for three genes taken from the list of  
799 adhesion genes shown in (A), maxstat thresholds defining high and low expression were:  
800 CXCL12: 81.08; DCN: 0.75; TGM2: 0.66 normalized counts. **C:** Gene expression (RNAseq, n  
801 = 873) measured in normalized counts (edgeR) of CXCL12, DCN in Bone Marrow Plasma  
802 Cell (BMPC), Monoclonal Gammopathy of Undetermined Significance (MGUS), smoldering  
803 Multiple Myeloma (sMM), Multiple Myeloma (MM), Multiple Myeloma Relapse (MMR), Human  
804 Myeloma Cell Lines (HMCL). The red dashed line marks one normalized read count.  
805 **Statistics** (A, B): Log-rank test; (C): Kruskal-Wallis, Mann–Whitney U Test. All p-values were  
806 corrected using the Benjamini-Hochberg procedure.

807

808 **Fig. 7:** Proposed model of “Detached Daughter Driven Dissemination” (DDDD) in  
809 aggregating multiple myeloma. **Heterotypic Interaction:** Malignant plasma cells colonize the

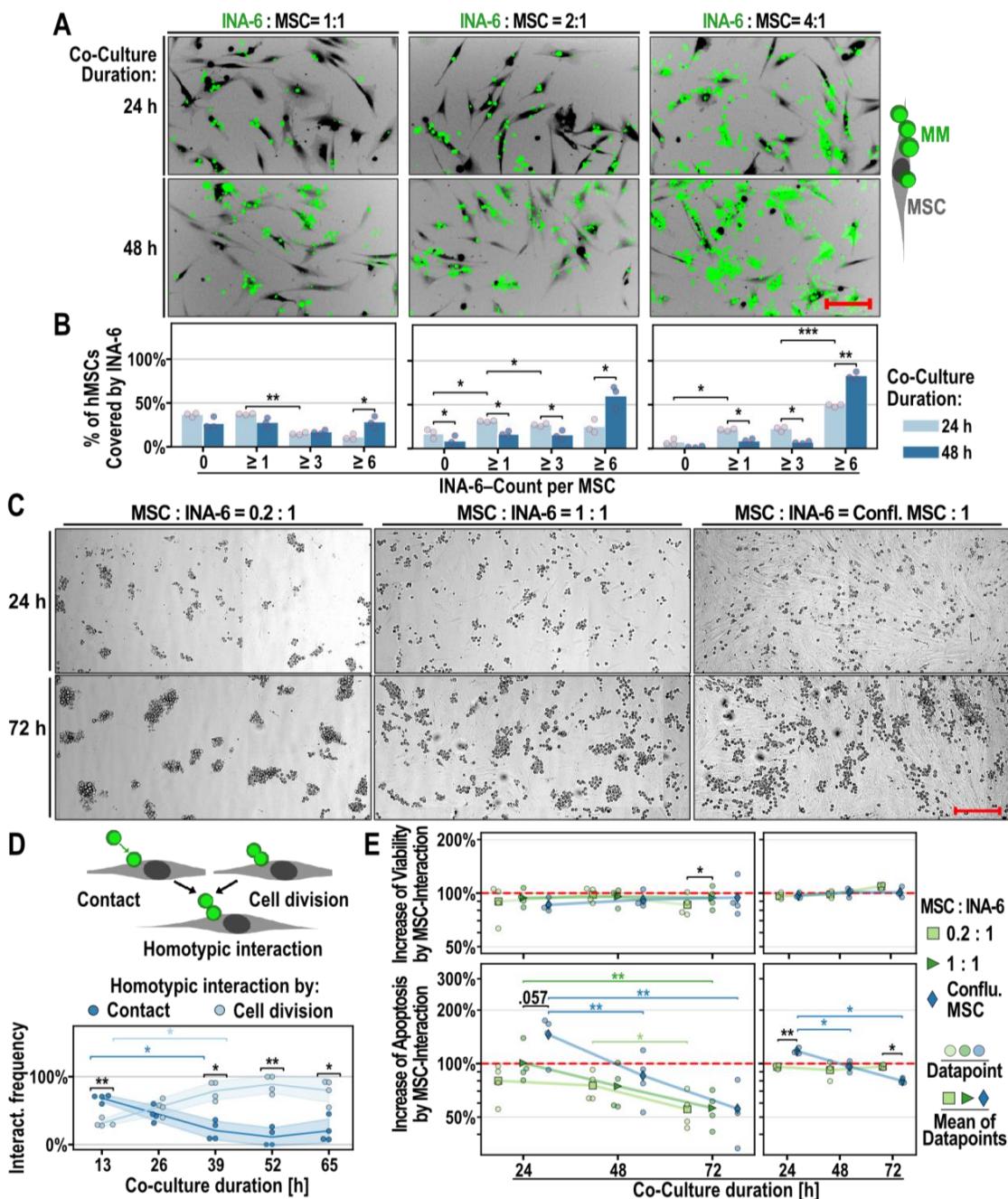
810 bone marrow microenvironment by adhering to an MSC (or osteoblast, ECM, etc.) to  
811 maximize growth and survival through paracrine and adhesion mediated signaling, even if  
812 contact may trigger initial apoptosis. Gene expression will focus on establishing a strong  
813 anchor within the bone marrow, but also on attracting other myeloma cells (via secretion of  
814 ECM factors and CXCL12/CXCL8, respectively). **Cell Division:** Cell fission can generate one  
815 daughter cell that no longer adheres to the MSC (nMA). **Homotypic Interaction:** If myeloma  
816 cells have the capacity to grow as aggregates, the daughter cell stays attached to their MSC-  
817 adhering mother cell (MA). **Re-Adhesion:** The daughter cell “rolls around” the mother cell  
818 until it re-adheres to the MSC. Our model estimates the rolling duration to be 1-10 h long.  
819 **Proliferation & Saturation:** We estimate that a single myeloma cell covers one MSC  
820 completely after roughly four population doublings. When heterotypic adhesion is saturated,  
821 subsequent daughter cells benefit from a homotypic interaction, since they stay close to  
822 growth-factor secreting MSCs and focus gene expression on proliferation (e.g. driven by  
823 E2F) and not adhesion (driven by NF- $\kappa$ B). **Critical Size:** Homotypic interaction is weaker  
824 than heterotypic interaction, and each cell fission destabilizes the aggregate. Hence,  
825 detachment of myeloma cells may depend mostly on aggregate size. **Dissemination:** After  
826 myeloma cells have detached, they gained a viability advantage through IL-6-independence  
827 (with unknown duration), which enhances their survival outside of the bone marrow and  
828 allows them to spread throughout the body.

829

830 **Tab 1:** Adhesion and ECM genes (shown in Fig. 6A) were filtered by their association with  
831 patient survival ( $p\text{-adj.} < 0.01$ ) and was categorized as continuous downregulation during  
832 disease progression. The complete list is presented in Table S2. Bone Marrow Plasma Cells  
833 (BMPC), Monoclonal Gammopathy of Undetermined Significance (MGUS), moldering  
834 Multiple Myeloma (sMM), Multiple Myeloma (MM), and Multiple Myeloma Relapse (MMR).  $p\text{-}$   
835  $\text{unc}$  = unadjusted  $p$ -values;  $p\text{-adj.}$ :  $p$ -values adjusted using the Benjamini-Hochberg method  
836 with 101 genes.

837

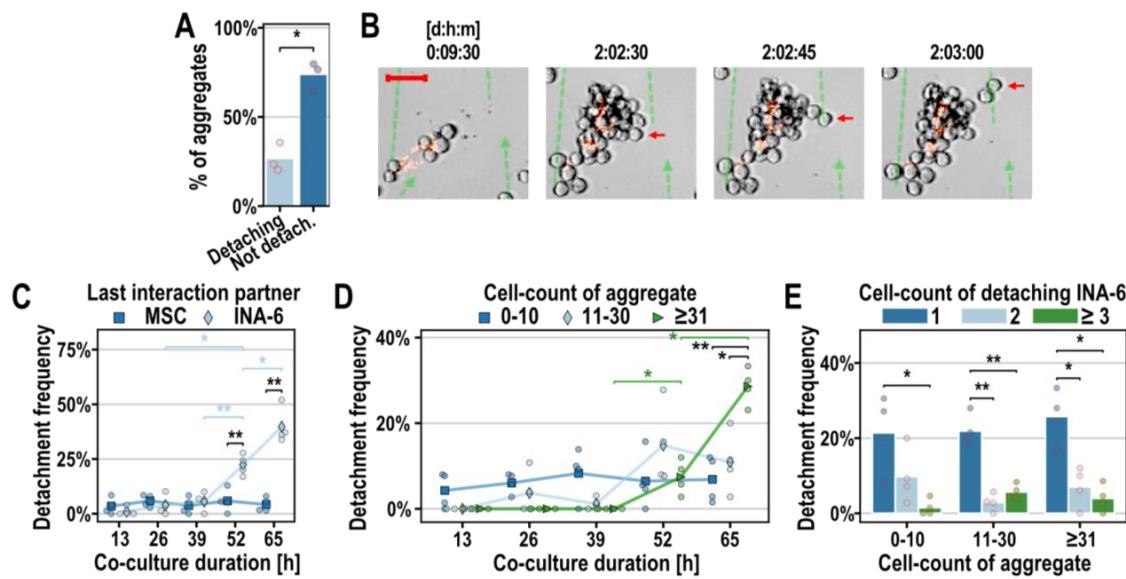
838 Fig. 1



839

840

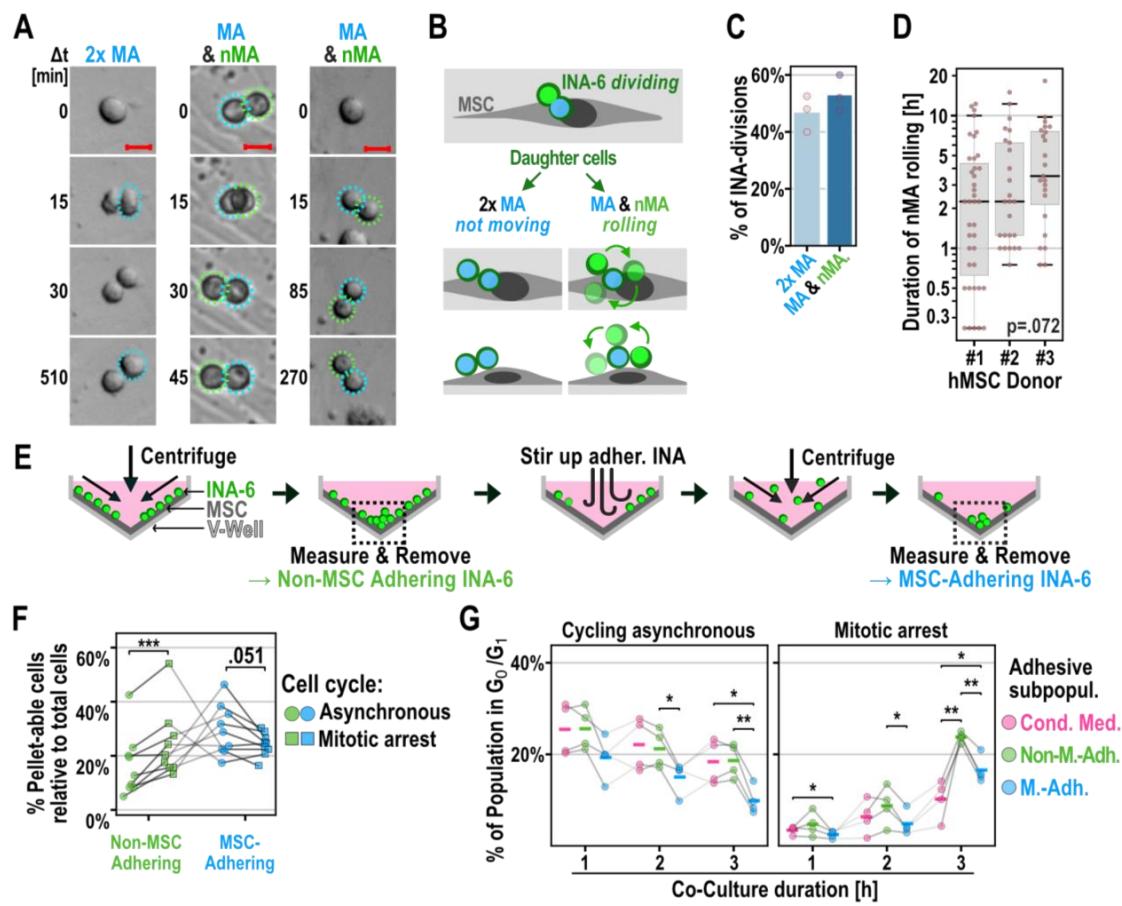
841 Fig. 2



842

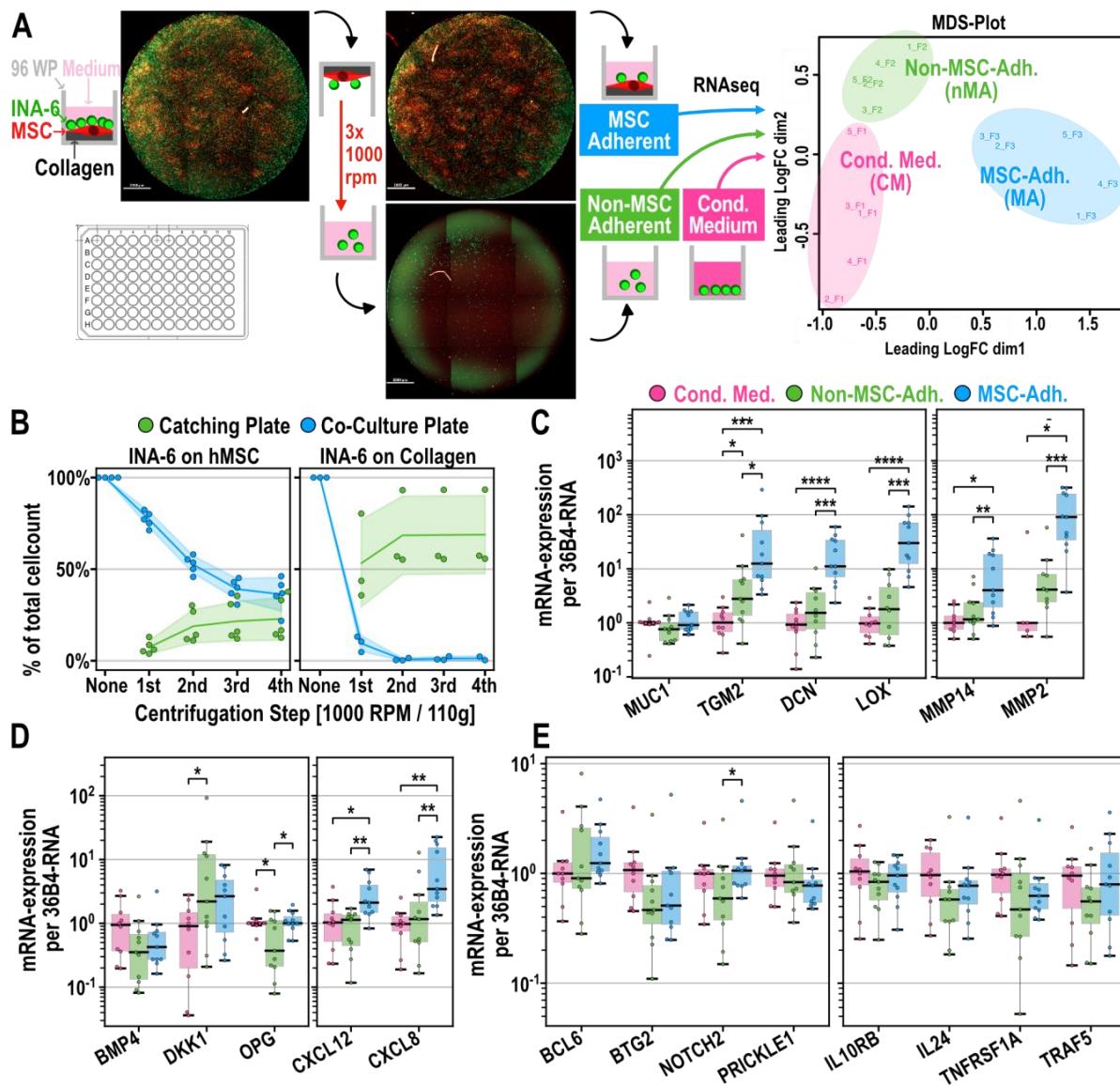
843

844 Fig. 3

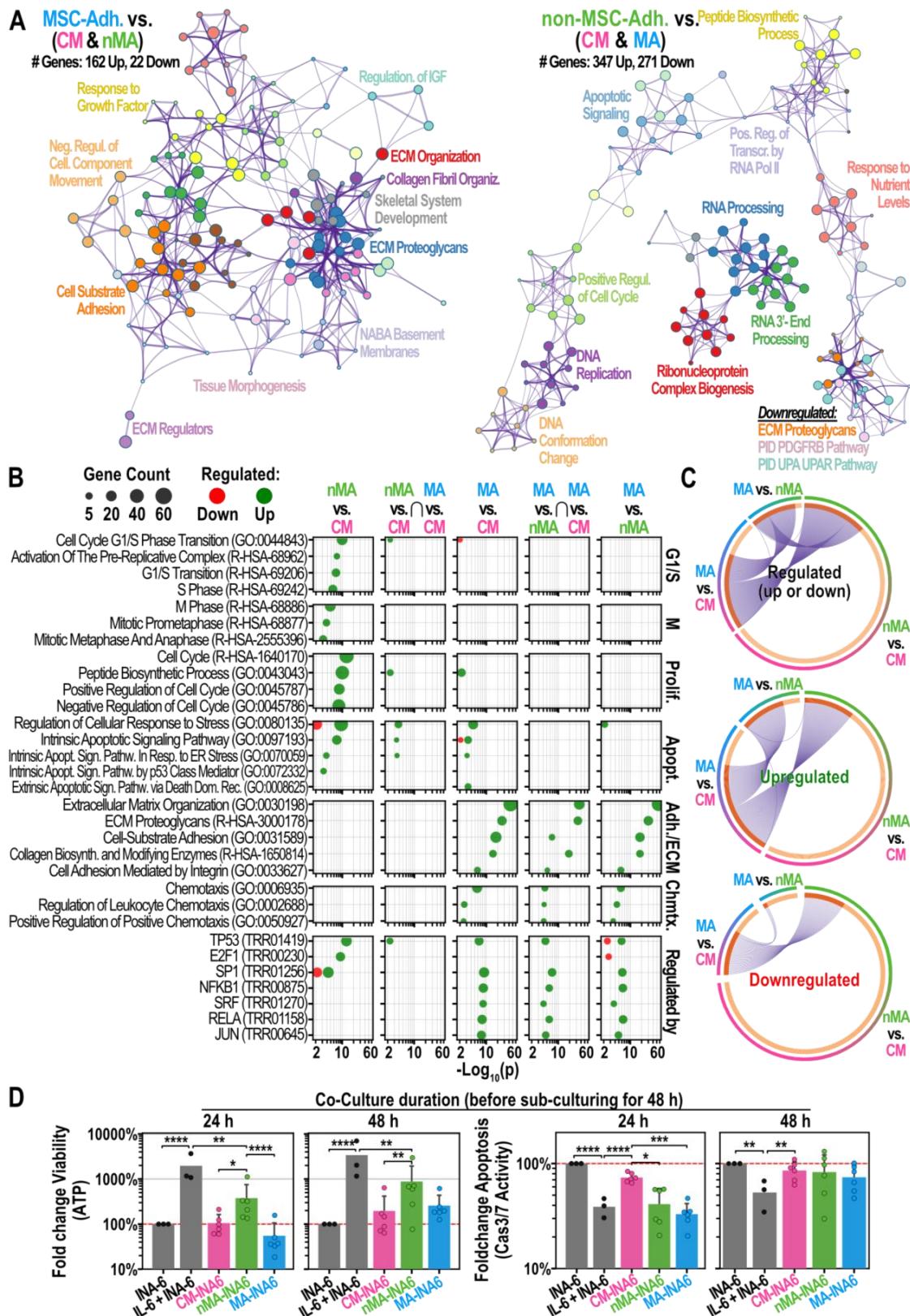


845

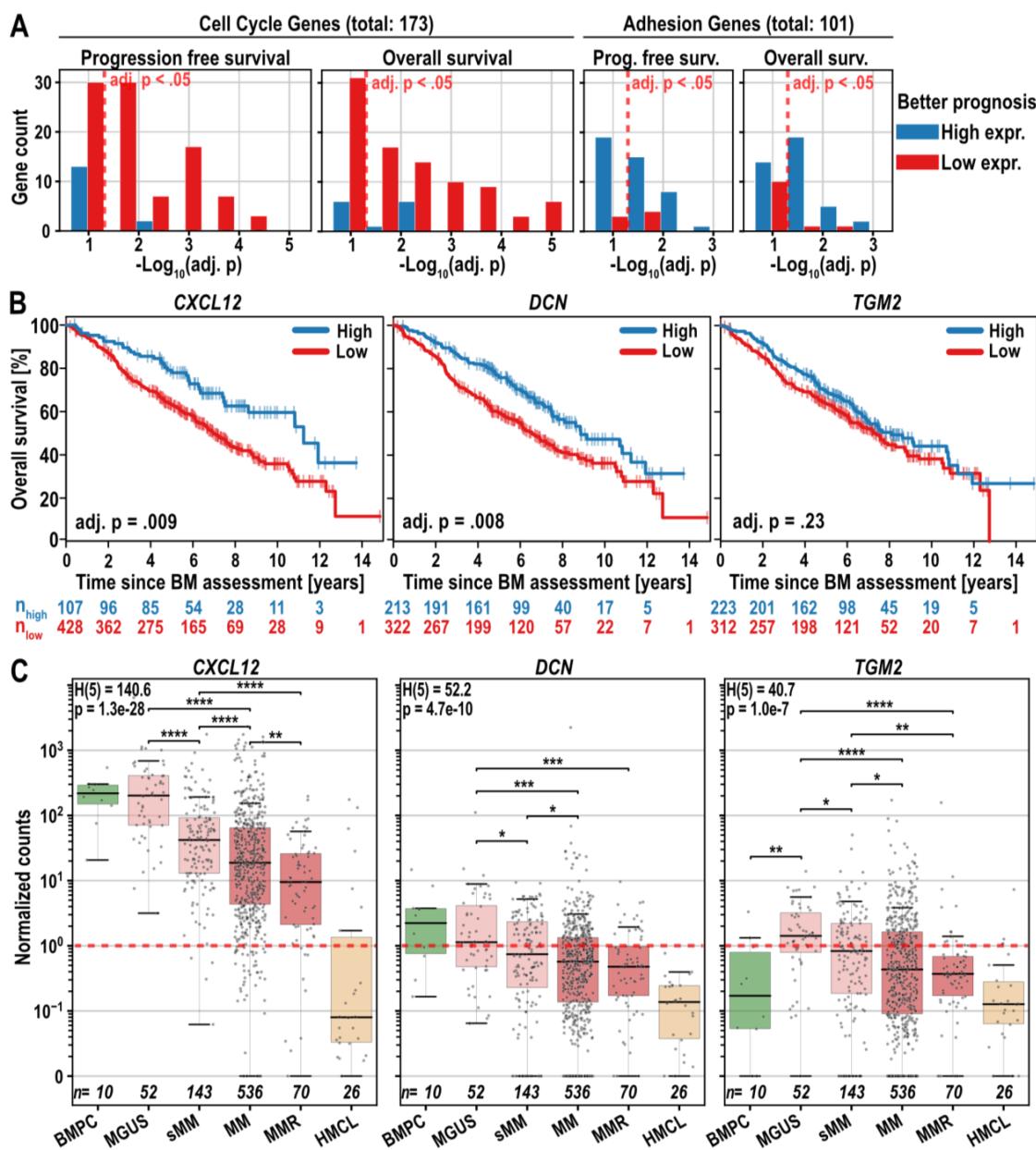
846



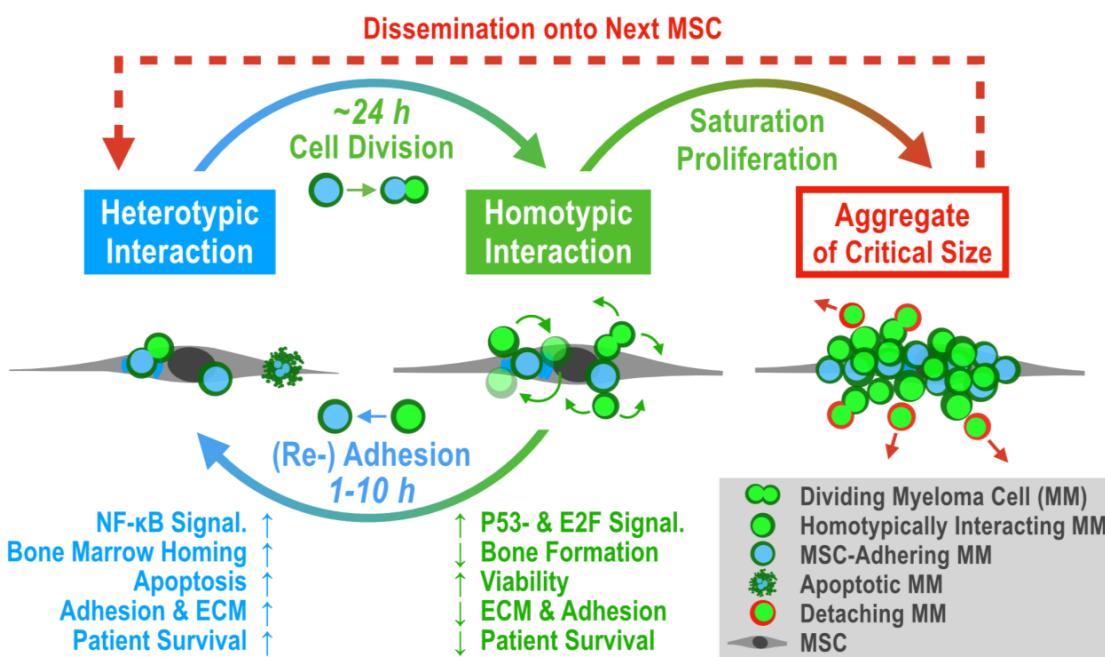
848 Fig. 5



851 Fig. 6

852  
853

854 Fig. 7



855

856

857 Tab. 1

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 > MGUS > sMM > MM > MMR)	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
	CXCL12	ENSG00000107562	Prog. Free	high	1.16E-04	2.93E-03
			Overall	high	6.48E-04	8.64E-03
	CYP1B1	ENSG00000138061	Overall	high	6.84E-04	8.64E-03
	DCN	ENSG00000011465	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
MYL9	ENSG00000101335	Prog. Free	high	1.46E-04	2.95E-03	
		Overall	high	1.56E-05	1.57E-03	

858

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## Supplementary Figures and Methods

### Keep it Together: Describing Myeloma Dissemination *in vitro* with hMSC-Interacting Subpopulations and their Aggregation/Detachment Dynamics

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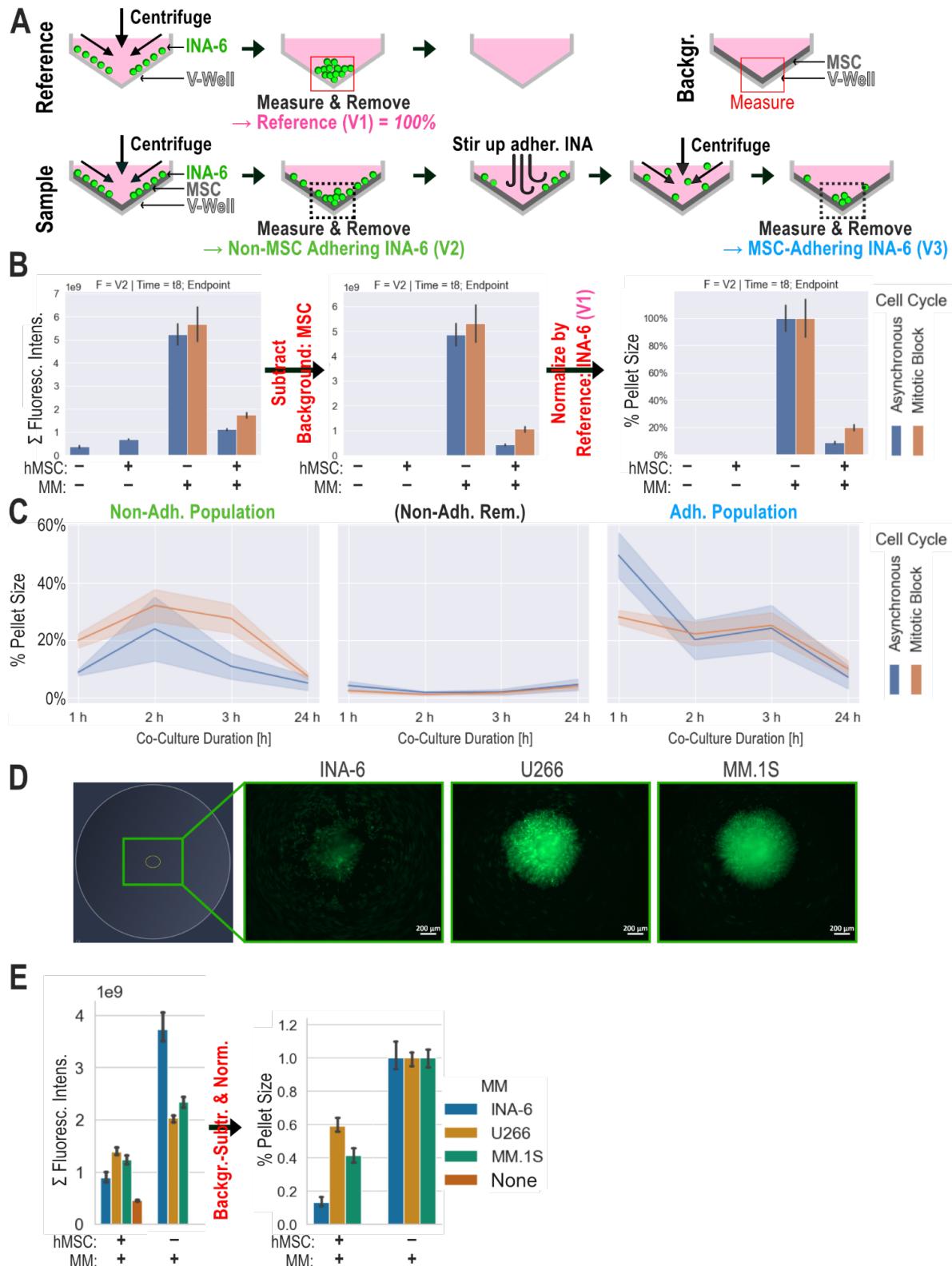
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<sup>8</sup>Vrije Universiteit Brussel, Department of Hematology and Immunology, Jette, Belgium

**Tab. S1:** List of hMSC donors, myeloma cell lines, and their mycoplasma test status. If no unique donors were available, hMSC donors were used twice for the same experiment at different passages. WPSC: Well plate sandwich centrifugation.

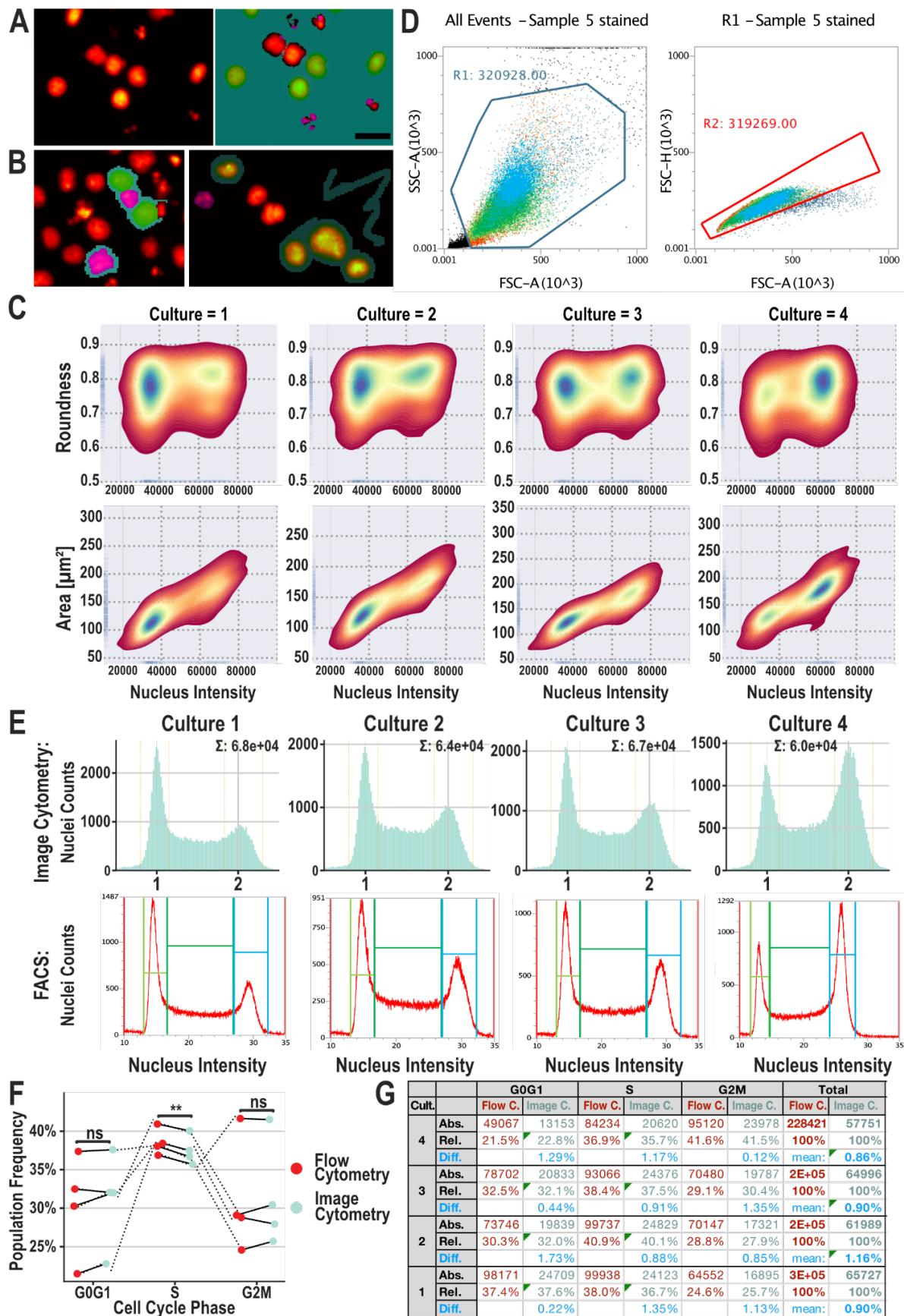
Cell Type	Donor / Line	Donor Ages	Donor Sex	Date of negative Mycoplasma test	Experiment(s)	Figures
Myeloma Cell Line	<b>INA-6</b>	80	m	09.02.22	All	All
	<b>U266</b>			10.10.22	- Validation of V-Well Adhesion Assay	S1E
	<b>MM1.S</b>			24.02.22		
hMSC	<b>1639</b>	49	m	not tested	- Validation of V-Well Adhesion Assay - Time-lapse: INA-6 on dispersed hMSC	S1E 1D; 2[A-E]
	<b>1571</b>	72	m	not tested	- Saturation of hMSCs	1[A-B]
	<b>1573</b>	47	m	not tested		
	<b>1578</b>	82	m	not tested		
	<b>1842</b>	63	m	not tested	- INA-6 Viability dep. on time and hMSC adhesion surface (INA not washed off)	1E right
	<b>1843</b>	60	m	not tested		
	<b>1537</b>	77	f	not tested		
	<b>1794</b>	82	m	not tested		
	<b>1779</b>	61	m	not tested	- INA-6 Viability dep. on time and hMSC adhesion surface (INA washed off)	1[C, E left]
	<b>1849</b>	69	m	not tested		
	<b>1854</b>	80	f	not tested		
	<b>1605</b>	71	f	not tested	- Time-lapse: INA-6 on dispersed hMSC	1D; 2[A-E]
	<b>1650</b>	57	m	not tested		
	<b>1859</b>	64	f	not tested	- Time-lapse: INA-6 on confluent hMSC	2[G-I]
	<b>1863</b>	79	f	not tested		
	<b>1861</b>	52	f	not tested		
	<b>1818</b>	81	f	not tested	- Cell Cycle Profiling after V-well assay	3C
	<b>1824</b>	82	f	not tested	(Donor measured twice, different passages) - V-well adhesion assay of mitotically blocked INA-6 followed by Cell Cycle Profiling after V-well assay	3[B,C]
	<b>1827</b>	56	m	not tested	- V-well adhesion assay of mitotically blocked INA-6 followed by Cell Cycle Profiling after V-well assay	

				assay	
<b>1501</b>	59	m	not tested	- INA-6 AI-assisted count during WPSC (INA-6 stained with celltracker green)	4B
<b>1643</b>	75	f	not tested		
<b>1718</b>	67	m	not tested		
<b>1720</b>	58	m	not tested		
<b>1653</b>	65	m	not tested		
<b>1591</b>	78	m	not tested	- WPSC (MACS) followed by RNAseq, Metascape analysis and qPCR validation - WPSC (Wash) followed by qPCR-Validation and Luminescent Viability assays	4[A,C,D,E] ; 5[A-C] 4[C-E], 4F
<b>1654</b>	74	m	not tested	- WPSC (MACS) followed by RNAseq, Metascape analysis and qPCR validation - WPSC (Wash) followed by qPCR-Validation and Luminescent Viability assays	4[A,C,D,E] ; 5[A-C] 4[C-E], 4F
<b>1655</b>	78	f	not tested	- WPSC (MACS) followed by RNAseq, Metascape analysis and qPCR validation	4[A,C,D,E] ; 5[A-C]
<b>1668</b>	80	f	not tested		
<b>1670</b>	66	f	not tested		
<b>1701</b>	81	m	not tested	- WPSC (Wash) followed by qPCR-Validation and Luminescent Viability assays	4[C-E], 4F
<b>1702</b>	79	f	not tested		
<b>1600</b>	77	m	not tested		
<b>1681</b>	56	m	not tested	- WPSC (Wash) followed by Luminescent Viability assays	4F
<b>1672</b>	65	m	not tested	- WPSC (Wash) followed by qPCR-Validation	4[C-E]

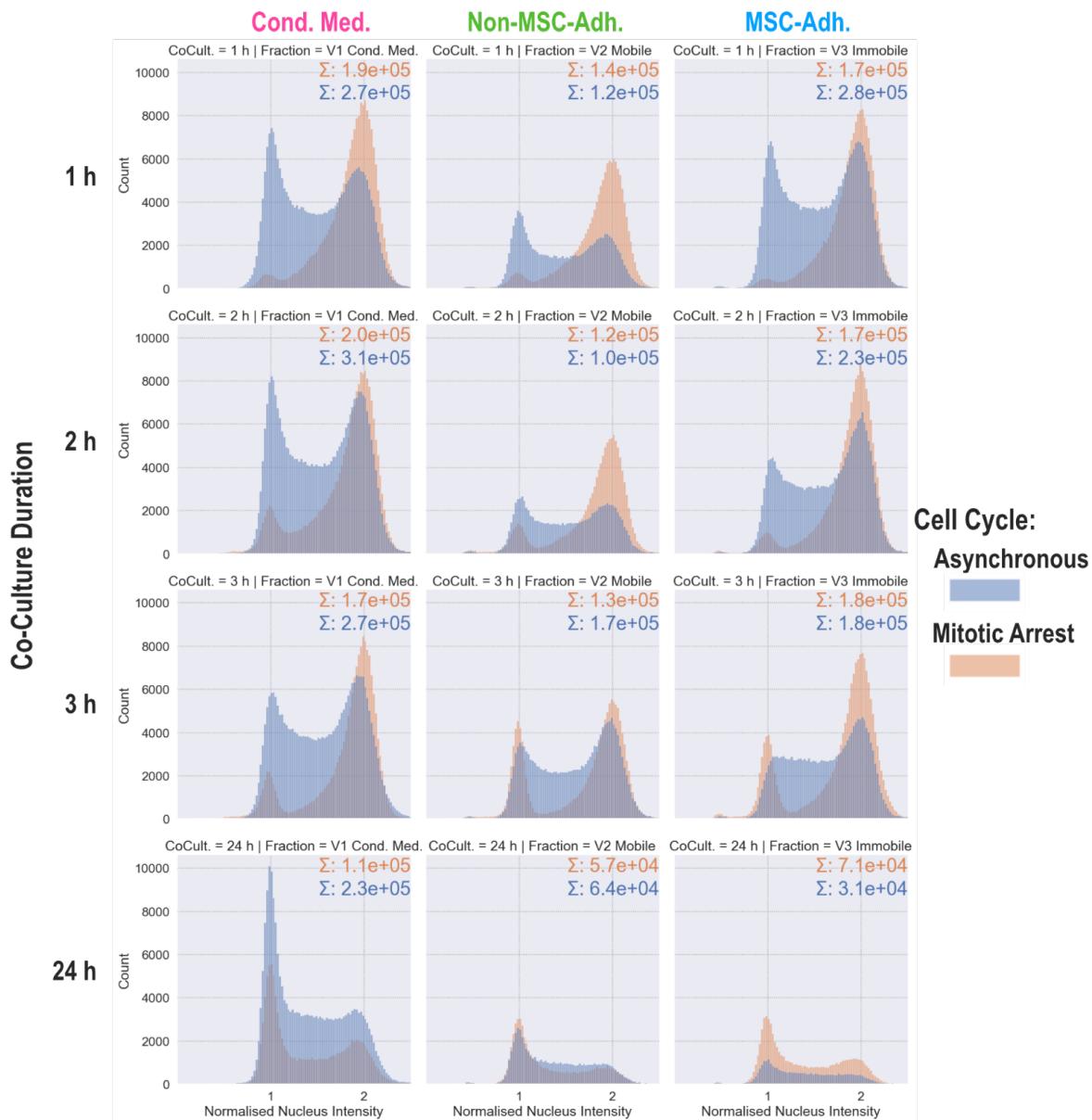
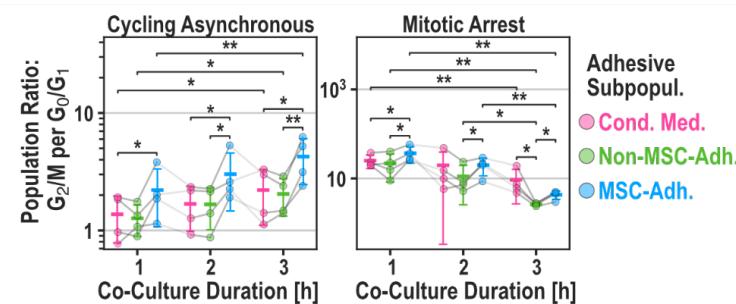


**Fig. S1:** Principle and quantification of the V-well adhesion assay of fluorescently labeled myeloma cells adapted by Weetall et al. 2001. **A:** Sample: Subsequent rounds of centrifugation and removal of cell pellet yielded the size of adhesive subpopulations. Fluorescently stained INA-6 cells were added to an hMSC monolayer. Non-adherent INA-6 cells (V2) were pelleted in the well-tip. Pellets were quantified by fluorescence brightness and isolated by pipetting. Immobile INA-6 cells (V3) were manually detached by forceful pipetting.

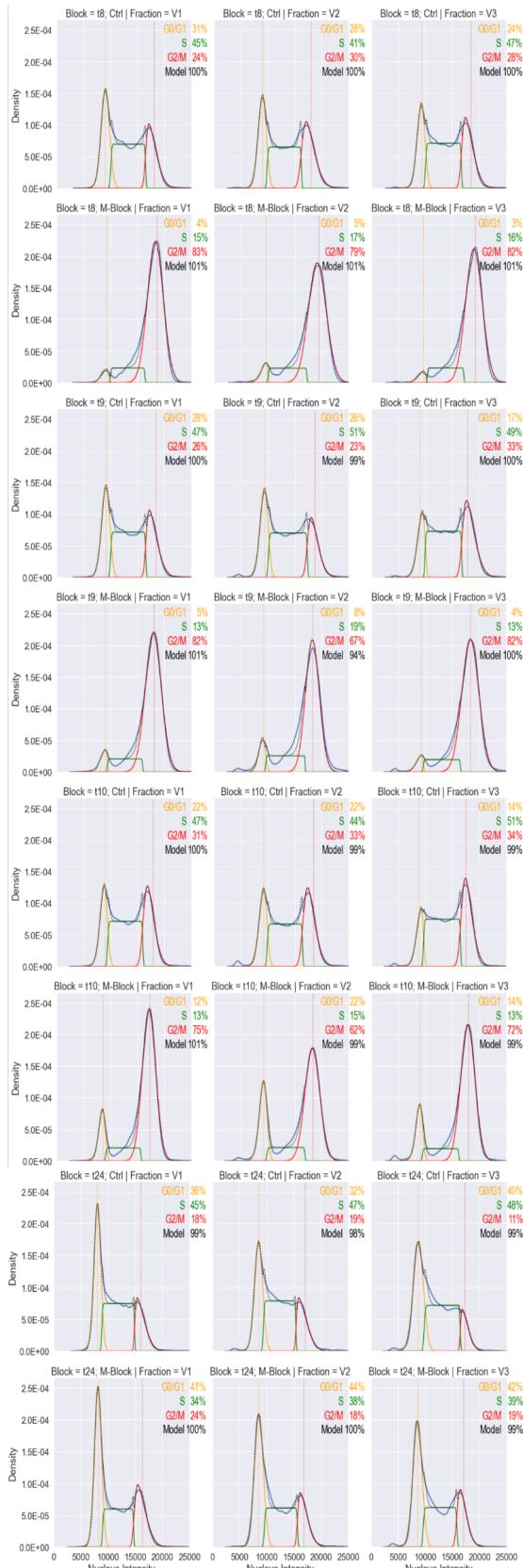
Reference: Omitting adhesive hMSC-layer yielded ~100% non-adherent cells (V1) after the first centrifugation step; Background: hMSC monolayer was used as background signal. **B:** Calculation of the population size relative to total cells starting with pellet intensity. The shown example is the pellet gained by centrifuging mobile subpopulation (V2) after 1 h of co-culture. (see Fig. 2 for context): Intensity values from pellet images were summarized. After subtracting the unlabeled hMSC signal and normalization by a full-size pellet (reference), the resulting values represented the fraction of the adhesive subpopulation. **C:** One of three biological replicates summarized in Fig. 2. Line range shows the standard deviation of four technical replicates. Non.Adh. Rem.: Fluorescence signal after removal of V2. **D:** Example images of myeloma cell lines (INA-6, U266, MM.1S) pelleted in the tip of V-wells. The leftmost image shows the recorded area in a complete V-well. Scale bar = 200  $\mu$ m. **E:** Results from (D) comparing adhesion strength of three myeloma cell lines to hMSC. Error bars represent technical deviation. MM=Multiple Myeloma.



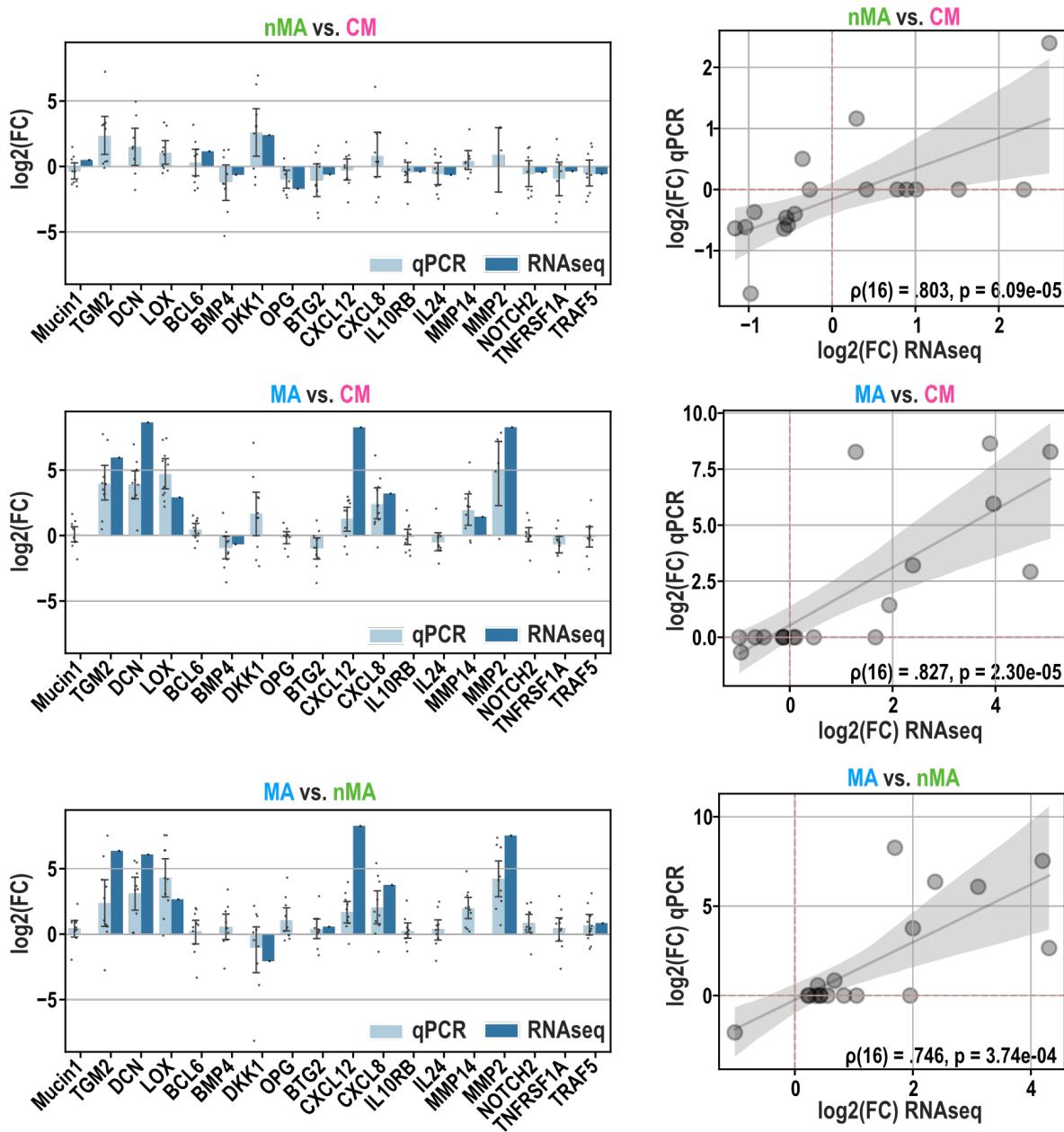
**Fig. S2:** Validation of image cytometric analysis of cell cycle in four INA-6 cultures **A:** Left: Example image cytometric scan: INA-6 cells were stained with Hoechst33342 and scanned by automated fluorescence microscopy. Right: The image was segmented using a convolutional neural network (ZEISS ZEN intellesis) trained to discern healthy nuclei (green) from fragmented ones (magenta). Doublets are excluded by setting an area- and roundness threshold. Scale bar: 20  $\mu$ m. **B:** Two example images from the training set. **C:** Quality of image cytometric data was ensured by plotting the distribution of nuclei brightnesses vs. the distribution of both nuclei-roundnesses and nuclei-areas. Nuclei with double fluorescence intensity have the same roundness while their area increases, as expected from a cell in G2 phase. **D:** The same samples from (C) were also measured with flow cytometry. Representative example of gating strategy: Left: Dead cells were excluded by setting a minimum threshold for side-scattering (SSC-A). Right: Doublets were excluded by setting a maximum threshold for forward scatter area (FSC-A) (sample “5” represents culture “4” in this figure). **E:** Cell cycle profiles of four independent INA-6 cultures were measured by both image cytometry (top) and flow cytometry (bottom). For both methods, frequencies of G0/G1, S, and G2M were summed up by setting fluorescence intensity thresholds. **F:** Image cytometry yields the same frequencies for G0/G1, S, and G2M when compared to flow cytometry. RM-ANOVA showed that the method has no significant effect on the frequencies of cell cycle populations [ $F(1,3)=1.421$ ,  $p\text{-unc}=.32$ ]. **G:** Results from (F) in tabular form. On average, frequencies for G0/G1, S, and G2M measured by Image cytometry differ by 0.95 percent points compared to flow cytometry measurement. Cult.: Culture; C.: Image cytometry; Abs.: Absolute cell count; Rel.: Relative cell count; Diff.: Difference between relative cell counts determined by flow cytometry and image cytometry.

**A****B**

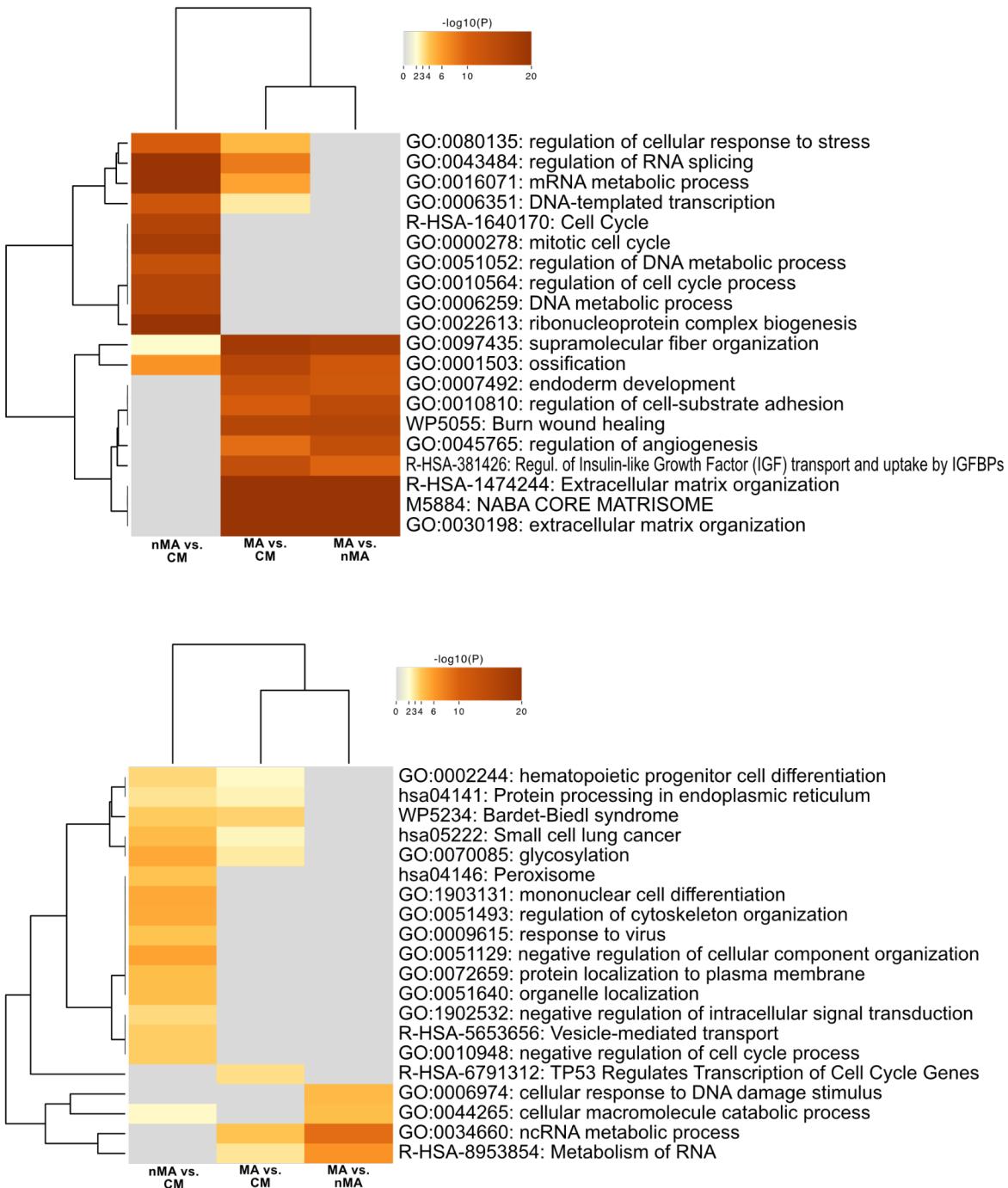
**Fig. S3:** Cell cycle analysis of INA-6 pellets gained from V-Well Adhesion assay (Fig. 3). **A:** Cell cycle profiles of MSC-adhering subpopulations. INA-6 cells were synchronized by double thymidine block followed by nocodazole. Cell cycle was released directly before addition to hMSCs. Histograms were normalized and summed up across all biological replicates ( $n=4$ ). Technical replicates (3) were pooled prior to cell cycle profiling. CoCult. = Co-culture duration. Fraction = Adhesion subpopulations. **B:** Similar figure to Fig. 3C displaying ratio of INA-6 populations ( $G_2/M$  to  $G_0/G_1$ ). **Statistics:** Paired t-test (B).



**Fig. S4:** Representative (one of the four independent sample sets as seen in Fig. S3) curve fitting analysis of cell cycle profiles generated by Image Cytometry. t8, t9, t10, and t24 refer to 1, 2, 3, and 24 hours after the addition of INA-6 cells to hMSCs.



**Fig. S5:** Correlation of RNAseq with qPCR **Left:** Validation of RNAseq results (Fig. 3) with qPCR showing the log<sub>2</sub>(foldchange expression) of 18 genes. For qPCR, Datapoints each represent one biological replicate (n=10), which is the mean of technical replicates (n=3). Bar height represents mean of biological replicates, error bars show standard deviation of biological replicates. **Right:** Correlation between qPCR and RNAseq in terms of log<sub>2</sub>(mean foldchange expression per gene). Each dot represents one gene shown in the barplot to the left. Genes measured with qPCR that showed no differential expression in RNAseq were set to have a log<sub>2</sub>(FC) = 0. Shaded area shows the confidence interval of linear regression. Correlation coefficient ( $\rho$ ) was calculated using Spearman's rank. N = 18 genes. FC = fold change expression.



**Fig. S6:** Functional enrichment analysis by Metascape using genes that are differentially expressed between MSC-interacting subpopulations. **Top:** Upregulated genes. **Bottom:** Downregulated genes.

**Tab. S2:** Adhesion genes (from Fig. 6A) filtered by association with patient survival ( $p < 0.05$ ) and categorized by a continuous downregulation across disease progression. The full table including non-significant associations is found in the supplementary data. Bone Marrow Plasma Cell (BMPC), Monoclonal Gammopathy of Undetermined Significance (MGUS), Smoldering Multiple Myeloma (sMM), Multiple Myeloma (MM), Multiple Myeloma Relapse (MMR). p-adj. = adjusted p-values (Benj.-Hoch.).

Regulation during disease progression	Gene	Ensemble ID	Progression on Free / Overall Survival	Better Prognosis with high/low expression	Association of expression with survival	
					[p-unc]	[p-adj]
Not downregulated or overall low expression	CCDC80	ENSG00000091986	Prog. Free	high	2.04E-03	1.58E-02
	CCN2	ENSG00000118523	Overall	high	2.89E-03	2.43E-02
	CCNE2	ENSG00000175305	Prog. Free	low	1.21E-02	4.62E-02
			Overall	low	5.34E-04	8.64E-03
	COL4A1	ENSG00000187498	Overall	high	9.47E-03	3.99E-02
	COL4A2	ENSG00000134871	Prog. Free	high	1.24E-02	4.62E-02
	F3	ENSG00000117525	Overall	high	9.18E-03	3.99E-02
	HTRA1	ENSG00000166033	Prog. Free	high	1.20E-02	4.62E-02
	IGFBP7	ENSG00000163453	Prog. Free	low	9.53E-03	4.38E-02
	MMP2	ENSG00000087245	Prog. Free	high	2.29E-05	2.32E-03
	OSMR	ENSG00000145623	Prog. Free	high	5.67E-04	7.15E-03
			Overall	high	1.29E-02	4.64E-02
Continuously downregulated (PC > MGUS > sMM > MM > MMR)	SERPINH1	ENSG00000149257	Prog. Free	low	1.83E-03	1.58E-02
			Overall	low	4.40E-03	2.61E-02
	ACTN1	ENSG00000072110	Overall	high	7.73E-03	3.55E-02
	AEBP1	ENSG00000106624	Prog. Free	high	1.08E-02	4.62E-02
	AXL	ENSG00000167601	Prog. Free	high	1.50E-03	1.51E-02
			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
	COL3A1	ENSG00000168542	Overall	high	1.08E-02	4.29E-02
	COL6A1	ENSG00000142156	Prog. Free	high	1.20E-02	4.62E-02
			Overall	high	1.10E-02	4.29E-02
	CXCL12	ENSG00000107562	Prog. Free	high	1.16E-04	2.93E-03
			Overall	high	6.48E-04	8.64E-03
	CYP1B1	ENSG00000138061	Prog. Free	high	8.64E-03	4.17E-02
			Overall	high	6.84E-04	8.64E-03
	DCN	ENSG00000011465	Prog. Free	high	4.83E-03	3.05E-02
			Overall	high	2.47E-04	8.33E-03
	FBLN1	ENSG00000077942	Prog. Free	high	2.68E-03	1.93E-02
			Overall	high	3.73E-03	2.61E-02
	GNB3	ENSG00000111664	Prog. Free	high	3.75E-03	2.52E-02
			Overall	high	5.73E-03	3.05E-02
	IGFBP4	ENSG00000141753	Prog. Free	high	8.68E-03	4.17E-02
			Overall	high	7.09E-03	3.41E-02
	ITGAX	ENSG00000140678	Prog. Free	high	6.72E-03	3.60E-02
			Overall	high	3.12E-03	2.43E-02
	LAMB2	ENSG00000172037	Overall	high	1.35E-03	1.39E-02
	LRP1	ENSG00000123384	Prog. Free	high	6.46E-03	3.60E-02
			Overall	high	4.34E-04	8.64E-03
	LTBP2	ENSG00000119681	Prog. Free	high	9.03E-05	2.93E-03
			Overall	high	1.17E-02	4.36E-02
	MAP3K8	ENSG00000107968	Prog. Free	high	9.58E-04	1.08E-02
	MFAP5	ENSG00000197614	Prog. Free	high	2.43E-04	4.09E-03
			Overall	high	4.27E-03	2.61E-02
	MMP14	ENSG00000157227	Prog. Free	high	6.93E-05	2.93E-03
			Overall	high	6.69E-03	3.38E-02
	MYL9	ENSG00000101335	Prog. Free	high	1.46E-04	2.95E-03
			Overall	high	1.56E-05	1.57E-03
	NRP1	ENSG00000099250	Prog. Free	high	1.89E-03	1.58E-02
			Overall	high	2.21E-03	2.03E-02
	TGFBI	ENSG00000120708	Overall	high	4.30E-03	2.61E-02
	TNC	ENSG00000041982	Prog. Free	high	1.28E-02	4.62E-02
			Overall	high	4.75E-03	2.67E-02
	TPM1	ENSG00000140416	Overall	high	1.37E-03	1.39E-02
	TUBA1A	ENSG00000167552	Prog. Free	low	6.78E-03	3.60E-02

## Supplementary Materials and Methods

### **Isolation and Culturing of Primary Human Bone Marrow-Derived Mesenchymal Stromal Cells**

Primary human MSCs were obtained from the femoral head of patients (S. Tab. 1) undergoing elective hip arthroplasty. Material was collected with the informed consent of all patients and the procedure was approved by the local Ethics Committee of the University of Würzburg (186/18). In brief, bone marrow was washed with MSC-Medium [Dulbecco's modified Eagle's medium (DMEM/F12) (Thermo Fisher Scientific, Darmstadt, Germany) supplemented with 10% Fetal Calf Serum (FCS) (Bio&Sell GmbH, Feucht, Germany (Fernandez-Rebollo et al., 2017), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 50 µg/ml ascorbate and 100 nmol/l sodium selenite (both Sigma-Aldrich GmbH, Munich, Germany)) and centrifuged at 250 g for 5 min. The pellet was washed four times with MSC-medium and resulting supernatants containing released cells were collected. Cells were pelleted and cultured at a density of  $1 \times 10^9$  cells per 175 cm<sup>2</sup> culture flask. After two days non-attached cells were washed away and adherent ones were cultivated in MSC-Medium until confluence. Then, they were either frozen in liquid nitrogen or directly utilized for experiments. hMSC cultures were sustained for a maximum of two passages. All cells were cultured at 37 °C and at 5% CO<sub>2</sub>.

### **Culturing of Myeloma Cell Lines**

The plasmacytoma cell line INA-6 [RRID:CVCL\_5209; DSMZ, Braunschweig, Germany, authenticated by DSMZ 2014 (see supplemental); (Burger et al., 2001; Gramatzki et al., 1994) was cultivated in RPMI 1640 medium (Life Technologies GmbH) supplemented with 20 % (v/v) FCS, 100 µg/ml gentamicin, 2 mmol/l L-glutamine (both Life Technologies GmbH), 1 mmol/l sodium pyruvate, 100 nmol/l sodium selenite (both Sigma Aldrich GmbH, Schnelldorf, Germany) and 2 ng/ml recombinant human interleukin-6 (IL-6; Miltenyi Biotec, Bergisch Gladbach, Germany). INA-6 were passaged three times per week by diluting them to  $1 \times 10^5$ ,  $2 \times 10^5$ , or  $4 \times 10^5$  cells/mL for 3, 2 and 1 days of culturing, respectively. MM.1S (RRID:CVCL\_8792) (Greenstein et al., 2003), and U266 cells (CVCL\_0566), (Nilsson et al., 1970) were propagated and cultivated in RPMI1640 medium comprising 10 % (v/v) FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mmol/l L-glutamine, and 1 mmol/l sodium pyruvate. All cells were cultured at 37 °C and at 5% CO<sub>2</sub>.

**Co-Culturing of Primary hMSCs and INA-6 and MSC-Conditioning of Medium**

For each co-culture, hMSCs were seeded out 24 h prior to INA-6 addition to generate MSC-conditioned medium (CM). CM from different donors was collected separately and used immediately when adding INA-6. To ensure that CM was free of hMSCs, it was strained (40 µm) and centrifuged for 15 minutes at 250 g. INA-6 cells were washed with PBS (5 min, 1200 rpm), resuspended in MSC-medium and added to hMSCs such that co-culture comprised 33% (v/v) of CM gathered directly from the respective hMSC-donor. Co-cultures did not contain IL-6 (Chatterjee et al., 2002).

**Collagen I Coating**

Collagen I solution (isolated from rat tail, Corning, NY, USA) was diluted 1:2 (75 ng/mL) in acetic acid (0.02 N), applied to 96 well plates (30 µL in each well) and incubated for 2 h at room temperature. Acetic acid was removed and wells were washed once with 100 µL of PBS. Coated plates were stored dry at 4 °C.

**Fluorescent Staining of Cells**

For each live staining, cells were strained (70 µm) to remove clumps and washed (5 min, 250 g) once with the respective media (without FCS) and then resuspended in staining reagents.

For CellTracker™ Green CMFDA Dye and CellTracker™ Deep Red Dye (Thermo Fisher Scientific) staining, 1 mL staining solution for a maximum of  $1 \times 10^6$  cells was prepared. Staining was done at RT for 15 min using 5 µM CMFDA (5-Chlormethyl-fluoresceindiacetat) and 5 min of 1-2 µM DeepRed. To reduce background, stained cells were pelleted, resuspended in cell medium (containing FCS), incubated for 30 min (37 °C, 5% CO<sub>2</sub>), washed in cell medium, resuspended in 100 µL - 1 mL and counted.

For PKH26 staining (Sigma Aldrich), a maximum of  $1 \times 10^4$  cells was resuspended in 500 µL diluent C before swiftly adding 500 µL of staining solution (1 µL diluted in 500 µL diluent C) and incubating cells for 5 min at RT. The staining reaction was stopped by adding 1 mL of FCS-containing medium and adding 3 mL of FCS-free medium. Cells were washed with 10 mL of FCS-containing medium, resuspended in 100 µL - 1 mL cell medium, and counted.

For Calcein-AM (Calcein-O,O'-diacetat-tetrakis-(acetoxymethyl)-ester) (Thermo Fisher Scientific) staining, end concentrations of 0.5 µM were used. 12.5 µL of diluted stock solution (2.5 µM) was carefully added to 50 µL of the co-culture and incubated for 10 minutes at 37 °C.

For Hoechst33342 staining, cells were washed once with PBS, resuspended in a maximum of 500 µL of PBS, and fixed with 5 mL of ice-cold ethanol (70% v/v) by vigorously pipetting up and down to dissociate aggregates. Cells were washed once with PBS and stained with 2.5 µg/mL Hoechst33342 (Thermo Fisher Scientific) diluted in PBS for 1 h at 37 °C.

### **Automated Fluorescence Microscopy**

To remove clumps for microscopic applications, we cultured cells in 40 µm strained FCS. To reduce background fluorescence and phototoxicity, we used phenol-red free versions of the respective medium, if available.

All microscopy equipment was acquired from ZEISS. The microscope was an Axio Observer 7 with confocal Apotome.2 equipped with a motorized reflector revolver and motorized scanning table (130x100 mm). The microscope was mounted on an Antivibrations-Set [Axio Observer (D)] with two antivibration carrier plates, each equipped with two vibration dampening feet. The light source was a microLED 2 for transmission light and (for fluorescence) Colibri 7 (R[G/Y]B-UV) for five channels of incident light (385, 475, 555, 590, 630 nm). For excitation (EX) and emission (EM) light filtering and beam splitting (BS) we used the following reflectors: 96 HE BFP shift free (E) (EX: 390/40, BS: 420, EM: 450/40), 43 HE Cy 3 shift free (E) (EX: 550/25, BS: 570, EM: 605/70), 38 HE eGFP shift free (E) (EX: 470/40, BS: 495, EM: 525/50) and 90 HE LED (E) (EX: 385, 475, 555 und 630 nm, BS: 405 + 493 + 575 + 653, EM: 425/30 + 514/30 + 592/30 + 709/100). We used the black and white camera Axiocam 506 mono (D) and if not stated otherwise, 2x2 binning was used for fluorescence imaging. For mosaic acquisitions (“tiles”) we used a tiling overlap of 8-10% and image tiles were not stitched. Images were magnified 5x and 10x (Fluar 5x/0.25 M27 and EC Plan-Neofluar 10x/0.3 Ph1 M27).

### **Cell Viability and Apoptosis Assay**

To examine cell viability and apoptosis, cells were seeded in a 96-well plate ( $1 \times 10^4$  cells per well) to be measured inside culture well after respective incubation time immediately. ATP-amount and Caspase 3/7 activity were used as a proxy for viability and apoptosis rates, respectively. They were assessed using the CellTiter-Glo Luminescent Cell Viability Assay and the Caspase-Glo 3/7 Assay, respectively (Promega GmbH, Mannheim, Germany), according to the manufacturer's instructions.

Luminescence was measured with an Orion II Luminometer (Berthold Detection Systems, Pforzheim, Germany).

### **Microscopic Characterization of MSC Saturation**

For saturating hMSC with INA-6, hMSCs were stained with CellTracker Green, plated out on 384-Well plates (Greiner) at  $5 \times 10^3$  hMSC/cm<sup>2</sup> and cultured for 24 h. INA-6 cells were stained with CellTracker DeepRed, resuspended in MSC-medium, added to adhering hMSCs in different amounts ( $5 \times 10^3$ ,  $1 \times 10^3$ ,  $2 \times 10^3$  INA-6/cm<sup>2</sup>) and co-cultured for 24 h and 48 h. The complete co-culture was scanned and the number of INA-6 cells adhering on one MSC was counted manually for 100 MSCs for each technical replicate. Fluorescent images were digitally re-stained (INA-6 green, hMSC inverse black).

### **Analysis INA-6 Survival and Aggregation Depending on hMSCs Confluence**

To describe aggregate growth and survival of INA-6 depending on hMSC density, unstained hMSCs were seeded out into 96-well plates (white, clear bottom, Greiner) at different densities (Tab. S3). To ensure nutrient supply, we used lower cell densities for longer co-culturing durations while maintaining constant ratios of INA-6 to adhesion surface provided by hMSCs. Those plates that are to be assessed after 72 h of co-culturing received further 100 µL of fresh MSC-medium after 24 h of co-culturing (total volume of 300 µL), and after 48 h of co-culturing, 100 µL was removed gently from the co-culture and (carefully not to stir up co-culture on bottom) replaced with fresh MSC-Medium after 48 h of co-culturing.

To describe aggregate growth, complete wells were scanned using 10x magnification, phase contrast, 2x2 binning, and autofocus focusing on each tile both before and after harvesting. Afterwards, INA-6 cells were harvested for measuring viability and apoptosis.

**Tab. S3:** Seeding densities for describing growth and survival of INA-6 depending on hMSC density. Co-cult. dur. = Co culturing duration; MSC-adh. surface = adhesion surface provided by hMSCs; vol. = volume.

Co- cult. dur. [h]	hMSC density [1000 hMSC/cm <sup>2</sup> ]			INA-6 density [1000 INA-6/cm <sup>2</sup> ]	Ratios INA : MSC (adh. surface)			Seeding vol. [ $\mu$ L]	End vol. [ $\mu$ L]
24	2	10	40	10	1 : 0.2	1 : 1	1 : confluent	200	200
48	1	5	40	5	1 : 0.2	1 : 1	1 : confluent	200	200
72	1	5	40	5	1 : 0.2	1 : 1	1 : confluent	200 [after 24 h: + 100] [after 48 h: exchange 100]	300

For luminescent assessment of cell survival, INA-6 were harvested by removing co-culture medium, adding 150  $\mu$ L of MSC-Medium, and then stirred by strongly pipetting up and down twice while aiming the pipette tip at the upper corner, lower left and lower right of the well bottom ('Mercedes star'). Washing and stirring was repeated once before washing wells again with 150 mL MSC-Medium. Harvested INA-6 cells were strained (40  $\mu$ m), pelleted, and resuspended in 200  $\mu$ L MSC-Medium. Cells were counted using Neubauer chambers, re-distributed into 96-well plates (white, clear bottom) with  $1 \times 10^5$  INA-6 cells per well, and then subjected to viability and apoptosis assays.

To minimize the loss of sensitive apoptotic cells, another approach was used to measure viability and apoptosis without harvesting INA-6 cells. hMSCs and INA-6 were seeded out individually in parallel to the co-cultures (S. Tab. 02). Prior to measuring viability and apoptosis, culture volume was adjusted to 150  $\mu$ L by removing 50  $\mu$ L or 150  $\mu$ L for the timepoints 48 h or 72 h, respectively (carefully not to stir up culture on bottom). 100  $\mu$ L of luminescent reagents were then added directly to 150  $\mu$ L of co-culture. The fold change of viability or apoptosis that is due to MSC interaction ( $FC_{MSC\ interaction}$ ) was then calculated using the following formula, with  $L$  being the mean of four technical replicates measured in relative luminescent units per seconds [RLU/s],  $L_{Co\ Culture}L_{MSC}$ ,  $L_{INA\ 6}$  the luminescence measured in the co-culture, hMSCs alone and INA 6 alone, respectively.

$$FC_{MSC\ Interaction} = \frac{L_{Co\ Culture}}{L_{MSC} + L_{INA\ 6}}$$

#### Time-Lapse Characterization of INA-6 Aggregation, Detachment and Division

In order to record the aggregation and detachment of INA-6 in contact with hMSCs, hMSCs (5e3 cells/cm<sup>2</sup>) were fluorescently stained with PKH26 and plated onto 8-well  $\mu$ -Slides (ibidi, Gräfelfing,

Germany). hMSCs were incubated for 24 h before being placed into an ibidi Stage Top Incubation System and were equilibrated to the incubation system for a minimum of 3 h (80% humidity and 5% CO<sub>2</sub>). INA-6 cells ( $2 \times 10^4$  cells/cm<sup>2</sup>) were washed and resuspended in 33% (v/v) MSC-conditioned medium before adding them directly before acquisition start in a small volume (10 µL). Brightfield and fluorescence images of 13 mm<sup>2</sup> of co-culture were acquired every 15 minutes for 63 h. Movement speed of the motorized table was adjusted to the lowest setting that allows acquisition of the complete region within 15 minutes.

Respective events of interest were analyzed manually and categorized into defined event parameters. Events were binned across the time axis using these boundaries: [0.0, 12.85, 25.7, 38.55, 51.4, 64.25]. We collected a minimum of events per recording and analysis so that each time bin contained at least 5 values, except when analyzing detachment events, since these did not appear before 20 h of incubation for some replicates. For each recording and event parameter, the event count was normalized by dividing by the total number of events per time bin.

We determined the frequency and the cause of aggregation by looking for two interacting INA-6 cells and went backward in time to see if they were two daughter cells or if two independent INA-6 cells had collided.

We determined the frequency of aggregates with detaching cells by tracing their growth across the complete time-lapse and looking for detachment events. We picked random 100 aggregates by including aggregates from both the border and center of the well.

We characterized detachment events by noting multiple parameters manually: Time point of detachment, aggregate size (at the time of detachment), the last interaction partner, and the number of detaching INA-6 cells.

For characterizing cell division events, we recorded a new set of time-lapse videos using unstained hMSCs that were grown to confluence for 24 h ( $4 \times 10^4$  hMSCs/cm<sup>2</sup>) to provide for unlimited adhesion surface. We categorized daughter cells in terms of their mobility (mobility being the speed of putative movements or “rolling”). The mobility criteria were met if one INA-6 daughter cell moved farther than half a cell radius within one frame (15 min) relative to the MSC-adherent INA-6 cell which was required to stand still in-between respective frames. We measured the “rolling” duration by subtracting the time point of the last perceived movement from the time point of division. We

excluded those division events from the measurement of rolling duration, if INA-6 cells underwent apoptosis shortly after division.

### **Cell Cycle Synchronization at M-Phase**

INA-6 cells were arrested at mitosis by double thymidine (2 mM) treatments followed by 5 h of nocodazole (500 ng/mL) incubation. In detail:  $3 \times 10^5$ /mL INA-6 in 4 mL were treated with 2 mM thymidine (Sigma) for 16.5 h. Cells were released by washing them in INA-6 medium once and allowed to cycle for 9 h before treating them with 2 mM thymidine for 18 h a second time. Afterwards, cells were released and allowed to cycle for 2 h before treating them with 100 ng/ mL nocodazole (Sigma) for 5 h. Arrested INA-6 were released by washing them once and resuspending them in MSC-medium with 33% MSC-conditioned medium. Cell cycle profile was checked using image cytometry (Fig S2).

### **V-Well Adhesion Assay**

This assay was modified from (Weetall et al., 2001). 96 v-well plates were coated with collagen I (rat tail, Corning). Collagen coating ensures that confluent hMSCs withstand centrifugation even after hMSCs in the well tip were removed. hMSCs ( $4 \times 10^4$  cells/cm<sup>2</sup>) were seeded out and grown to confluence for 24 h in collagen-coated v-well plates. To ensure that only INA-6 are pelleted in the v-well tip, hMSCs were removed from the well-tip by touching the well-ground with a 10 µL pipette and roughly pipetting hMSCs away.

Arrested INA-6 ( $1 \times 10^4$  cells/cm<sup>2</sup>) were released by washing them once in PBS and resuspending them in 33% (v/v) MSC-conditioned medium before adding them on top of confluent hMSCs. INA-6 adhered for 1, 2, 3 and 24 h before the complete co-culture was stained with 0.5 µM Calcein-AM (10 min at 37 °C).

Non-adherent INA-6 were pelleted by centrifugation using a Hettich 1460 rotor (*r* = 124 mm) at 2000 rpm (555 g) for 10 min.

The well tip was imaged by fluorescence microscopy with 5x magnification, 96 HE emission filter, autofocus configured for maximum signal intensity, 2x2 binning and 14 bit grayscale depth. Pellet brightness was analyzed in ZEN 2.6 (Zeiss) by summing up pixel brightnesses across the complete pellet image. Background brightness was acquired from a cell culture with only hMSCs. Reference brightness was acquired from a cell culture with only INA-6, defining 100% pellet brightness without

adhesion. Background intensity was subtracted before normalizing by reference. Outliers were removed from technical replicates ( $n=4$ ) if their z-score was larger than  $1.5 \sigma$  technical variation.

After measuring pellet brightnesses, the cell pellet was removed by pipetting 10  $\mu\text{L}$  from the well tip.

Pellets of the same technical replicates were pooled, washed in PBS, resuspended in 200  $\mu\text{L}$  PBS, added to 1.8 mL ice-cold 70% ethanol and stored at -20 °C.

Remaining non-MSC-adhering INA-6 cells were removed by replacing culture medium with 100  $\mu\text{L}$  of medium. MSC-adherent INA-6 were manually detached by rapid pipetting and equally pelleted, analyzed, and isolated.

### **Cell Cycle Profiling**

INA-6 cells were fixed in 70% ice-cold ethanol, washed, resuspended in PBS, distributed in 96-well plates and stained with Hoechst-33342 (2.5  $\mu\text{g/mL}$  in PBS) for 1 h at 37 °C.

For image cytometric cell cycle profiling, plates were scanned completely using automated fluorescence microscopy with 5x magnification, 96 HE emission filter, 1x1 binning, 14 bit depth and an illumination time that fills 70% of grayscale range. The autofocus was configured to re-adjust every second tile. A pre-trained convolutional neural network (“DeepFeatures 2 reduced”, Intellesis, Zeiss) was fine-tuned to segment scans into background, single nuclei and fragmented nuclei. Nuclei were filtered to exclude fragmented nuclei and those nuclei with extreme size (within the range of 50-500  $\mu\text{m}^2$ ) and roundness (within the range of 0.4-1.0). Cell cycle profiles were normalized by the mode of the nucleus intensities within the G0/G1 peak. To retrieve frequencies of cells cycling in G0/G1, S, and G2 phase, the brightness distribution of all single nuclei was fitted to the sum of three Gaussian curves (“Skewed Gaussian Model” for G0G1 and G2 phase, and “Rectangle Model” for S phase) using the python package LMFIT (Newville et al., 2014) (Fig. S4). The gaussian curves were used to calculate the cell frequencies for each cell cycle phase by integration using the composite trapezoidal rule implemented by numpy.trapz (Harris et al., 2020).

For validation of image cytometry, 5 mL of INA-6 stock culture was removed and ethanol fixed as described above. Flow cytometry analyses were performed using an Attune Nxt Flow Cytometer (Thermo Fisher, USA). Data analyses were performed using FlowJo V10 software (TreeStar, USA).

**Protocol: Well Plate Sandwich Centrifugation (WPSC)**

96 well plates (flat bottom, clear) were coated with collagen I (rat tail, Corning). Collagen coating ensures that confluent hMSCs withstand centrifugation and repeated washing. hMSCs ( $2 \times 10^4$  cells/cm<sup>2</sup>) were seeded out and grown to confluence for 72 h in collagen-coated 96-well plates.

To remove aggregates from the medium and prevent clogging of magnetic columns, we strained any FCS-containing fluid with a 40 µm cell strainer.

Collect MSC-conditioned medium and add INA-6:

1. Collect hMSC-conditioned medium (CM) from the well plates and replace it with 100 µL of fresh hMSC medium. Collect CM from different donors separately
2. Strain CM (40 µm) and centrifuge it for 15 minutes at 250 g to ensure that CM does not contain hMSCs
3. Dilute CM by mixing 2 parts of CM with 1 part of MSC-medium (dilute 1.5 fold)
4. Count INA-6 cells and retrieve enough cells to fill all 96 wells with  $2 \times 10^4$  INA-6/cm<sup>2</sup> ( $6.8 \times 10^4$  cells per well, covering ~65% of the well bottom).
5. Centrifuge INA-6 (5 min, 250 g) and resuspend them in a volume of diluted CM to reach a concentration of  $6.8 \times 10^5$  INA-6/mL
6. Add 100 µL INA-6 suspension to hMSCs (end volume: 200 µL; end concentration: 33% (v/v) hMSC-conditioned medium)
7. Incubate for 24 h at 37 °C and 5% CO<sub>2</sub>

Prepare CM-INA6 reference:

8. Add 100 µL of fresh MSC-medium into each well of an empty 96-well plate (not coated)
9. Add 100 µL of INA-6 suspension ( $6.8 \times 10^5$  INA-6/mL in diluted CM)
10. Incubate for 24 h at 37 °C and 5% CO<sub>2</sub>

Collect CM-INA6 and nMA-INA6

11. Pre-warm well plate centrifuge to 37 °C
12. Prepare a counter-weight by filling 200 µL of water into all wells of an empty 96-well plate
13. Prepare well-plate sandwiches:

- a. Turn an empty 96-well plate (“catching plate”) upside down and place one on top of the co-culture-plate, the CM-IN6 reference plate, and the counter-weight so that all well openings align.
  - b. Fix well plates using tape with reusable adhesive (e.g. Leukofix)
14. Turn both plates around. Medium will spill from the co-culture plate into the catching plate
  15. Centrifuge plate for 40 seconds at 1000 rpm with the catching plate facing the ground
  16. Remove the adhesive tape and the co-culture plate.
  17. Turn the co-culture plate around and add 30 µL of washing medium (MSC-Medium 0% FCS, 3 mM EDTA) gently by touching the wall of each well and pressing the pipette slowly.
    - a. *Work quickly to ensure that co-culture does not dry. We recommend using a multipette (Eppendorf).*
    - b. *Many nMA-IN6 are removed by physical force applied by adding 30 µL of medium and not just by centrifugation. Hence, it is critical to apply the same dispensing technique across all replicates. We recommend using a multipette (Eppendorf) that can apply 30 µL with controllable pressure, since its push-button retains a long pushing path even for dispensing small volumes, unlike push-buttons from the usual 100 µL pipettes that reduce the pushing-path for smaller volumes.*
    - c. *Centrifugation minimizes technical variability by replacing one step of manual pipetting. Also, it ensures that confluent MSCs remain unharmed. Manual pipetting on the other hand would require touching the well-bottom to remove all fluids which damages the adhesive hMSC layer.*
  18. Turn the co-culture plate upside down, place it onto the catching plate and re-apply adhesive tape to fix the wellplate sandwich
  19. Repeat steps 14-18 two more times until the catching plate contains 290 µL of medium in each well
  20. Pool CM-IN6 from the catching plate that was fixed to the reference plate
  21. Pool nMA-IN6 from the catching plate that was fixed to the co-culture plate
  22. Collect remaining IN6 by adding 100 µL of PBS into each well of the catching plates, collect and pool with CM-IN6 or nMA-IN6.

23. Strain CM-INA6 and nMA-INA6 using 40 µm cell strainer
  24. Isolate MA-INA6 by continue with either accutase dissociation or rough pipetting
- Collect MA-INA6 by accutase dissociation followed by MAC sorting
25. Block 2 mL tubes with sorting buffer (PBS, 2 mM EDTA, 1% BSA) for 1 h at 4 °C
  26. Dilute accutase (Sigma A6964) (400-600 units/mL) 4-fold in cold PBS. Always keep accutase on ice, since accutase loses activity at room temperature.
  27. Add 50 µL of cold accutase (directly after the last centrifugation step) and incubate co-culture plate for 5 minutes at 37 °C.
  28. Place a co-culture plate onto a shaker and shake for 1 minute at 300 rpm.
  29. Collect cell suspension from wells and stop the reaction by adding 500 µL of FCS to pooled cell suspension.
  30. Evaluate presence of adherent INA-6 cells and the integrity of confluent hMSCs under the microscope.
  31. Repeat steps 24-27 until all INA-6 cells have dissociated or until confluent hMSCs start to tear.
  32. Strain cell suspension (30 µm). This yields MA-MSC.
  33. Pellet MA-INA6, nMA-INA6 and CM-INA6 (1200 rpm, 10 min).
  34. Resuspend MA-INA6 in 86 µL sorting buffer (PBS, 2 mM EDTA, 1% BSA)
  35. Resuspend CM-INA6 and nMA-INA6 in 300 µL cold diluted accutase and incubate for 3 min at 37 °C to ensure equal treatment for all samples.
  36. Stop accutase by adding 200 µL of FCS (100%)
  37. Pellet CM-INA6 and nMA-INA6 (1200 rpm, 10 min) and resuspend in 86 µL sorting buffer (PBS, 2 mM EDTA, 1% BSA).
  38. Transfer samples into 2 mL tubes that were blocked with sorting buffer
  39. Add 10 µL of CD45 coated magnetic beads (Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach)
  40. Place tubes into rotator and incubate for 15 minutes at 4 °C
  41. Continue with MAC sorting according to the manual. Use an MS column and wash 3 times.

42. Improve purity of eluted MA-INA6 by straining eluate ( $30\ \mu\text{m}$ ) (wash strainer using 1 mL of sorting buffer) and applying it onto an MS column a second time. Wash three times.

43. Collect 20  $\mu\text{L}$  per eluate and apply it onto a 96-well plate to evaluate purity

a. Incubate plate for 24 h

b. Count the number of adherent cells (hMSCs) per INA-6 using phase contrast microscopy

c. *We reached a mean purity of  $3.2 \times 10^{-4}$  ( $\pm 2.2 \times 10^{-4}$ ) hMSCs per MA-INA6.*

d. *hMSC contamination did not have an impact on RNAseq, since those genes that are highly expressed in hMSCs (VCAM1, ALPL, FGF5, FGFR2), did not appear as differentially expressed in MA-INA6 (Data not shown). RNAseq detected  $0.44 \pm 0.16$  CPM-normalized counts of VCAM1 transcripts in MA-INA6, however, it was excluded like all genes with less than 1 count in at least 2 of 5 replicates.*

44. Count cells using a Neubauer chamber

45. Pellet samples (250 g for 5 min)

46. Resuspend in respective medium or lysis buffer (e.g. RA1 for RNA extraction)

Collect MA-INA6 by rough pipetting (no MAC sorting)

47. After the last centrifugation step, add hMSC-medium to each well of the co-culture plate to reach a volume of 150  $\mu\text{L}$

a. *Since the yield of MA-INA6 was large, we dissociated MA-INA-6 cells from hMSCs by vigorous pipetting (for further samples after RNAseq, see Tab. S1). Since no enzymatic digestion is used, we reckoned that there would be no need for MAC sorting. Confluent hMSCs withstand this procedure and don't dissociate as single cells, which can be removed by straining cells ( $30\ \mu\text{m}$ ). We reached similar purities as for MAC-sorting (Data not shown).*

48. Using a multi-channel pipette (100  $\mu\text{L}$ ), gently raise 90  $\mu\text{L}$  into the tips

49. Lean pipette tip on the upper well-border and roughly pipette up and down once

50. Repeat step 48 at the lower right and lower left well border (Total of 3 pipetting steps  
“Mercedes Star”)

51. Attach a catching plate onto the co-culture and centrifuge for 40 seconds at 500 rpm (28 g)

52. Repeat steps 46-50 until a sufficient amount of MA-INA6 is removed
53. Control purity of MA-INA-6 by placing out aliquot onto an empty 96-well plate.
54. Collect MA-INA6 from catching plate
55. Remove hMSCs by straining cell suspension (30 µm)
56. Count cells using a Neubauer chamber
57. Pellet MA-INA6 (250 g for 5 min)
58. Resuspend in respective medium or lysis buffer

Centrifugal force: We used a Hettich 1460 rotor ( $r = 124$  mm) (Hettich GmbH & Co. KG, Tuttlingen, Germany). For calculating the centrifugal force that acts onto the co-culture within well plate sandwiches, we subtracted the height of the catching plate (14.4 mm, Greiner 96 well plate) and the depth of each well (10.9 mm). This yields a radius of 98.7mm, which translates to the following centrifugal forces: 500 rpm: 28 g; 1000 rpm: 110 g; 2000 rpm: 441 g.

Washing medium with EDTA: EDTA removes calcium from integrins which are required for adhesion. It is not strong enough to dissociate INA-6 from hMSCs, but could help with removing INA-6 from other INA-6. For generating samples for RNAseq, we added 3 mM of EDTA to washing medium. For further samples, we did not add EDTA to the washing medium, since we found that it does not increase yield for all biological replicates consistently (Data not shown). We suspect that integrin-mediated adhesion depends on hMSC donor or internal variance of INA-6. We recommend using 3 mM of EDTA, however, this requires further optimizations like including an incubation time at 37 °C after the addition of washing medium to account for biological variance. However, this could take long incubation times of up to 60 minutes (Lai et al., 2022).

### Track Cell Number During WPSC

To track the cell count during WPSC, INA-6 were stained with CellTracker green and both in co-culturing- and catching plates were scanned after each centrifugation step. For each round of centrifugation, an empty catching plate was used. A pre-trained convolutional neural network (Intellesis, Zeiss) was fine-tuned to segment the scans into background, cells, and cell borders. Single cells were counted and the cumulative sum for each catching plate was calculated.

### **Sub-Culturing After WPSC of MSC-Interacting INA-6 Subpopulations**

After CM-INA6, nMA-INA6, and MA-INA6 were isolated, they were counted with a Neubauer chamber using all nine quadrants and diluted to  $10^5$  cells/mL in MSC-medium (10% FCS, no IL-6 except for control). 100  $\mu$ L of cell suspension was applied to 96-well plates, incubated for 48 h at 37 °C and 5% CO<sub>2</sub> and then subjected to viability and apoptosis assays.

### **RNA Isolation**

Total RNA was isolated from INA-6 cells by using the NucleoSpin RNA II Purification Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

### **RNAseq, Differential Expression and Functional Enrichment Analysis of INA-6 cells**

FASTQ files were merged to the respective sample. The quality of FASTQ files was assessed with FastQC (Andrews, 2010) tool, and a joint report was created with MultiQC (Ewels et al., 2016) tool. Fastq files were aligned with STAR (Dobin et al., 2013) to the GRCh38 reference genome build (Zerbino et al., 2018). Quality and alignment statistics of final BAM files were assessed with samtools stats (Li et al., 2009), and a joint report with FastQC reports by MultiQC was generated.

Raw read counts were generated with HTSeq (Anders et al., 2015) with the union method. HTSeq runs internally in STAR. Differential gene expression analysis was done with edgeR (Robinson et al., 2010) in R 3.6.3 (R Core Team, 2018), according to the edgeR manual.

Counts were merged and genes with zero counts in all samples were removed (number of genes: 36380).

The whole count table was annotated with R Bioconductor (Gentleman, n.d.) (Gentleman et al. 2004) human annotation data package org.Hs.eg.db (Carlson, 2016).

A DGEList Element was created with the raw counts, gene information, i.e. Ensembl GenelIDs, HUGO Symbol, Genename, and ENTREZ GenelIDs and a sample grouping meta data table.

```
y <- DGEList(counts=ct2[,-1:4], group=meta.data$group, genes=ct2[,1:4])
```

Counts were filtered to keep only those genes which have at least 1 read per million in at least 2 samples (number of genes: 14136). Afterwards normalization factors were recalculated.

```
keep <- rowSums(cpm(y)>1) >=2  
y <- y[keep, , keep.lib.size = FALSE]  
y1 <- calcNormFactors(y)
```

A design matrix was created with grouping factor by treatment condition (group=F1, F2, F3, which are abbreviations for CM-INA6, nMA-INA6, MA-INA6, respectively)

```
design = model.matrix(~0+group)
```

Dispersion was estimated, the resulting coefficient of biological variation (BCV) is 0.135, i.e. BCV expression values vary up and down by 13.5% between samples.

```
y1.1 <- estimateDisp(y1, design)  
BCV <- sqrt(model.F$y1.1$common.dispersion)
```

A generalized linear (glmQLFit function) model was fitted.

```
fit <- glmQLFit(y1.1, design)
```

and pairwise comparisons were made, e.g.

```
F1vsF2 <- glmQLFTest(fit, contrast = makeContrasts(groupF1 - groupF2,  
levels = design))
```

top significant differential expressed genes were written to a table

```
DE.F1vsF2 <- topTags(F1vsF2, n=nrow(F1vsF2), p.value = 0.05)
```

Afterwards, gene list of differentially expressed genes were used for functional enrichment analysis with metascape (Zhou et al., 2019).

### RT-qPCR

For cDNA synthesis 1 µg of total RNA was reverse transcribed with Oligo(dT)15 primers and Random Primers (both Promega GmbH, Mannheim, Germany) and Superscript IV reverse transcriptase (Thermo Fisher Scientific) according to the manufacturer's instructions. For quantitative PCR the cDNA was diluted 1:10 and qPCR was performed in 20 µl by using 2 µl of cDNA and 10 µl of GoTaq qPCR Master Mix (Promega GmbH) and 5 pmol of sequence-specific primers obtained from biomers.net GmbH (Ulm, Germany) or Qiagen GmbH (Hilden, Germany) (see Tab. S4 for primer sequences and PCR conditions). qPCR conditions were as follows: 95°C for 3 min; 40 cycles: 95°C for 10 s; respective annealing temperature for 10 s; 72°C for 10 s; followed by melting curve analysis for the specificity of qPCR products by using the qPCR thermal cycler Professional Thermocycler Biometra (Analytik Jena AG, Jena, Germany). Samples that showed unspecific byproducts were discarded. Ct values were measured in three technical replicates (triplicates). Non-detects were discarded. One of three technical replicates was treated as an outlier and excluded if

its z-score crossed  $1.5\sigma$  technical variation. We normalized expression by the housekeeping gene 36B4. Efficiencies were determined in each reaction by linear regression of log transformed amplification curve (Ramakers et al., 2003). Differential expression was calculated based on a modified  $\Delta\Delta Ct$  formula that separated exponents to apply individual efficiencies to each Ct value:

$$\text{Fold Change} = \frac{E_{tar}^{\Delta Ct_{tar}(co-treated)}}{E_{ref}^{\Delta Ct_{ref}(co-treated)}} = \frac{E_{tar,co}^{Ct_{tar,co}} : E_{tar,treated}^{Ct_{tar,treated}}}{E_{ref,co}^{Ct_{ref,co}} : E_{ref,treated}^{Ct_{ref,treated}}}$$

$E_{tar,co}$  = Efficiency of the target gene measured in the control sample

$Ct_{tar,co}$  = Ct value of the target gene measured in the control sample

$tar$  = Target Gene;  $ref$  = Reference Gene

$treated$  = Treated sample;  $co$  = Control Sample

Fold change expression was normalized by the median of CM-INA6 (and not samplewise, as commonly used in  $\Delta\Delta Ct$ ) since some genes were not expressed without direct MSC contact s (e.g. MMP2), and also in order to display variation of CM-INA6 next to nMA-INA6 and MA-INA6.

**Tab. S4:** List of primers. Some primers required a melting step to be performed before fluorescent readout to remove byproducts.

Primer	Sequence 5' - 3'	base pairs [bp]	annealing temp. [°C]
36B4_s	tgcatcagtacccatttatcat	122	60
36B4_as	aggcagatggatcagccaaga		
BCL6_s	tagagcccataaaacggtcctcat	221	55 + Melting Step at 77 °C
BCL6_as	cgc当地点attgagccgagatgtgt		
BMP4_s	tacatgcgggatcttaccg	132	58
BMP4_as	atgttcttcgtggtaagc		
BTG2_s	gtattcttgtagggccacactaa	264	60 + Melting Step at 78 °C
BTG2_as	tcttaagggtgattcggtttggaa		
CXCL8_s	actgagagtgattgagagtggacc	251	55 + Melting Step at 77 °C
CXCL8_as	ccctacaacagacccacacaatac		
CXCL12_s	gattcttcgaaagccatgtgcc	119	56

CXCL12_as	caatgcacacttgtctgttgtgt		
DCN_s	caacaacaagcttaccagagtacct	160	57
DCN_as	tgaaaagactcacacccgaataaga		
DKK1_s	gcactgatgagttactgcgcttag	129	56
DKK1_as	ttttgcagtaattccggggc		
IL10RB_s	gagtgagcctgtctgtgagcaa	139	55
IL10RB_as	cttgtaaacgcaccacagcaag		
IL24_s	caaacagttggacgtagaaggcagc	149	55
IL24_as	tgaaatgacacagggAACAAACCA		
LOX_s	ctgctcagattccccaaag	125	57
LOX_as	tggcatcaagcaggcatag		
MMP2_s	ttgtatttgatggcatcgctcaga	155	56
MMP2_as	cgtataccgcatcaatctttccg		
MMP14_s	cgacaagattgtatgctgctc	140	57
MMP14_as	tcccttcccagactttgatg		
MUC1_s	gcagcctctcgatataacctg	200	58
MUC1_as	gtaggtgggtactcgctca		
NOTCH2_s	gtgcttgttgaacacttgtgcc	185	55
NOTCH2_as	cactcgcatctgtatccaccaatg		
OPG (TNFRSF11B)	no sequence available (Proprietary primers from Qiagen: QT00014294 TNFRSF11B_1_SG)		60
PRICKLE1_s	cagaggtatatcatgaaggacggc	102	56
PRICKLE1_as	gtcccacaccaatatgttccccac		
TGM2_s	caaccttctcatcgagttacttccg	100	58
TGM2_as	tcatccacgactccacccag		
TNFRSF1A_s	ctccttcaccgcttcagaaaacc	153	55
TNFRSF1A_as	ttcactccaataatgccggtaactg		
TRAF5_s	tgcctgttagataaagaggcatca	177	56
TRAF5_as	aacactgcacaggttcaaataagc		

**Statistics**

For molecular analyses, each data point represents one biological replicate, which we define as the mean of all technical replicates of co-cultures that were seeded out from the same batch of hMSCs and/or INA-6 cells on the same day. For analyses of time-lapse recordings, each datapoint represents the normalized event count from a recording of one co-culture. We prioritized unique hMSCs for each biological replicate or recording (Tab. S1). Bars and lines represent the mean and error bars represent the standard deviation of all hMSC donors or recordings (= all biological replicates).

Metric, normal distributed, dependent data was analyzed using factorial RM-ANOVA and paired Student's t-test. Results of RM-ANOVA are reported as such:  $[F(df_1, df_2) = F; p = p\text{-value}]$ , with  $df_1$  being the degrees of freedom of the observed effect,  $df_2$  being the degrees of freedom of the error and  $F$  being the F-statistic (Vallat, 2018). If sphericity was met, p-values were not corrected with the Greenhouse-Geisser method (p-unc).

$$df_1 = k - 1 \quad k = \text{The number of groups (of a factor, if factorial RM-ANOVA)}$$

$$df_2 = (k - 1)(n - 1)n = \text{The number of samples in each group}$$

$$F = \frac{SS_{Effect} \div df_1}{SS_{Error} \div df_2} \quad SS = \text{Sums of squares for effect or error}$$

If datapoints within dependent sample pairs were missing, such pairs were excluded from paired t-test while other pairs of the same subject remained.

Metric non-normal distributed, independent data was analyzed using Kruskal-Wallis H-test and Mann-Whitney U tests. Results of Kruskal-Wallis H-test was reported as such:  $[H(df) = H]$ , with  $df$  being the degrees of freedom and  $H$  being the Kruskal-Wallis H statistic, corrected for ties (Vallat, 2018).

$$df = k - 1 \quad k = \text{The number of groups}$$

Metric bivariate non-normal distributed data was correlated using spearman's rank correlation and reported as such:  $[\rho(df) = \rho, p = p\text{-value}]$ , with  $\rho$  being Spearman's rank correlation coefficient.  $df$  is calculated as such:

$$df = n - 2 \quad n = \text{The number of observations}$$

These test were applied using the python (3.10) -packages pingouin (0.5.1). For three-factor RM-ANOVA we used statsmodels (0.14.0) (Seabold & Perktold, 2010; Vallat, 2018). Data was plotted

using seaborn (Waskom, 2021). Sphericity was ensured by Mauchly's test. Normality was checked with the Shapiro-Wilk test for  $n > 3$ .

Datapoints were log10 transformed to convert the scale from multiplicative ("foldchange") to additive, or in order to fulfill sphericity requirements.

P-values derived from patient survival data were corrected using the Benjamini-Hochberg procedure. For other post-hoc analyses, p-values were not adjusted for family-wise error rate in order to minimize type I errors. To prevent type II errors, the same conclusions were validated by different experimental setups and through varying hMSCs donors across experiments (Tab. S1).

Significant p-values from pairwise tests were annotated as stars between data groups (p-value: 0.05 > \* > 0.01 > \*\* >  $10^{-3}$  > \*\*\*  $10^{-4}$  > \*\*\*\*). If too many significant pairs were detected, we annotated only those pairs of interest.

No power calculation was performed to determine sample size since samples were limited by availability of primary hMSC donors. Experiments were repeated until a minimum of three biological replicates were gathered.

### **Patient Cohort, Analysis of Survival and Expression**

Patient samples ( $n=873$ ) were collected at the UKHD and processed as described (Seckinger et al., 2017, 2018), and are available at the European Nucleotide Archive (ENA) via accession numbers PRJEB36223 and PRJEB37100. Consecutive patients with monoclonal gammopathy of unknown significance (MGUS) ( $n = 62$ ), asymptomatic ( $n = 259$ ), symptomatic, therapy-requiring ( $n = 764$ ), and relapsed/refractory myeloma ( $n = 90$ ), as well as healthy donors ( $n = 19$ ) as comparators were included in the study approved by the ethics committee (#229/2003, #S-152/2010) after written informed consent.

Gene expression was measured by RNA sequencing as previously described (Seckinger et al., 2018). Gene expression is defined as the log2 transformed value of normalized counts + 1 (as pseudocount). Progression-free (PFS) and overall survival (OS) was analyzed for the subset of previously untreated symptomatic MM patients. For delineating "high" and "low" expression of target adhesion ( $n=101$ ) and cell cycle ( $n=173$ ) genes, thresholds per gene were calculated with maximally selected rank statistics by the maxstat package in R (Hothorn & Lausen, n.d.). PFS and OS were

analyzed for high vs. low expression with the Kaplan-Meier method (Kaplan & Meier, 1958). Significant differences between the curves were analyzed with log-rank tests (Harrington & Fleming, 1982). P-values were corrected for multiple testing by the Benjamini-Hochberg method. Analyses were performed with R version 3.6.3 (R Core Team, 2018).

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## Chapter 2: Semi-Automation of Data Analysis

### Introduction

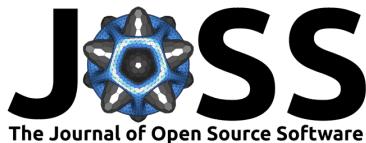
Why did I need plotastic?

Why do biologists need plotastic? - Assays output more data in shorter time, e.g. multiplex qPCR - example: 20 genes, 3 timepoints, 11 biological replicates, (all 3 technical replicates already averaged) -  $20 * 3 * 11 = 660$  data points

this is multidimensional data: 660 data points spread across two dimensions: time and gene

shortly Describe Main Packages in more detail: - seaborn: It multidimensional data - pingouin: It's a statistical package

-in manual analysis e.g. in Excel, the user has to manually select the data, copy it, paste it into a new sheet, and then perform the statistical test. In Prism, the user has to select the data, click on the statistical test, and then select the data again. This is not only time-consuming, but also prone to



## plotastic: Bridging Plotting and Statistics in Python

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## Summary

plotastic addresses the challenges of transitioning from exploratory data analysis to hypothesis testing in Python's data science ecosystem. Bridging the gap between seaborn and pingouin, this library offers a unified environment for plotting and statistical analysis. It simplifies the workflow with a user-friendly syntax and seamless integration with familiar seaborn parameters (y, x, hue, row, col). Inspired by seaborn's consistency, plotastic utilizes a `DataAnalysis` object to intelligently pass parameters to pingouin statistical functions. The library systematically groups the data according to the needs of statistical tests and plots, conducts visualisation, analyses and supports extensive customization options. In essence, plotastic establishes a protocol for configuring statistical analyses through plotting parameters. This approach streamlines the process, translating seaborn parameters into statistical terms, allowing researchers to focus on correct statistical testing and less about specific syntax and implementations.

## Statement of need

Python's data science ecosystem provides powerful tools for both visualization and statistical testing. However, the transition from exploratory data analysis to hypothesis testing can be cumbersome, requiring users to switch between libraries and adapt to different syntaxes.

seaborn has become a popular choice for plotting in Python, offering an intuitive interface. Its statistical functionality focuses on descriptive plots and bootstrapped confidence intervals ([Waskom, 2021](#)). The library pingouin offers an extensive set of statistical tests, but it lacks integration with common plotting capabilities ([Vallat, 2018](#)). statannotations integrates statistical testing with plot annotations, but uses a complex interface and is limited to pairwise comparisons. ([Charlier et al., 2022](#)).

plotastic addresses this gap by offering a unified environment for plotting and statistical analysis. With an emphasis on user-friendly syntax and integration with familiar seaborn parameters, it simplifies the process for users already comfortable with seaborn. The library ensures a smooth workflow, from data import to hypothesis testing and visualization.

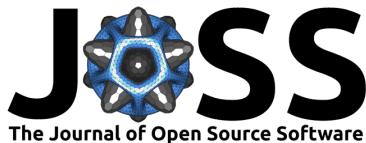
## Example

The following code demonstrates how plotastic analyzes the example dataset "fmri", similar to Waskom (2021) (Figure 1).

```
### IMPORT PLOTASTIC
import plotastic as plst

# IMPORT EXAMPLE DATA
```

Kuric, & Ebert. (1970). plotastic: Bridging Plotting and Statistics in Python. *Journal of Open Source Software*, *1* VOL? (ISSUE?), PAGE? 1  
<https://doi.org/N/A>.



```

DF, _dims = plst.load_dataset("fmri", verbose = False)

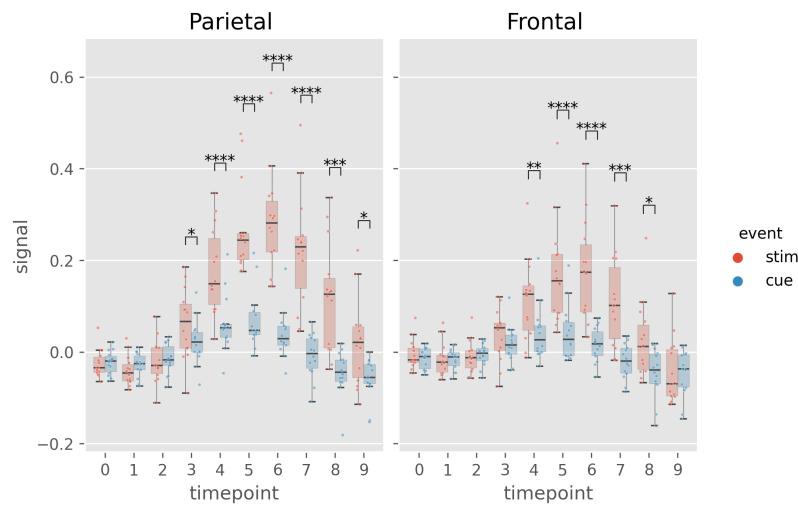
# EXPLICITLY DEFINE DIMENSIONS TO FACET BY
dims = dict(
    y = "signal",      # y-axis, dependent variable
    x = "timepoint",   # x-axis, independent variable (within-subject factor)
    hue = "event",     # color, independent variable (within-subject factor)
    col = "region"     # axes, grouping variable
)

# INITIALIZE DATAANALYSIS OBJECT
DA = plst.DataAnalysis(
    data=DF,           # Dataframe, long format
    dims=dims,         # Dictionary with y, x, hue, col, row
    subject="subject", # Datapoints are paired by subject (optional)
    verbose=False,     # Print out info about the Data (optional)
)

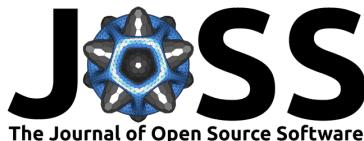
# STATISTICAL TESTS
DA.check_normality()    # Check Normality
DA.check_sphericity()   # Check Sphericity
DA.omnibus_rm_anova()   # Perform RM-ANOVA
DA.test_pairwise()       # Perform Posthoc Analysis

# PLOTTING
(DA
    .plot_box_strip()   # Pre-built plotting function initializes plot
    .annotate_pairwise() # Annotate results from DA.test_pairwise()
        include="__HUE" # Use only significant pairs across each hue
    )
)

```



**Figure 1:** Example figure of `plotastic` (version 0.1). Image style was set by `plt.style.use("ggplot")`



**Table 1:** Results from `DA.check_sphericity()`. `plotastic` assesses sphericity after grouping the data by all grouping dimensions (`hue`, `row`, `col`). For example, `DA.check_sphericity()` grouped the ‘fmri’ dataset by “region” (`col`) and “event” (`hue`), performing four subsequent sphericity tests for four datasets.

'region', 'event'	spher	W	chi2	dof	pval	group count	n per group
'frontal', 'cue'	True	3.26e+20	-462.7	44	1	10	[14]
'frontal', 'stim'	True	2.45e+17	-392.2	44	1	10	[14]
'parietal', 'cue'	True	1.20e+20	-452.9	44	1	10	[14]
'parietal', 'stim'	True	2.44e+13	-301.9	44	1	10	[14]

**Table 2:** Results of `DA.omnibus_rm_anova()`. `plotastic` performs one two-factor RM-ANOVA per axes (grouping the data by `row` and `col` dimensions) using `x` and `hue` as the within-factors. For this example, `DA.omnibus_rm_anova()` grouped the ‘fmri’ dataset by “region” (`col`), performing two subsequent two-factor RM-ANOVAs. Within-factors are “timepoint” (`x`) and “event” (`hue`). For conciseness, GG-Correction and effect sizes are not shown.

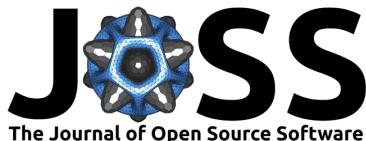
'region'	Source	SS	ddof1	ddof2	MS	F	p-unc	stars
'parietal'	timepoint	1.583	9	117	0.175	26.20	3.40e-24	****
'parietal'	event	0.770	1	13	0.770	85.31	4.48e-07	****
'parietal'	timepoint *	0.623	9	117	0.069	29.54	3.26e-26	****
	event							
'frontal'	timepoint	0.686	9	117	0.076	15.98	8.28e-17	****
'frontal'	event	0.240	1	13	0.240	23.44	3.21e-4	***
'frontal'	timepoint *	0.242	9	117	0.026	13.031	3.23e-14	****
	event							

## Overview

The functionality of `plotastic` revolves around a seamless integration of statistical analysis and plotting, leveraging the capabilities of `pingouin`, `seaborn`, `matplotlib` and `statannotations` (Charlier et al., 2022; Hunter, 2007; Vallat, 2018; Waskom, 2021). It utilizes long-format `pandas` DataFrames as its primary input, aligning with the conventions of `seaborn` and ensuring compatibility with existing data structures (McKinney, 2011; Wickham, 2014).

`plotastic` was inspired by `seaborn`’s intuitive and consistent usage of the same set of parameters (`y`, `x`, `hue`, `row`, `col`) found in each of its plotting functions (Waskom, 2021). These parameters intuitively delineate the data dimensions plotted, yielding ‘faceted’ subplots, each presenting `y` against `x`. This allows for rapid and insightful exploration of multidimensional relationships. `plotastic` extends this principle to statistical analysis by storing these `seaborn` parameters (referred to as dimensions) in a `DataAnalysis` object and intelligently passing them to statistical functions of the `pingouin` library. This approach is based on the impression that most decisions during statistical analysis can be derived from how the user decides to arrange the data in a plot. This approach also prevents code repetition and streamlines statistical analysis. For example, the `subject` keyword is specified only once during `DataAnalysis` initialisation, and `plotastic` selects the appropriate paired or unpaired version of the test. Using `pingouin` alone requires the user to manually pick the correct test and to repeatedly specify the `subject` keyword in each testing function.

In essence, `plotastic` translates plotting parameters into their statistical counterparts. This translation minimizes user input and also ensures a coherent and logical connection between plotting and statistical analysis. The goal is to allow the user to focus on choosing the



correct statistical test (e.g. parametric vs. non-parametric) and worry less about specific implementations.

At its core, `plotastic` employs iterators to systematically group data based on various dimensions, aligning the analysis with the distinct requirements of tests and plots. Normality testing is performed on each individual sample, which is achieved by splitting the data by all grouping dimensions and also the `x`-axis (hue, row, col, `x`). Sphericity and homoscedasticity testing is performed on a complete sampleset listed on the `x`-axis, which is achieved by splitting the data by all grouping dimensions (hue, row, col) ([Table 1](#)). For omnibus and posthoc analyses, data is grouped by the row and col dimensions in parallel to the `matplotlib` axes, before performing one two-factor analysis per axes using `x` and hue as the within/between-factors. ([Table 2](#)).

`DataAnalysis` visualizes data through predefined plotting functions designed for drawing multi-layered plots. A notable emphasis within `plotastic` is placed on showcasing individual datapoints alongside aggregated means or medians. In detail, each plotting function initializes `matplotlib` figure and axes using `plt.subplots()` while returning a `DataAnalysis` object for method chaining. Axes are populated by `seaborn` plotting functions (e.g., `sns.boxplot()`), leveraging automated aggregation and error bar displays. Keyword arguments are passed to these `seaborn` functions, ensuring the same degree of customization as in `seaborn`. Users can further customize plots by chaining `DataAnalysis` methods or by applying common `matplotlib` code to override `plotastic` settings. Figures are exported using `plt.savefig()`.

`plotastic` also focuses on annotating statistical information within plots, seamlessly incorporating p-values from pairwise comparisons using `statannotations` ([Charlier et al., 2022](#)). This integration simplifies the interface and expands options for pair selection in multidimensional plots, enhancing both user experience and interpretability.

`plotastic` also focuses on annotating statistical inside the plot. It integrates `statannotations` for annotating p-values from pairwise comparisons inside the plot ([Charlier et al., 2022](#)), and simplifies the interface and offering further options for pair selection in multidimensional plots.

For statistics, `plotastic` integrates with the `pingouin` library to support classical assumption and hypothesis testing, covering parametric/non-parametric and paired/non-paired variants. Assumptions such as normality, homoscedasticity, and sphericity are tested. Omnibus tests include two-factor RM-ANOVA, ANOVA, Friedman, and Kruskal-Wallis. Posthoc tests are implemented through `pingouin.pairwise_tests()`, offering (paired) t-tests, Wilcoxon, and Mann-Whitney-U.

To sum up, `plotastic` stands as a unified and user-friendly solution catering to the needs of researchers and data scientists, seamlessly integrating statistical analysis with the power of plotting in Python. It streamlines the workflow, translates `seaborn` parameters into statistical terms, and supports extensive customization options for both analysis and visualization.

## Acknowledgments

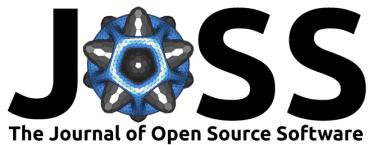
This work was supported by the Deutsche Forschungsgemeinschaft (DFG) SPP microBONE grants EB 447/10-1 (491715122), JA 504/17-1, HO 4462/1-1 (401358321). We thank the Elite Netzwerk Bayern and the Graduate School of Life Sciences of the University of Würzburg.

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Kuric, & Ebert. (1970). plotastic: Bridging Plotting and Statistics in Python. *Journal of Open Source Software*, *5* VOL? (ISSUE?), PAGE? 5  
<https://doi.org/N/A>.

## Discussion

Is plotastic useful for biologists? - Yes but use is limited by minimal knowledge of Python  
- However, that is subject to change as Python is becoming more popular in biology and AI assisted coding decreased the barrier to entry significantly. Tools like github copilot are able to generate code, fix bugs and suggest improvements. This is a game changer for biologists that are not familiar with programming. - Furthermore, installing and using plotastic for biologists is overestimated. These steps are needed:  
- Install anaconda from the internet  
- Open the terminal  
- Type `pip install plotastic`  
- Check Rea

# Summarising Discussion

## Time Lapse

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## Myeloma

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## Semi-Automated Analysis Improves Agility During Establishing new *in vitro* Methods

Was plotastic useful for me? - Yes incredibly. I was able to perform the statistical tests and visualize the data in a fraction of the time that I would have needed manually. This allowed me to focus on the interpretation of the results and the writing of the manuscript. There was one particular example where my analysis was so fast, that I fed raw datatables during microscopy into python scripts and was able to adapt the experimental technique during the experiment. This allows for an agile and adaptive work environment that is not possible with manual analysis and proved invaluable during development of *in vitro* methods. - These experiments benefited from the use of plotastic, as the

Further research is needed to assess the true impact of semi-automated analysis on the agility

of establishing new *in vitro* methods.

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## Appendix



**Statement of individual author contributions and of legal second publication rights to manuscripts included in the dissertation**

**Manuscript 1: Research Article (submitted, under revision)**

Martin Kuric (MK), Susanne Beck, Doris Schneider, Wyonna Rindt, Marietheres Evers, Jutta Meißner-Weigl, Sabine Zeck, Melanie Krug, Marietta Herrmann, Tanja Nicole Hartmann, Ellen Leich, Maximilian Rudert, Denitsa Docheva, Anja Seckinger, Dirk Hose, Franziska Jundt, Regina Ebert (RE) (2024): Keep it Together: Describing Myeloma Dissemination *in vitro* with hMSC-Interacting Subpopulations and their Aggregation/Detachment Dynamics, **Cancer Research Communications**

Participated in	<b>Author Initials</b> , Responsibility decreasing from left to right				
Study Design	<u>MK</u>	Regina Ebert	Wyonna Rindt		
Methods Development	<u>MK</u>	Doris Schneider			
Data Collection	<u>MK</u>	Doris Schneider			
Data Analysis and Interpretation	<u>MK</u>	Susanne Beck	Regina Ebert		
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	<u>MK</u>	Regina Ebert			

**Explanations:** The content of this publication exceeds the usual scope (~29 pages Supplemental). It includes not only research findings but also survival data and protocols of new, established methods and their validations. The contribution of Martin Kuric was pivotal and predominant in all aspects of this work. Doris Schneider assisted in the experimental procedures. Susanne Beck analyzed the raw data from RNAseq and survival data, which were interpreted, depicted, and summarized by Martin Kuric.

**Manuscript 2: Data Analysis Software (submitted, passed peer-review, under revision)**

Martin Kuric (MK), Regina Ebert (2024): plotastic: Bridging Plotting and Statistics in Python, **Journal of Open Source Software**

Participated in	<b>Author Initials</b> , Responsibility decreasing from left to right				
Idea, Architectural Design	<u>MK</u>				
Software Development Feature Implementation Testing	<u>MK</u>				
Distribution of Software Documentation Version Control (GitHub) Deployment (PyPi)	<u>MK</u>				
Manuscript Writing Writing of Statement of Need Writing of Example Writing of Overview	<u>MK</u>	Regina Ebert			

**Explanations:** The software was entirely created by Martin Kuric, comprising more than 8000 total lines (including ~2000 testable lines) and is comparable in size to a typical web application. The release of this software involved version control using GitHub, packaging and deployment on PyPi. Regina Ebert gave feedback on submitted manuscript.

**Manuscript 3: Research Letter (published)**

Daniela Simone Maichl, Julius Arthur Kirner, Susanne Beck, Wen-Hui Cheng, Melanie Krug, Martin Kuric (MK), Carsten Patrick Ade, Thorsten Bischler, Franz Jakob, Dirk Hose, Anja Seckinger, Regina Ebert & Franziska Jundt (2023): Identification of NOTCH-driven matrisome-associated genes as prognostic indicators of multiple myeloma patient survival, **Blood Cancer Journal 13:134**

Participated in	Author Initials, Responsibility decreasing from left to right				
Study Design Methods Development	Daniela Simone		Franziska Jundt		
Data Collection	Daniela Simone		Franziska Jundt		
Data Analysis and Interpretation	Daniela Simone	Susanne Beck	Franziska Jundt	<u>MK</u>	
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	Daniela Simone		Franziska Jundt	<u>MK</u>	

**Explanations:** This co-authorship is not a chapter in this dissertation. Martin Kuric produced figures of processed but complex-to-visualize data and gave feedback on submitted manuscript.

**Manuscript 4: Research Paper (under peer-review)**

Wyonna Rindt, Melanie Krug, Shuntaro Yamada, Franziska Sennefelder, Louisa Belz, Wen-Hui Cheng, Azeem Muhammad, Martin Kuric (MK), Marietheres Evers, Ellen Leich, Tanja Nicole Hartmann, Ana Rita Pereira, Marietta Herrmann, Jan Hansmann, Mohammed Ahmed Yassin, Kamal Mustafa, Regina Ebert, and Franziska Jundt (2024): A 3D bioreactor model to study osteocyte differentiation and mechanobiology under perfusion and compressive mechanical loading, **Acta Biomaterialia**

Participated in	Author Initials, Responsibility decreasing from left to right				
Study Design Methods Development	Wyonna Rindt	Franziska Jundt		<u>MK</u>	
Data Collection	Wyonna Rindt	Franziska Jundt		<u>MK</u>	
Data Analysis and Interpretation	Wyonna Rindt	Franziska Jundt		<u>MK</u>	
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	Wyonna Rindt	Franziska Jundt		<u>MK</u>	

**Explanations:** This co-authorship is not a chapter in this dissertation. Martin Kuric contributed by counseling during weekly meetings in tight collaboration with Franziska Jundt's group, assisting Wyonna Rindt during laboratory experiments, image analysis and giving feedback on submitted manuscript.

**Manuscript 5: Research Paper (under revision)**

Marietta Herrmann, Jutta Schneidereit, Susanne Wiesner, Martin Kuric (MK), Maximilian Rudert, Martin Lüdemann, Mugdha Srivastava, Norbert Schütze, Regina Ebert, Denitsa Docheva, Franz Jakob (2024): Peripheral blood cells enriched by adhesion to CYR61 are heterogenous myeloid modulators of tissue regeneration with early endothelial progenitor characteristics, **European Cells and Materials**

Participated in	Author Initials, Responsibility decreasing from left to right				
Study Design Methods Development	Marietta Herrmann				
Data Collection	Marietta Herrmann			<u>MK</u>	

Data Analysis and Interpretation	Marietta Herrmann				
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	Marietta Herrmann			<u>MK</u>	

**Explanations:** This co-authorship is not a chapter in this dissertation. Martin Kuric contributed by establishing and measuring large automated microscopy scans of stained cells for quantifying osteogenic differentiation and giving feedback on submitted manuscript.

<b>Manuscript 6: Research Letter (published)</b>					
<b>Participated in</b>	<b>Author Initials, Responsibility decreasing from left to right</b>				
Study Design Methods Development	Marietheres Evers				
Data Collection	Marietheres Evers				
Data Analysis and Interpretation	Marietheres Evers			<u>MK</u>	
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	Marietheres Evers			<u>MK</u>	

**Explanations:** This co-authorship is not a chapter in this dissertation. Martin Kuric contributed by counseling during regular meetings with Ellen Leich's group and giving feedback on submitted manuscript.

If applicable, the doctoral researcher confirms that she/he has obtained permission from both the publishers (copyright) and the co-authors for legal second publication.

The doctoral researcher and the primary supervisor confirm the correctness of the above mentioned assessment.

Würzburg

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Doctoral Researcher's Name	Date	Place	Signature
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Würzburg

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Primary Supervisor's Name	Date	Place	Signature
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**Statement of individual author contributions to figures/tables of manuscripts included in the dissertation**

**Manuscript 1: Research Article (submitted, under revision)**

Martin Kuric (MK), Susanne Beck, Doris Schneider, Wyonna Rindt, Marietheres Evers, Jutta Meißner-Weigl, Sabine Zeck, Melanie Krug, Marietta Herrmann, Tanja Nicole Hartmann, Ellen Leich, Maximilian Rudert, Denitsa Docheva, Anja Seckinger, Dirk Hose, Franziska Jundt, Regina Ebert1 (2024): Keep it Together: Describing Myeloma Dissemination *in vitro* with hMSC-Interacting Subpopulations and their Aggregation/Detachment Dynamics, **Cancer Research Communications**

Figure	<b>Author Initials</b> , Responsibility decreasing from left to right				
1	<u>MK</u>	Doris Schneider			
2	<u>MK</u>	Doris Schneider			
3	<u>MK</u>	Doris Schneider	Sabine Zeck	Wyonna Rindt	Melanie Krug
4	<u>MK</u>	Doris Schneider	Susanne Beck		
5	<u>MK</u>	Susanne Beck			
6	<u>MK</u>	Susanne Beck			
7	<u>MK</u>				
S1	<u>MK</u>	Doris Schneider	Sabine Zeck	Wyonna Rindt	Melanie Krug
S2	<u>MK</u>	Doris Schneider	Marietta Herrmann		
S3	<u>MK</u>	Doris Schneider	Sabine Zeck		
S4	<u>MK</u>				
S5	<u>MK</u>				
S6	<u>MK</u>	Susanne Beck			
Table	<b>Author Initials</b> , Responsibility decreasing from left to right				
1	<u>MK</u>	Susanne Beck			
2	<u>MK</u>	Susanne Beck			
S1	<u>MK</u>	Doris Schneider			
S2	<u>MK</u>	Susanne Beck			
S3	<u>MK</u>				
S4	<u>MK</u>	Doris Schneider			

**Manuscript 2: Data Analysis Software (submitted, passed peer-review, under revision)**

Martin Kuric, Regina Ebert (2024): plotastic: Bridging Plotting and Statistics in Python, **Journal of Open Source Software**

Figure	<b>Author Initials</b> , Responsibility decreasing from left to right				
1	<u>MK</u>				
Table	<b>Author Initials</b> , Responsibility decreasing from left to right				
1	<u>MK</u>				
2	<u>MK</u>				

Documentation	<b>Author Initials</b> , Responsibility decreasing from left to right				
README	<u>MK</u>				
Example Gallery	<u>MK</u>				
Features	<u>MK</u>				
Testing	<b>Author Initials</b> , Responsibility decreasing from left to right				
Test-Code (Pytest)	<u>MK</u>				
Continuous Integration	<u>MK</u>				

**Explanations:** All files are available on GitHub (<https://github.com/markur4/plotastic>) and installable via pypi.com. Documentations are found in the Readme, including example gallery and feature explanation. Software tests was written using pytest. Coverage of code by tests is reviewable with codecov (<https://app.codecov.io/gh/markur4/plotastic>). Continuous Integration is implemented using GitHub actions.

#### Manuscript 3: Research Letter (published)

Daniela Simone Maichl, Julius Arthur Kirner, Susanne Beck, Wen-Hui Cheng, Melanie Krug, Martin Kuric, Carsten Patrick Ade, Thorsten Bischler, Franz Jakob, Dirk Hose, Anja Seckinger, Regina Ebert & Franziska Jundt (2023): Identification of NOTCH-driven matrisome-associated genes as prognostic indicators of multiple myeloma patient survival, **Blood Cancer Journal** **13:134**

Figure	<b>Author Initials</b> , Responsibility decreasing from left to right				
1 a	Daniela Simone			Susanne Beck	
1 b	Daniela Simone			Susanne Beck	
1 c	Daniela Simone			Susanne Beck	<u>MK</u>
1 d	Daniela Simone			Susanne Beck	<u>MK</u>
Table	<b>Author Initials</b> , Responsibility decreasing from left to right				
1	Daniela Simone				

**Explanations:** Martin Kuric plotted multidimensional diagrams using python and fine-adjusted them using professional design software (Affinity Publisher, Serif Ltd).

#### Manuscript 4: Research Paper (under peer-review)

Wyonna Rindt, Melanie Krug, Shuntaro Yamada, Franziska Sennefelder, Louisa Belz, Wen-Hui Cheng, Azeem Muhammad, Martin Kuric (MK), Marietheres Evers, Ellen Leich, Tanja Nicole Hartmann, Ana Rita Pereira, Marietta Hermann, Jan Hansmann, Mohammed Ahmed Yassin, Kamal Mustafa, Regina Ebert, and Franziska Jundt (2024): A 3D bioreactor model to study osteocyte differentiation and mechanobiology under perfusion and compressive mechanical loading, **Acta Biomaterialia**

Figure	<b>Author Initials</b> , Responsibility decreasing from left to right				
1	Wyonna Rindt				<u>MK</u>
2	Wyonna Rindt				
3	Wyonna Rindt				
4	Wyonna Rindt				
5	Wyonna Rindt				<u>MK</u>
6	Wyonna Rindt				<u>MK</u>
7	Wyonna Rindt				<u>MK</u>

**Explanations:** Martin Kuric contributed by counseling on experimental procedures and data analysis, such as quantifying normalized fluorescence intensity of immunohistochemistry and qPCR.

**Manuscript 5: Research Paper (under revision)**

Marietta Herrmann, Jutta Schneidereit, Susanne Wiesner, Martin Kuric (MK), Maximilian Rudert, Martin Lüdemann, Mugdha Srivastava, Norbert Schütze, Regina Ebert, Denitsa Docheva, Franz Jakob (2024): Peripheral blood cells enriched by adhesion to CYR61 are heterogenous myeloid modulators of tissue regeneration with early endothelial progenitor characteristics, **European Cells and Materials**

<b>Figure</b>	<b>Author Initials</b> , Responsibility decreasing from left to right				
1	Marietta Herrmann				
2	Marietta Herrmann				
3	Marietta Herrmann				
4	Marietta Herrmann				
5	Marietta Herrmann				
6	Marietta Herrmann				
7	Marietta Herrmann				<u>MK</u>

**Explanations:** Martin Kuric scanned osteogenically differentiated MSCs in Fig. 7 for quantification of alizarin red staining.

**Manuscript 6: Research Letter (published)**

Marietheres Evers, Martin Schreder, Thorsten Stühmer, Franziska Jundt, Regina Ebert, Tanja Nicole Hartmann, Michael Altenbuchinger, Martina Rudelius, Martin Kuric (MK), Wyonna Darleen Rindt, Torsten Steinbrunn, Christian Langer, Sofia Catalina Heredia-Guerrero, Hermann Einsele, Ralf Christian Bargou, Andreas Rosenwald, Ellen Leich (2023): Prognostic value of extracellular matrix gene mutations and expression in multiple myeloma, **Blood Cancer J.** 13(1):43

<b>Figure</b>	<b>Author Initials</b> , Responsibility decreasing from left to right				
1	Marietheres Evers				
2	Marietheres Evers				

**Explanations:** Martin Kuric contributed indirectly through counseling and feedback on submitted manuscript.

I also confirm my primary supervisor's acceptance.

Doctoral Researcher's Name

Date

Place

Signature

## Affidavit

I hereby confirm that my thesis entitled "Development and Semi-Automated Analysis of an in vitro Model for Myeloma Cells Interacting with Mesenchymal Stromal Cells" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg  
Place, Date

Signature

## Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation "Entwicklung und semi-automatisierte Analyse eines in vitro-Modells für Myelomzellen in Interaktion mit mesenchymalen Stromazellen" eigenständig, d.h. insbesondere selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg  
Ort, Datum

Unterschrift

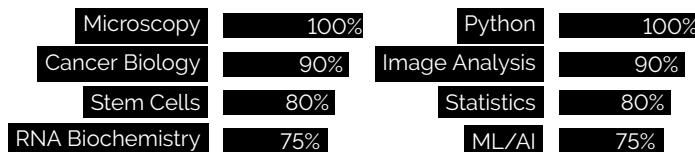
# MARTIN KURIC

Cell Biologist | Data Scientist



## WHO AM I?

As a cancer cell biologist with a strong passion for data analysis and machine learning, I am seeking a position where I can utilize my creativity to automate tasks, solve complex problems or handle big data.



## SELECTED PROJECTS

2024	<b>Python Software “ImageP”</b> Accelerates batch processing of images of different sizes and types by >100%. <code>numpy</code> / <code>skimage</code> / <code>scipy</code>	<a href="#">GitHub Repository</a> ⚡
2020-2024	<b>Python Software “plotastic”</b> Published a statistical library that self-configures based on intuitive plotting parameters. <code>pandas</code> / <code>matplotlib</code> / <code>pingouin</code> / <code>seaborn</code>	<a href="#">Journal of Open Source Software</a>   <a href="#">GitHub Repository</a> ⚡
2018-2024	<b>Cancer Research Project</b> Worked in a team with up to three technical assistants and published a list of genes with relevance for survival of myeloma patients (under peer-review). <code>Time-Lapse Microscopy</code> / <code>RNAseq</code> / <code>Analysis of Patient Survival</code>	<a href="#">Journal: Cancer Research Communications</a>
26.05.2022	<b>Deep-Learning Assisted Image Cytometry</b> Measurement of per-cell parameters from large automated microscopy scans. <code>Convolutional Neural Networks</code> / <code>Image Segmentation</code>	<a href="#">Poster at "Achilles Conference"</a>

## EDUCATION

28.01.2019 – 2024	<b>Dr. rer. nat. in Biomedicine</b> Research focus: Dissemination of multiple myeloma & mesenchymal stromal cell interactions	<b>Prof. Dr. Regina Ebert</b>   University of Würzburg
01.04.2017 – 2024 parallel to M.Sc. & PhD	<b>Elite Biological Physics</b> Interdisciplinary & international study program for exceptional students of physics or biology.	<b>University of Bayreuth</b>
01.10.15 – 15.08.18	<b>M.Sc. in Biochemistry &amp; Molecular Biology</b> Research focus: RNA biochemistry, small RNAseq, stem cells & piRNAs in <i>S. mediterranea</i>	<b>Prof. Dr. Claus-D. Kuhn</b>   University of Bayreuth
01.10.12 – 14.12.15	<b>B.Sc. in Biochemistry</b> Research focus: Cell biology, mitochondrial inheritance in <i>S. cerevisiae</i>	<b>Prof. Dr. Benedikt Westermann</b>   University of Bayreuth

## LANGUAGES

German, English - C2  
Slovakian - passive  
French, Spanish - A2

## SOFT SKILLS

Quality Management  
Project Management  
Violent Free Communication

## HOBBIES

Coding - Python  
Music - Piano & Guitar  
Gym - Lift. Grow. Repeat

Würzburg

05.03.2024

Location

Date

Martin Kuric

Signature