
Preisverleihung 2018

# STIFTUNG PROFESSOR DR. MAX CLOËTTA

Heft Nr. 46

**Prof. Dr. Timm Schroeder**

«Long-term single-cell quantification: New tools for old questions»

**Prof. Dr. Johanna Joyce**

«Exploring and Therapeutically Exploiting the Tumor Microenvironment»

# STIFTUNG
# PROFESSOR DR. MAX CLOËTTA

## *fünfundvierzigste Preisverleihung*

9. November 2018
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Heft Nr. 46 der Schriftenreihe

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INHALT

# VORWORT

*Prof. Dr. Fritjof Helmchen*

So wie Sterne in einer Galaxie erst entstehen, sich dann umwandeln und schliesslich sterben, so durchlaufen Zellen in unserem Körper einen Lebensweg von ihrer Geburt bis zu ihrem Tod beziehungsweise ihrer Teilung. Das Schicksal der Zellen wird dabei sowohl von den molekularen Prozessen und Signalkaskaden innerhalb der Zelle gelenkt als auch durch den Austausch und die Wechselwirkungen mit den umgebenden Zellen. Innere wie äussere Faktoren bestimmen zum Beispiel wie aus einer Stammzelle spezialisierte Zellen hervorgehen, die sich normal entwickeln und zur gesunden Funktion eines Gewebes beitragen, aber auch wie sich Krebszellen heranbilden, im Körper ausbreiten, festsetzen und zerstörerisch verhalten. Diejenigen Prozesse im Detail aufzuklären, die der fortschreitenden Entwicklung von Zellen und ihren Schicksalsentscheidungen zugrunde liegen, Methoden zu entwickeln, die diese Prozesse messbar, sichtbar und manipulierbar machen, und schliesslich die neu gewonnenen Erkenntnissen in innovative Therapieansätze zur Behandlung von Krankheiten umzusetzen, dies sind Grundpfeiler der Forschung der beiden diesjährigen Cloëtta-Preisträger.

**Prof. Dr. Timm Schroeder** konzentriert sich dabei in seiner Forschung besonders auf die enorme «innere» Komplexität der molekularen Netzwerke, die die Entwicklung von Stammzellen steuern. Durch bahnbrechende methodische Entwicklungen, die es ermöglichen, mit Filmaufnahmen einzelne fluoreszenzmarkierte Zellen bei ihrer Teilung und der Erzeugung von Nachkommenschaft über lange Zeiträume zu verfolgen, gelang es ihm die Aktivität von entscheidenden Signal- und Kontrollmolekülen zu messen und zu analysieren. Dadurch konnten wichtige neue Erkenntnisse über verschiedene Arten von Stammzellen gewonnen werden. Die dabei gesammelten grossen Datenmengen erfordern anspruchsvolle Methoden der Bildverarbeitung und Bioinformatik.

**Mit Prof. Dr. Johanna Joyce** wird als zweite Preisträgerin eine herausragende Krebsforscherin ausgezeichnet, die sich insbesondere für die «äussere» Komplexität der Wechselwirkungen von Krebszellen mit ihrer

unmittelbaren Umgebung interessiert. Diese «Mikro-Umgebung» besteht aus einer Vielzahl unterschiedlichster Zelltypen, die durch ihr kompliziertes Zusammenwirken entscheidenden Einfluss darauf haben, ob Krebszellen sich weiter ausbreiten oder in Schach gehalten und zurückgedrängt werden können. Eine verbesserte Kenntnis der Nische, in der sich Krebszellen aufhalten, kann daher zu neuen Ideen bezüglich therapeutischer Ansätze führen, wie Prof. Joyce am Beispiel von Hirntumoren eindrucksvoll zeigen konnte.

Im Grossen wie im Kleinen ist die Welt dynamisch und komplex, auf kosmischer Skala im Weltall, aber auch im Mikrokosmos der biologischen Zellverbände. Die Anerkennung dieser Komplexität und gleichzeitig die Neugier, Ordnungsmuster in ihr aufzudecken, sind Grundlage jeglicher Wissenschaft.

Mit der Verleihung des Cloëtta-Preises wird die beeindruckende wissenschaftliche Leistung von Prof. Dr. Johanna Joyce und Prof. Dr. Timm Schroeder gewürdigt. Die Stiftung Prof. Dr. Max Cloëtta freut sich, die Preisträger am 9. November 2018 in Lausanne zu feiern.

*Brigitt Küttel*
*Geschäftsführerin*

*Stiftungsrat*

Die Gemeinnützige Hertie Stiftung mit Sitz in Berlin fokussiert ihre Fördertätigkeit auf zwei Gebiete: «Gehirn erforschen» und «Demokratie stärken». Eine äusserst interessante Spannweite – mit einem persönlichen Bezug zur Stiftung Prof. Dr. Max Cloëtta.

Seit 2006 vergibt die Hertie Stiftung rund alle zwei Jahre eine Senior-Forschungsprofessur, mit der sie das Lebenswerk verdienter Wissenschafter ehrt und diesen ermöglicht, auch über die ordentliche Pensionierung hinaus weiter tätig zu sein.

Als bislang einziger Forscher ausserhalb Deutschlands erhielt Prof. Adriano Fontana im Jahr 2009 eine Senior-Forschungsprofessur dieser verdienten Stiftung zugesprochen, rund ein Jahr nach seiner Wahl in unseren Stiftungsrat. Er gehöre, so die Hertie Stiftung, als internationaler Experte im Bereich der Neuro- und Infektionsimmunologie zu den 100 weltweit am häufigsten zitierten Immunologen, der unter anderem 1999 mit dem deutschen Aids-Forschungspreis und 1997 dem Hoechst Marion Roussel-Multiple-Sklerose-Forschungspreis ausgezeichnet wurde. Die Stiftung Prof. Dr. Max Cloëtta ehrte ihn bereits 1993 mit ihrem Preis.

Die Hertie-Professur von Professor Fontana endete im Juni 2017, und ein halbes Jahr später, auf Ende 2017, gab er das Präsidium unserer Stiftung ab. Wir freuen uns, dass er uns aber als Mitglied des wissenschaftlichen Ausschusses sein immenses Wissen auch weiterhin zur Verfügung stellt, und danken ihm sehr herzlich für seinen grossen, immer sorgfältigen und wertschätzenden Einsatz für die Stiftung.

Mit Professor Fritjof Helmchen, Professor für Neurowissenschaften und Co-Direktor des Instituts für Hirnforschung an der Universität Zürich, hat auf den 1. Januar 2018 ein weiterer hervorragender Forscher und ehemaliger Cloëtta-Preisträger (2015) das Präsidium übernommen. Wir freuen uns sehr auf die weitere Zusammenarbeit.

Auf Ende 2018 muss die Stiftung ihr amtsältestes Stiftungsratsmitglied verabschieden. Dr. Hans Bollmann wurde im Februar 1997 in den Stiftungsrat gewählt und amtete seit 2009 als dessen Vizepräsident. 22 Jahre unterstützte er die Stiftung als selbstständiger Rechtsanwalt mit seinem profunden juristischen Wissen, seiner grossen Erfahrung in der Verwaltung der der Stiftung anvertrauten Vermögen und seinem feinen Humor. Für seine grossen Verdienste ist ihm die Stiftung ausserordentlich dankbar.

Die Stiftung schätzt sich glücklich, dass die Nachfolge von Dr. Bollmann nahtlos geregelt werden konnte. Herzlich im Stiftungsrat willkommen heissen wir Rechtsanwalt Martin Wipfli. Seit dem Erlangen des Anwaltspatents war er in verschiedenen Funktionen im Bereich Steuerberatung tätig, bis er 1998 zusammen mit Partnern eine Firma für Steuerberatung, Unternehmens- und Rechtsberatung sowie Vermögensverwaltung gründete.

*Cloëtta-Preis*

Stiftungsrat und Geschäftsstelle freuen sich, auch dieses Jahr zwei herausragende Forscher mit dem Cloëtta-Preis auszeichnen zu können: Der erste Preis geht an Prof. Dr. Timm Schroeder, Leiter des Departments of Biosystems Science and Engineering der ETH Zürich, Basel. Mit Frau Prof. Dr. Johanna Joyce wird eine herausragende Wissenschafterin der Universität Lausanne und des Ludwig Instituts für Krebsforschung in Lausanne geehrt. Wir freuen uns auf spannende Vorträge und gratulieren den beiden Preisträgern herzlich.

*Forschungsstellen*

Die Research Positions der Stiftung Prof. Dr. Max Cloëtta sind für den akademischen Mittelbau in der Schweiz von grosser Bedeutung. Finanziert werden Stellen an schweizerischen Hochschulen, Kliniken oder Instituten für bereits ausgebildete und selbstständig arbeitende Forscherinnen und Forscher bis max. 40 Jahre. Mit diesem Programm will die Stiftung einem Mangel an Forschernachwuchs in der Schweiz entgegenwirken und den Stelleninhabern helfen, die manchmal nicht einfache Phase bis zur Beru-

fung auf eine ordentliche Professur zu überbrücken. Die Stipendien werden alle zwei Jahre vergeben, das nächste Mal wieder 2020.

2018 finanzierte die Stiftung Prof. Dr. Max Cloëtta folgende Forschende an Schweizer Universitäten mit fünfjähriger Unterstützungsperiode:

**Dr. Mathias Hauri-Hohl,** 1975, Universitäts-Kinderspital Zürich, Abteilung Stammzellentransplantation. Projekt: «Improving T-Cell Reconstitution and Enhancing Central Tolerance Mechanism in Hematopoietic Stem Cell Transplantation». Unterstützungsdauer: 1.1.2016–31.5.2021 (Sistierung 1.4.2018–31.8.2018)

**Dr. Alexandre Theocharides,** 1975, Universitätsspital Zürich, Klinik für Hämatologie. Projekt: «The Role of Cell-Extrinsic Factors in Hematopoietic Stem Cell Malignancies». Unterstützungsdauer: 1.6.2015–31.5.2020

**Dr. Wei Lynn Wong,** 1976, Universität Zürich, Institut für experimentelle Immunologie. Projekt: «The Role of IAPs and RIPKs in Hematopoiesis and Disease, Specifically in Tumor Formation and Metastasis». Unterstützungsdauer: 1.1.2016–31.12.2020

mit dreieinhalbjähriger Unterstützungsdauer:

**Dr. Grégory Verdeil,** 1976, Universität Lausanne, Abteilung für fundamentale Onkologie und Ludwig Cancer Centre. Projekt: «Finding and characterizing new targets to overcome T cell exhaustion for immunotherapy of cancer». Unterstützungsdauer: 1.8.2017–31.1.2021

**Dr. Britta Maurer,** 1976, Universitätsspital Zürich, Klinik für Rheumatologie und Zentrum für experimentelle Rheumatologie. Projekt: «Early diagnosis in disease monitoring of systemic autoimmune disorders with molecular targeted imaging». Unterstützungsdauer: 1.4.2018–30.9.2021

*Klinische Medizin Plus*

Seit 2010 vergibt die Stiftung Prof. Dr. Max Cloëtta in Zusammenarbeit mit der Uniscientia Stiftung, Vaduz, Stipendien «Klinische Medizin Plus». Medizinerinnen und Medizinern werden während oder unmittel-

bar nach Abschluss ihrer Facharztausbildung Stipendien von drei bis maximal zwölf Monaten für die Absolvierung einer Spezialausbildung an einer renommierten, vornehmlich ausländischen Institution ausgerichtet. Die Uniscientia Stiftung finanziert das Programm, der Stiftung Prof. Dr. Max Cloëtta obliegt die wissenschaftliche Verantwortung. Ende 2015 wurde der Vertrag über diese erfolgreiche Zusammenarbeit um weitere drei Jahre bis und mit 2018 verlängert.

2018 kommen folgende Medizinerinnen und Mediziner in den Genuss eines Stipendiums:

**Dr. med. Angéline Adam,** 1979, Postdoc am Department of Population Health, New York University School of Medicine. Projekt: Training in health services research methods focusing on unhealthy alcohol use screening and intervention approaches in primary care. Guest Institution: New York University School of Medicine, Section on Tobacco, Alcohol, and Drug Use, USA, 1.7.2018–31.12.2018

**Dr. med. Eleonora Seelig,** 1980, Clinical research fellow am Wellcome Trust MRC Institute of Metbolic Science, Addenbrooke's Hospital, Stanford UK. Projekt: Effects of metformin on the central nervous system. Guest Institution: Wellcome Trust MRC Institute of Metbolic Science, Addenbrooke's Hospital, Stanford UK, 1.4.2018–31.7.2018

**Dr. med. Arseny Sokolov,** 1986, Clinical and research fellow, Universitätsspital Lausanne. Projekt: Technology-based therapeutic approaches for cognitive neurology. Guest Institution: University of California, San Francisco, USA 1.9.17–31.8.18

**Dr. med. Marian Severin Wettstein,** 1989, Universitätsspital Zürich, Klinik für Urologie. Projekt: Underutilization of re-resection in T1 bladder cancer and the impact on oncological outcomes. Guest Institution: Princes Margaret Cancer Center, Toronto, CAN 11.1.2018–31.12.2018

Zusammen mit dem Team der Geschäftsstelle freue ich mich, die Stiftung Prof. Dr. Max Cloëtta auch weiterhin in eine aktive Zukunft für die Förderung der medizinischen Forschung in der Schweiz begleiten zu dürfen. Dem Stiftungsrat, der Uniscientia Stiftung, unseren Stipendiatinnen und Stipendiaten und den medizinischen Fakultäten danken wir herzlich für die jederzeit sehr angenehme Zusammenarbeit.

THE CLOËTTA PRIZE 2018
IS AWARDED TO

PROFESSOR

# TIMM SCHROEDER

BORN IN 1970 IN PRETORIA, SOUTH AFRICA
DEPARTMENT OF BIOSYSTEMS SCIENCE AND ENGINEERING
ETH ZURICH, BASEL

FOR HIS GROUND-BREAKING CONTRIBUTIONS
TO RESEARCH AND TECHNOLOGY DEVELOPMENT IN THE FIELD
OF MOLECULAR CONTROL OF MAMMALIAN STEM CELL FATE
AT THE INTERFACE OF BIOLOGY, MEDICINE AND INFORMATICS

LAUSANNE, 9TH NOVEMBER 2018

IN THE NAME OF THE FOUNDATION BOARD:

THE PRESIDENT

THE VICE PRESIDENT

A MEMBER

Timm Schroeder

CURRICULUM VITAE


| | |
|---|---|
| Name: | Timm Schroeder<br>born 3. September 1970 in Pretoria, South Africa<br>German, married |
| Languages: | man: German, English, French, Japanese, Latin<br>machine: VB, VB.NET, VBA, LUA, C++ |
| Position: | Professor of Cell Systems Dynamics, Department of Biosystems Science and Engineering, ETH Zürich, Mattenstr. 26, 4058 Basel, Switzerland<br>timm.schroeder@bsse.ethz.ch |


**Career**

| | |
|---|---|
| 2013– | **Full Professor, Cell Systems Dynamics**<br>Department of Biosystems Science and Engineering **(D-BSSE)** Swiss Federal Institute of Technology **(ETH) Zürich,** Basel, Switzerland<br>2015–17/2017–: Deputy-/**Head of Department** D-BSSE |
| 2011–13 | Director, research unit Stem Cell Dynamics, **Helmholtz Zentrum München** – German Research Center for Environmental Health, Neuherberg, Germany |
| 2004–11 | Principal Investigator (tenured 2009), Research group «Hematopoiesis», Institute of Stem Cell Research, **Helmholtz Zentrum München**<br>2006–13: Deputy Director, Institute of Stem Cell Research |
| 2002–04 | Visiting Researcher, Group for Stem Cell Biology, **RIKEN Center for Developmental Biology,** Kobe, Japan |
| 2000–02 | PostDoc, **GSF, Munich,** Germany<br>2001 and 2002: Research visits in Kyoto (groups Tasuku Honjo and Shinichi Nishikawa) and Harvard (group Dan Tenen) Universities, Japan and USA |

| | |
|---|---|
| 1997–2000 | Dr. rer. nat. thesis *(summa cum laude 2001),* Chair of Genetics, Friedrich Alexander **University Erlangen** and Institute for Clinical Molecular Biology and Tumour Genetics, **GSF, Munich,** Germany<br>1999 and 2000 Research visits, Kyoto University, Japan |
| 1991–97 | Studies in Biology, Friedrich Alexander **University Erlangen,** Germany |
| 1990-91 | Mandatory civilian service, research technician in cancer research, University hospital Grosshadern, Munich, Germany |

***Research awards***

| | |
|---|---|
| 2018 | Cloëtta Prize, Professor Dr. Max Cloëtta Foundation, Switzerland |
| 2017 | Erwin Schrödinger Prize – The Stifterverband Science Award for interdisciplinary research by the German Helmholtz Association, shared with Drs. Theis, Marr (Germany) and Haghverdi (UK) |
| 2012 | McCulloch&Till Award, International Society for Hematology and Stem Cells |
| 2002 | GSF Ph.D. student of the year award |
| 2001 | New Investigator Award, International Society for Experimental Hematology |

- **Selected committee memberships**
- International Society for Experimental Hematology (ISEH): Vice President/President Elect/**President/**Immediate Past President and Executive Committee. 2014–18
- Personalized Health Allianz Zürich - Basel Governing Board. 2017-
- European Hematology Association (EHA) Research Committee 2015–
- Swiss Stem Cell Network (SSCN) Steering Committee 2014–
- Basel Stem Cell Network (BSCN), Switzerland Steering Committee 2013–

# SELECTED PUBLICATIONS


1. Coutu DL*, Kokkaliaris KD, Kunz L and **Schroeder T* (2018).** Multicolor quantitative confocal imaging cytometry. ***Nature Methods*** 15, 39–46.

2. Coutu DL, Kokkaliaris KD, Kunz L and **Schroeder T (2017).** Three-dimensional map of nonhematopoietic bone and bone-marrow cells and molecules. ***Nature Biotechnology*** 35, 1202–1210.

3. Hoppe PS, Schwarzfischer M, Loeffler D, Kokkaliaris KD, Hilsenbeck O, Moritz N, Endele M, Filipczyk A, Gambardella A, Ahmed N, Etzrodt M, Coutu DL, Rieger MA, Marr C, Strasser MK, Schauberger B, Burtscher I, Ermakova O, Bürger A, Lickert H, Nerlov C, Theis FJ and **Schroeder T (2016).** Early myeloid lineage choice is not initiated by random PU.1 to GATA1 protein ratios. ***Nature*** 535, 299–302.

4. Hilsenbeck O, Schwarzfischer M, Skylaki S, Schauberger B, Hoppe PS, Loeffler D, Kokkaliaris KD, Hastreiter S, Skylaki E, Filipczyk A, Strasser M, Buggenthin F, Feigelman JS, Krumsiek J, van den Berg AJJ, Endele M, Etzrodt M, Marr C, Theis FJ* and **Schroeder T* (2016).** Software tools for single-cell tracking and quantification of cellular and molecular properties. ***Nature Biotechnology*** 34, 703–706.

5. Filipczyk A, Marr C, Hastreiter S, Feigelman J, Schwarzfischer M, Hoppe PS, Loeffler D, Kokkaliaris KD, Endele M, Schauberger B, Hilsenbeck O, Skylaki S, Hasenauer J, Anastassiadis K, Theis FJ* and **Schroeder T* (2015).** Network plasticity of pluripotency transcription factors in embryonic stem cells. ***Nature Cell Biology*** 17, 1235–1246.

6. Filipczyk A, Gkatzis K, Fu J, Hoppe PS, Lickert H, Anastassiadis K* and **Schroeder T* (2013).** Biallelic expression of nanog protein in mouse embryonic stem cells. ***Cell Stem Cell*** 13, 12–13.

7. Eilken HM, Nishikawa S-I and **Schroeder T (2009).** Continuous single-cell imaging of blood generation from haemogenic endothelium. ***Nature*** 457, 896–900.

8. Rieger MA, Hoppe PS, Smejkal BM, Eitelhuber AC and **Schroeder T (2009).** Hematopoietic cytokines can instruct lineage choice. ***Science*** 325, 217–218.

9. **Schroeder T (2008).** Imaging stem-cell-driven regeneration in mammals. ***Nature*** 453, 345–351.

10. **Schroeder T (2005).** Tracking hematopoiesis at the single cell level. ***Annals of the New York Academy of Sciences*** 1044, 201–209.

# MOLECULAR STEM CELL FATE CONTROL: QUANTIFICATION OF CELLULAR AND MOLECULAR DYNAMICS AT THE SINGLE-CELL LEVEL

*Timm Schroeder*
*Department of Biosystems Science and Engineering, ETH Zurich, Basel, Switzerland*

*Summary*

**Despite decades-long intensive research, surprisingly many long-standing questions in stem cell research remain disputed. One major reason is the fact that we usually analyze only populations of cells, rather than individual cells, and at very few time points of an experiment, rather than continuously. My group therefore develops imaging systems including the required software to long-term image, segment and track individual cells, and to quantify e.g. divisional history, position, interaction, and protein expression or activity of all observed individual cells over many generations. Dedicated software, machine learning and computational modeling enable data acquisition, curation and analysis. Custom-made microfluidics devices improve cell handling, observation, dynamic manipulation and molecular analysis. The resulting continuous single-cell data is used for analyzing the dynamics, interplay and functions of signaling pathway and transcription factor networks in controlling hematopoietic, pluripotent, skeletal and neural stem cell fate decisions. After the first 1.5 decades of my independent research group, I here review these technological developments, and some of the long-standing biological questions in stem and progenitor cell biology they have contributed to answer.**

*Introduction: The need for long-term single-cell quantification of cellular and molecular dynamics*

How do cells behave to generate and regenerate healthy tissues? What has changed in disease? How do molecular machineries control these cell behaviors, and how can we manipulate them to control cell fates for therapy? These questions are at the core of most biological and biomedical

research projects. However, as discussed for some examples below, surprisingly many obvious questions remain without satisfying and accepted answers despite decades of intensive research. This is certainly the case in hematopoietic stem cell biology, the classical mammalian stem cell system. Many of the concepts, terms and questions of stem cell research have been defined in this system since the middle of last century, but remain unresolved controversies. During my doctoral thesis, where I worked on the possible effects of Notch activation on hematopoietic progenitor cell fates, I realized that many basic conceptual questions in the field remain disputed. I felt that the lack of adequate technologies for quantifying the dynamics of cellular and molecular behaviors is one important reason for this lack of satisfying answers.

Our blood system produces millions of cells every second of our life. As in other cell systems, the number and type of cells produced must be tightly regulated, and also adapted over time to changing needs. Failure to produce the right number of the right cells at the right time and location can quickly lead to deadly diseases like anemia or leukemia. It seems obvious that the first step in analyzing the molecular control of the underlying cell fate choices (Fig. 1) must be to know what cells actually do in health and disease, and how cell fate choices change upon molecular mutation and manipulation.

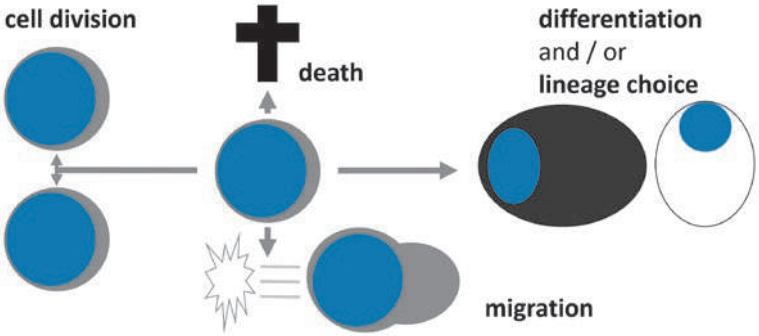


***Figure 1: Cell fate options of multipotent stem and progenitor cells.*** *These are chosen in close reciprocal dynamic interaction with the microenvironment of individual cells.*

However, surprisingly, we typically lack the precise knowledge about what our cells of interest really do. This is because cell and molecular behavior is usually analyzed as population averages and / or by snapshot analyses – and not continuously at the single-cell level. This snapshot average approach is dangerous, since it will mask properties and behaviors of individual cells, and typically leaves too much room for interpretation when generating conclusions from primary data (Schroeder, Annals Of The New York Academy Of Sciences 2005; Nature 2008; Nature Methods 2011; Etzrodt et al., Cell Stem Cell 2014; Hoppe et al., Nature Cell Biology 2014; Skylaki et al., Nature Biotechnology 2016). While the need for single cell analyses has long been recognized, and commercial off-the-shelf solutions like FACS, colony assays and more recently single-cell sequencing are increasingly available and used, the continuous live single-cell quantification of dynamics remains challenging and missing in most studies. As illustrated in Figure 2, even simple questions like *"How did one cell generate four cells?"* allow many competing interpretations about the underlying cell fate choices, even when analyzed at the single-cell level but with data only from the start and end of the experiment (Fig. 2).

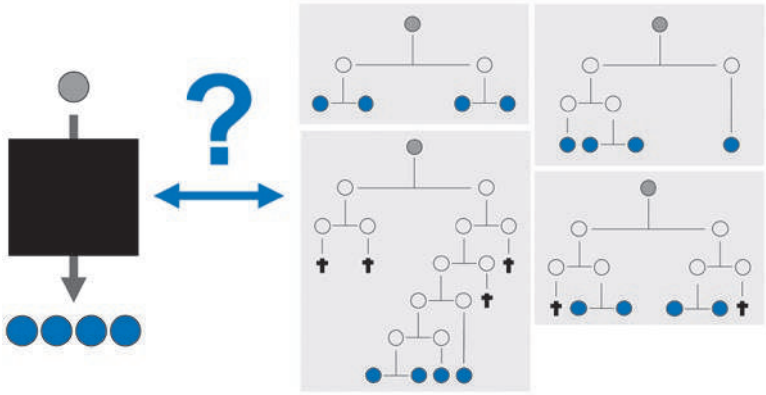

***Figure 2: Continuous single-cell fate quantification is required to understand the cellular dynamics underlying normal and pathological phenotypes.***

As shown in this example, snapshot data allows very different assumptions about the involved cell fate choices which are all compatible with the measured data. For example, half of the cells might have died, or none of the cells could have died. Obviously, the assumed reasons e.g. for normal or pathological tissue (re)generation, possible interventions in disease, and the molecular machineries picked to the analyze for many years would fundamentally differ depending on whether one picks the first or second (of many possible additional) interpretation.

The same not only holds true for quantifying cell fates, but also for analyzing the dynamics of the molecules involved in their control. Depending on how frequent and long the e.g. expression, activation or subcellular location of molecules of interest is analyzed, one will come to very different conclusions about possible dynamics like oscillations, step functions or transient peaks. However, this knowledge is crucial to understand how the molecular machineries leading to normal or diseased cell behavior are wired and implemented, and how to manipulate them for therapy. Again, typical snapshot average data is too ambiguous and usually allows different competing interpretations (Schroeder, Nature Methods 2011; Etzrodt et al., Cell Stem Cell 2014; Skylaki et al., Nature Biotechnology 2016).

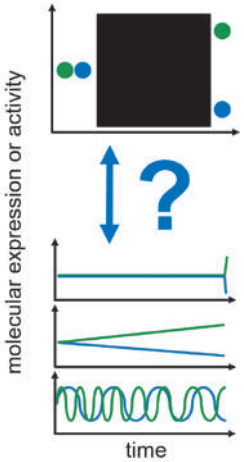


***Figure 3: Continuous single-cell molecular quantification is required to understand the molecular dynamics underlying cell fate control.***

Continuous single-cell quantification of molecular dynamics is therefore essential. This often requires much higher frequencies of image acquisition to temporally resolve fast molecular dynamics, posing even bigger technical challenges to data acquisition and analysis. This would be balanced by shorter required imaging durations, since the molecular events are much shorter than cell fate choices – minutes to hours versus days. However, since the functional relevance of specific molecular dynamics in individual cells only becomes clear when being able to link them to the future cell fate decisions of the same cell or its progeny, the combination of both the high-frequency shorter molecular imaging at the beginning of the experiment, and the following lower-frequency long-term cell fate imaging over days is required.

With this comprehensive novel kind of data, the confusing heterogeneous effect of e.g. signaling inputs on cell fate choices of individual cells, or possibly on the same cell with changing intracellular molecular states over time (Fig. 4) can likely be better understood.

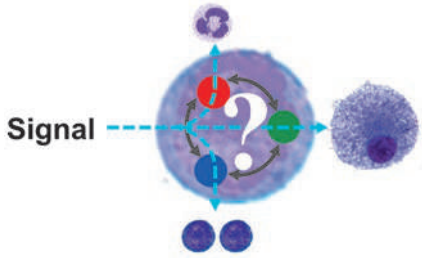


***Figure 4: Changing or cycling intracellular molecular states, e.g. due to cell cycle progression, could lead to changed modulation of signaling inputs and thus altered or cycling effects on cell fate of the same signaling pathway in the same cell over time.***

In conclusion, quantification of cell fate choices and molecular dynamics at the single-cell level and continuously over time is essential for a precise understanding of the cellular and molecular mechanisms underlying health and disease. Here, I will discuss some of the technologies developed in my group to enable these quantifications, and how we used them to try to answer some of the long-standing disputes in the field.

The purpose of this manuscript is the review of some of my own group's work honored with the Cloëtta Prize, not a comprehensive review of the literature. The listed references are therefore restricted to my own publications. For a more comprehensive and balanced representation of the relevant literature, I refer to the references in my listed publications.

*Development of technologies for long-term single-cell quantification of cellular and molecular dynamics*

For the reasons discussed above, we develop novel technologies allowing the continuous long-term imaging, single-cell tracking and quantification of cells.

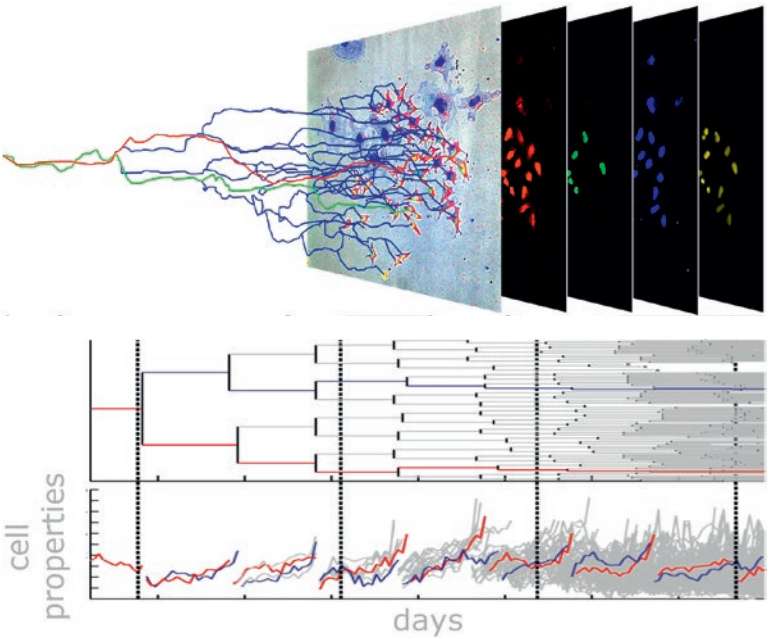


***Figure 5: Long-term imaging, segmentation and tracking enables the single-cell quantification of cellular and molecular dynamics over up to weeks.*** *Adapted from (Hilsenbeck et al., Nature Biotechnology 2016; Skylaki et al., Nature Biotechnology 2016).*

Mammalian cells, including rare primary stem and progenitor cells, are purified, cultured, manipulated and observed by time-lapse video microscopy over up to weeks. The mobility of cells requires high temporal imaging frequency to prevent the confounding of cell identities when tracking individual cells. This requirement of reliably taking frequent pictures over long periods of time brings many technological challenges. The imaging hardware has to be much more robust and reliable than for normal imaging experiments. We typically take around one picture per second, which means that mechanical parts like shutters "click" one million times in less than two weeks, and thus sometimes even within one single experiment. The typical guaranteed hardware cycle times for two-year warrantee periods are thus used within days to weeks. Mechanic wear and tear are not the only problem. Given that we have to observe our cells of interest with high temporal resolution to not lose track of their identities, failures of acquiring even individual pictures can render a movie of 10 000s of pictures useless. While failing e.g. every 100$^{th}$ image acquisition when manually taking pictures is not problematic – one can just click a button again – it is catastrophic for high frequency time-lapse imaging where it would lead to loss of every single experiment conducted. However, while these are challenging problems, they can be solved with the right combination of (usually not off-the-shelf) commercially available hardware.

The biggest challenge is data processing, storage and analysis. Not only are the shear amounts of data scary. The imaging capacity in my laboratory can currently produce about one petabyte of primary data per month. Just the storage (not analysis) of this one month worth of data on the cheapest storage hardware available in academic IT service departments will cost more per year every year than typical research grants pay for annual consumables of individual research projects. More importantly, both, the reliable and efficient acquisition, and the meaningful and statistically sound analysis of this kind and volume and data remains impossible with commercially available software. Still in 2018, and certainly in the early 2000s when I started working on these challenges. Commercially available custom software by reputable software companies for cell tracking in time-lapse data existed then and looked promising. However, after wasting 10 000s of US dollars – my apologies to my

Japanese mentor Shinichi Nishikawa – it turned out that in routine day-to-day use, it was neither a match for the data volumes at hand, nor for the required usability, reliability and specific functionality.


***Figure 6: Software tools for single-cell segmentation (fasTER), tracking (tTt) and quantification (qTfy, XiT) developed in the Schroeder group.*** *All our published software is open-sourced and can be downloaded at www.bsse.ethz.ch/csd/software.html.*

I therefore had to start programming myself, the result of which (tTt, Fig. 6) (Hilsenbeck et al., Nature Biotechnology 2016) has meanwhile been much further developed by contributions of many, and proven useful for many published and ongoing studies in groups on 4 continents. Together with self-programmed software for microscope hardware control, computer vision and machine learning for cell recognition and segmentation (Hilsenbeck et al., Bioinformatics 2017), automated cell tracking, image correction (Schwarzfischer et al., Proceedings Microscopic Image Analysis With Applications In Biology 2011; Buggenthin et al., BMC Bioinformatics 2013; Peng et al., Nature Communications 2017) and quantification (Hilsenbeck et al., Nature Biotechnology 2016), as well as statistical analysis of pedigree structures (Stadler et al., Journal Of Theoretical Biology 2018) and machine learning for high-dimensional pattern recognition and cell fate predictions (Buggenthin et al., Nature Methods 2017), it is now part of a continuously growing software pipeline.

This pipeline enables the long-required continuous long-term single-cell quantification of many dimensions of cellular and molecular properties, dynamics and kinship. For example, divisional history, position, interaction, and protein expression or activity are recorded and quantified for all observed individual cells over many generations (Fig. 5). As discussed above, this is a crucial prerequisite for the improved understanding of

molecular cell fate control (Eilken et al., Nature 2009; Rieger et al., Science 2009; Filipczyk et al., Nature Cell Biology 2015; Hoppe et al., Nature 2016; Kokkaliaris et al., Blood 2016).

Software is a crucial, but not the only required technology required to custom-develop for this approach to work efficiently. All components of a single-cell tracking experiment must be perfectly adjusted to each other, from the cells and molecular reporter and manipulation materials used, over the culture and liquid handling systems at hand, the imaging hard- and software to the data acquisition, storage and analysis pipeline. All these components will have to be adjusted with each new biological question to be analyzed, a process often requiring many iterations of optimizations. One important missing part for us was the lack of commercially available custom micro- or macro-fluidic devices optimized for efficient liquid handling, cell culture and imaging. With the help of our engineering colleagues and facility at the Department of Biosystems Science and Engineering of the ETH Zurich in Basel, we have therefore begun to develop and produce our own custom-made components addressing the specific experimental needs of different biological systems analyzed (Fig.7).

***Figure 7: The flow from computationally aided design of microfluidic chip to the produced real-world device.*** *Adapted from (Dettinger et al., Analytical Chemistry 2018).*

These combined solutions routinely and robustly work in my and other groups. However, they still require dedicated specialists who understand the value of the new kind of generated data to be willing to invest their time. Many challenges remain, and generic one-fits-all solutions do not exist. My disappointing answer to the typical question of interested colleagues "How do I best do these experiments" unfortunately remains (and will likely also remain in many instances in the future) "It depends". Each novel combination of specific biological question, available biological material, reporters and culture system, required imaging frequency, dimensionality and duration, and optical properties of the observed structures will typically need rounds of optimization, and specialists' love and care in acquisition and analysis of data (Skylaki et al., Nature Biotechnology 2016). In many cases, automation of data analysis fails due to the lack of e.g. reliable computer vision solutions, and manual curation, error correction or even analysis and generation remain required. Given the new kind of continuous single-cell quantification and kinship data, the required mathematical tools often have not yet even been developed, let alone implemented into easy to use automated software tools, and a lot of groundwork is still required in this area.

Nevertheless, I am convinced that quantification of behaviors over time, as opposed to states at one timepoint, will be the future also of routine screening approaches e.g. in pharmaceutical industry. Indeed, we have begun to use long-term singe-cell fate quantification for mid-throughput screening for novel extracellular regulators of stem cell self-renewal expressed by their niche. By observing the behavior of individual stem cells in complex co-cultures with stromal cells, and the concurrent manipulation of 50 different candidate genes in the stem cells' environment, we were able to identify more novel regulators in a year than the field had in the previous 20 years of research using the same cell models (Kokkaliaris et al., Blood 2016). This well demonstrates that the conclusions yielded by this continuous observation approach are typically so much more robust and allow insights which would otherwise be missed, that higher investments into the more demanding technological approach will quickly pay off. In particular for recurring problems as in high-throughput screening with standardized cells and questions to be analyzed, the relevant steps can be automated with sufficient reliability. Most of the

current challenges in this area are simply engineering problems which can be solved with sufficient time and money.

Finally, in addition to the single-cell tracking approaches of cultured cells, we have started to also optimize quantitative high-dimensional large-volume 3D imaging in vivo. Through optimization of existing, and development of novel materials, protocols and custom software, large volumes of tissues, e.g. over the total length and width of a full mouse femur in thick sections, can now be imaged with sub-cellular resolution in up to 10 colors and quantitatively analyzed (Fig. 8).

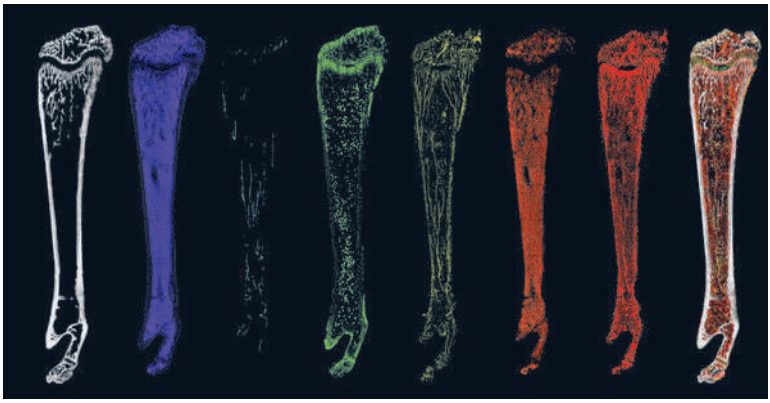

***Figure 8: Large-volume multi-color 3D imaging of bone and bone marrow.*** *A thick section of a full mouse femur imaged for different molecular and cellular components in seven colors is shown (Kunz, Coutu, Kokkaliaris and Schroeder, unpublished).*

Importantly, we developed these approaches for use on standard confocal microscopes available in many research institutions and with precise description of the relevant individual steps to help democratizing large tissue quantitative multicolor cytometry.

Along the same line, all data acquired during development of this technology with a lot of effort, testing hundreds of expensive antibodies, and imaging hundreds of bones over thousands of microscopy hours, was made openly available for the community (Fig. 9) to freely download and use (Coutu et al., Nature Biotechnology 2017).


***Figure 9: Nature Biotechnology cover depicting one view of the central sinus of mouse bone marrow from the voluminous open imaging data published in this issue.*** *Reprinted by permission from Springer Nature, Nature Biotechnology, Three-dimensional map of non-hematopoietic bone and bone-marrow cells and molecules, Daniel L Coutu, Konstantinos D Kokkaliaris, Leo Kunz, Timm Schroeder, Copyright 2017.*

We are now using this approach to better quantify the location e.g. of different hematopoietic and mesenchymal and skeletal stem and progenitor cell populations, their hierarchy, and their possible interactions with their microenvironments in the bone marrow and other tissues.

Most recently, by incorporating antibody-based proximity ligation into the approach, we also succeeded to improve its sensitivity to the single-molecule level (Kunz et al., unpublished). This now allows the precise quantification of the location and concentration of many relevant molecular regulators and their interaction with other molecules, in space and simultaneously in relation to multiple cell types of interest. It will be exciting to see the individual molecular players in their specific locations and in relation to their producing and target cells, as opposed to the diffuse idea of an average even distribution throughout a tissue.

Of course, while this approach yields important quantitative single-cell data, it uses fixed tissue, thus not allowing the quantification of dynamics in live cells. Developing 3D live cell imaging approaches with the required depth, throughput, and importantly the duration to observe cellular processes for longer than a few hours remains an important technological problem for the community to solve in the future.

*Some biological questions solved by long-term single cell quantification*

Developing these approaches sent me on a 1.5 decade long detour and has been an important part of my group's work. However, technology development was always motivated and guided by the need of the biological questions to be solved. Here, I discuss a few of those long-standing questions in the hematopoietic system solved by long-term single cell imaging and quantification.

*Finding the missing link: Hemogenic endothelium caught in the act*

What is the origin of the first blood cells during development, and does hemogenic endothelium exist? This question remained controversial for more than a century.

Since the 1800s, it had been observed that the first blood cells in vertebrate embryos appear next to endothelial cells in all sites of de novo hematopoiesis – in the blood islands of the extraembryonic yolk sac, in the aorta of the aorta-gonad-mesonephros region within the developing embryo, and in the placenta. This led to several competing hypotheses about the specific embryonic cell type differentiating into the first hematopoietic cells. One possible explanation was a common precursor of endothelial and hematopoietic cells, the hemangioblast, which would simultaneously give rise to both cell types (Fig. 10) hence explaining their neighborhood (Hoppe et al., Nature Cell Biology 2014). Alternatively, the first blood cells could be generated from cell types close to, but different from, endothelium, e.g. in the embryonic subaortic mesenchymal patches and then transmigrate the endothelium into blood vessels (Hoppe et al., Nature Cell Biology 2014). The same could be true for cellular sources somewhere in the embryo far away from the first sites of appear-

ance of detectable blood cell numbers, with subsequent migration of the early blood cells to these sites either by the circulation within blood vessels, or by active migration outside the vessels (Tanaka et al., Cell Reports 2014). Finally, another explanation was the existence of hemogenic endothelium. In this case, endothelial cells would first be generated, and a subset would later differentiate into blood (Fig. 10). It would explain why nascent blood cells are found next to endothelium, and why endothelial and early hematopoietic cells share many molecular markers. However, this explanation could also hold true for all other above-mentioned hypotheses.

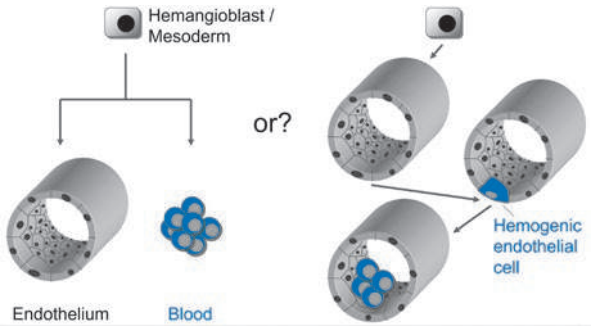


***Figure 10: Possible cellular sources for the first blood cells during embryo-genesis.*** *Possible relationships between endothelium and blood. Left: endothelium and blood are independently created from one progenitor (hemangioblast). Right: blood is generated from specialized hemogenic endothelial cells. The existence of hemogenic endothelium could be proven by continuous long-term single-cell imaging of murine endothelial to hematopoietic transition in mesodermal cells derived from embryonic stem cells or primary embryonic mesoderm (Eilken et al., Nature 2009).*

Why was it so difficult to prove the existence of hemogenic endothelium? Since this process happens within the embryo, in mammals also deep in the uterus, it could never be observed live and at the single-cell level. The available snap-shot data from e.g. fixed and sectioned embryos could not exclude the other hypotheses discussed above as the sole and sufficient explanation. The existence of hemogenic endothelium thus remained disputed until 2009.

We therefore set out to establish a culture system for the relevant developmental processes, which would be optically accessible to be observed by time-lapse imaging. By using a 2-dimensional stromal co-culture system allowing the generation of blood and endothelium (as well as perivascular cells and cardiomyocytes) from mouse mesodermal cells, we could observe their differentiation at the single-cell level for many days. We used mesodermal cells derived either from embryonic stem cells differentiated into mesoderm in vitro, or directly from embryos at day 7.5 post fertilization. The use of embryonic stem cell derived mesoderm enabled the easier generation and use of fluorescent molecular reporter lines for the identification of specific endothelial and blood developmental and functional stages. The use mesoderm from the embryo on the other hand then allowed confirmation of observations with primary material directly from the embryo. By long-term imaging and tracking all progeny of individual mesodermal cells throughout their hemogenic differentiation, we were able to show that they indeed first go through endothelial stages – defined by morphology, molecular and functional markers – before further differentiating into blood cells. This provided prove for the long-disputed existence of hemogenic endothelium (Eilken et al., Nature 2009).

The provided evidence together with 3 simultaneously published studies with supporting evidence from alternative approaches indeed satisfied the field to accept the existence of hemogenic endothelium. It not only solved a long-standing dispute in developmental biology and provided some insight into the timing and control of a curios differentiation event at the birth of the hematopoietic system. It also defined a critical step in the generation of immature hematopoietic stem and progenitor cells with great potential for clinical therapy. Knowing that hemogenic endothelium exists guides the development of culture systems for the stepwise generation of desired cell types eventually leading to blood cell generation. It also guides the identification of the relevant molecular machineries and their manipulation for the induction of the hemogenic program in endothelial or other cells, e.g. through direct reprogramming. Indeed, the field saw a surge of activity leading to the confirmation of our findings in different vertebrates, improved understanding of endothelial to hemogenic transition and its molecular control (Swiers et al., Nature Communications 2013), and transfer of this knowledge to the continuously im-

proving attempts of generating definitive blood stem and progenitor cells from pluripotent and endothelial cells in vitro.

*Lineage choice: Controlled by cell-intrinsic stochastic switches or instructed by cell-extrinsic signals?*

How are lineage choice decisions made in differentiating multipotent progenitor cells? Are they made cell-autonomously by cell-intrinsic mechanisms or instructed by cell-extrinsic signals? This central and seemingly trivial question in hematopoietic stem and progenitor cell biology is discussed since the 1950s, and two major schools of thought with opposing basic concepts remain under discussion until today. The more obvious hypothesis assumes that lineage choice is instructed by signals from the microenvironment, which activate signaling pathways controlling the molecular programs inducing lineage choice, commitment and maturation (see also next chapter). However, colony assays in vivo and in vitro yield very heterogeneous lineage outputs with lineage choice frequencies which are constant only at the population level, but different and unpredictable between individual cells. This observation is hard to reconcile with the idea that lineage choice is under strict control of extracellular signals since all cells in the same culture medium should then behave the same. An alternative model of lineage choice therefore assumes cell intrinsic mechanisms which lead to different lineage choices with specific probabilities, respectively. In this case, the lineage choice of an individual cell is independent of its environment and cannot be predicted. However, at the population level, frequencies of a specific lineage are fixed. This is a very attractive model, since it would allow multipotent cells the required flexibility to differentiate into different cell types, while also being robust against dysregulated signals from the environment which would lead to overshooting uni-lineage differentiation (and thus the lack of required other lineages). In this model, the required adaptation of lineage output of the blood system depending on the body's need, in case of e.g. infections or lower oxygen environments, would be ensured by allowing survival and proliferation only of the required cell types after their lineage commitment, but not by influencing the lineage choice itself (see also selective model in the next chapter).

How would such a cell-intrinsic mechanism work to allow different outputs with specific frequencies? The most prominent hypothesis assumes lineage choice to be made by transcription factor networks, which are wired by protein-protein or protein-DNA interactions between its members. These circuits would lead to stochastic output, i.e. stable molecular end states with different defined probabilities, respectively, which would then drive different lineage choices. The stochasticity would be driven by random noise, e.g. from transcriptional bursts, and channeled into fixed probabilities for different outcomes by the wiring of the network with different interactions, feedback, feed-forward and dampening motives between different transcription factors and their genes. Indeed, molecular interactions between transcription factors involved in controlling hematopoietic lineage choice exist, making this an attractive and plausible model. However, none of these networks, their dynamics and their actual involvement in hematopoietic lineage choice could ever be quantified at the single cell level and linked to actual future cell fate choices – leaving the possibility that this is not more than a nice idea, and the use of the term *"stochastic"* here is just a euphemism for *"we have no clue what is actually going on"*.

### *The PU.1/GATA1 stochastic toggle switch does not initiate hematopoietic lineage choice*

We therefore set out to quantify the actual dynamics of one paradigmatic central molecular switch of the hematopoietic transcription factor network, which was assumed to be responsible for lineage choice (Fig. 11).

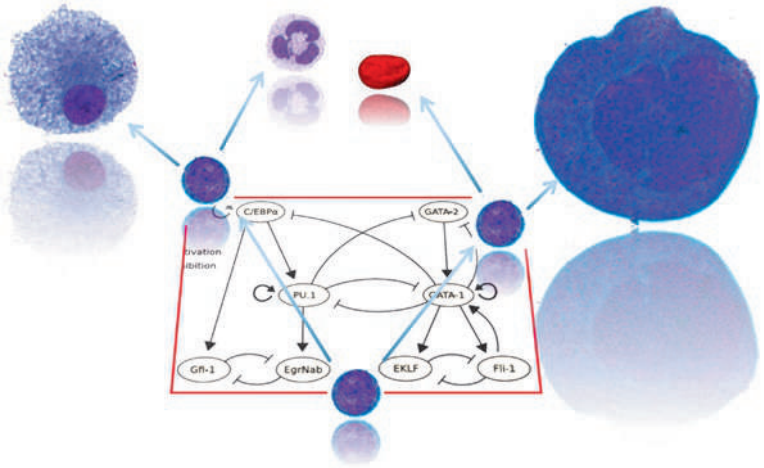


***Figure 11: The putative transcription factor network underlying cell intrinsic stochastic lineage decision making during myeloid differentiation of hematopoietic stem cells.*** *Parts adapted from (Krumsiek et al., PloS One 2011).*

We non-invasively quantified the protein numbers of two transcription factors, PU.1 and GATA1, in living cells throughout their differentiation. These two proteins have long been described as lineage specific transcription factors for the monocytic/granulocytic and megakaryocytic/erythroid lineages of the hematopoietic system, respectively. Their overexpression can reprogram cells from one to the other lineage, respectively. Both protein can bind each other to inhibit the other's activity, and can auto-activate the transcription of their own genes, respectively. This wiring constitutes a toggle switch, where higher expression of one would lead to ever stronger expression and dampening of the stronger and weaker factor, respectively. A cell initially expressing both factors (e.g. before lineage choice), would thus flip into a state where only one of the factors would be expressed, leading to the lineage decision driven by this factor.

After years of technological optimizations, generating the required reporter mouse lines and manually tracking, we finally were able to simultaneously quantify the dynamics of protein expression for both transcription factors in living differentiating hematopoietic stem cells and all their

progeny over a week and up to 12 cell generations. By quantifying morphologies and molecular marker expression, we were able to detect lineage choice of the generated cells, and compare it to the previous molecular dynamics of the quantified factors.

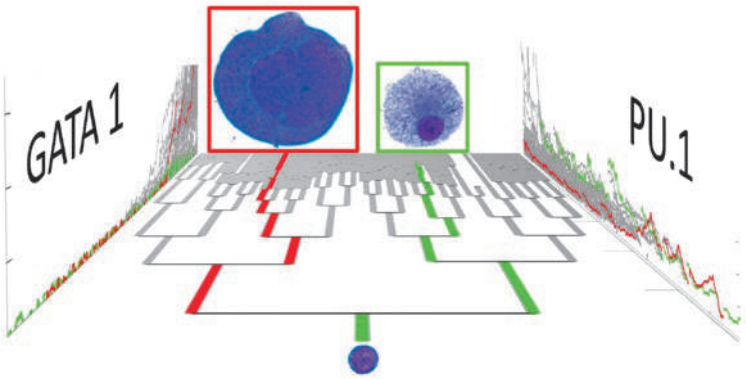


***Figure 12: Non-invasive live single-cell quantification of intracellular protein numbers*** *of the lineage specific transcription factors PU.1 and GATA1 throughout the differentiation of hematopoietic stem cells into megakaryocytes, erythrocytes, monocytes or neutrophil granulocytes over up to 7 days and 12 generations.*

The average expression data found for both proteins at the end of the experiments was identical to what had been described in the literature. However, surprisingly, the expression dynamics we found before and during the time where lineage decisions were made were not compatible with what had been assumed in the field for decades (Hoppe et al., Nature 2016; Strasser et al., Nature Communications 2018). We could therefore show that the presumed stochastic PU.1/GATA1 switch is not used to initiate the monocytic/granulocytic versus megakaryocytic/erythroid lineage choice of differentiating hematopoietic stem cells. However, it would be premature to generalize this finding to conclude that stochastic molecular network switches are never responsible for cell-intrinsic cell fate control. It will be interesting to analyze whether this switch might be used to control the differentiation of other cell types, or if the same wiring, but of other molecules may be relevant for these cell fate choices.

*Nature versus nurture: Lineage selection or instruction by hematopoietic cytokines?*

Can cell-extrinsic cytokine signals influence the lineage choice of multipotent hematopoietic progenitors? Related to the question discussed above, this central and obvious question in hematopoietic stem and progenitor cell biology was intensively discussed since the 1950s. It surprisingly remained without a definitive answer for more than half a century – and while billions of US dollars' worth of cytokines were used annually for clinical therapy.

It was well-known that the lineage composition of hematopoietic colonies is influenced by the specific microenvironment these colonies developed in in vivo, or by the presence of hematopoietic cytokines in defined culture conditions in vitro. The types of living cells ultimately produced from multipotent blood progenitor cells can therefore be influenced by cell-extrinsic signals. However, this could be explained by very different fundamental mechanisms – lineage instruction versus lineage selection – which would both lead to the same final experimental observations described above. One possibility is that cytokine signaling directly influences the genetic and epigenetic programs controlling lineage choice – lineage instruction. However, it could also be possible that cells make their lineage choice independently of signaling pathway activity (see previous chapter), and cytokine signaling would only influence the survival and/or proliferation of already lineage committed cells. In this case, signaling pathways activated by cytokine signaling would only influence the molecular programs involved in cell survival or proliferation control, but have no influence on molecular lineage choice control.

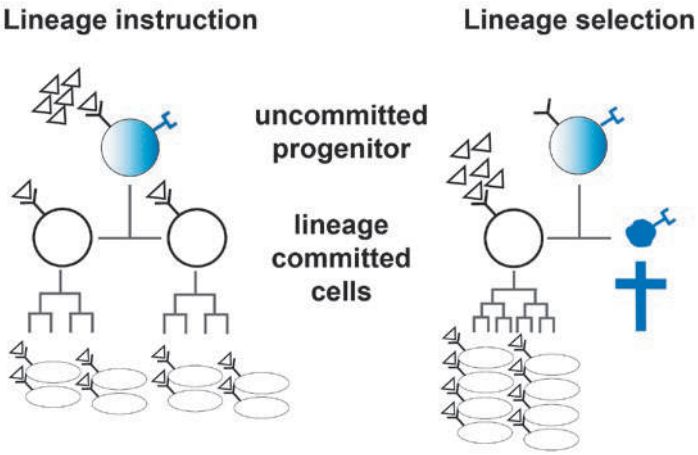


***Figure 13: Alternative models – lineage instruction versus lineage selection – explaining the influence of cytokine signaling on colony lineage output.*** *The existence of lineage instruction on hematopoietic progenitor cells could be proven by long-term single cell imaging and tracking (Rieger et al., Science 2009). Adapted from (Rieger and Schroeder, Cell Cycle 2009).*

While conceptually very different and based on completely distinct molecular mechanisms and effects, it proved difficult to experimentally demonstrate the existence of lineage instruction. This was due to the difficulty to exclude the possibility of lineage choice control exclusively by selection with snapshot data. Cytokines undoubtedly support hematopoietic progenitor cell survival and proliferation. They can therefore clearly contribute to the enrichment of cells of a specific lineage by selection, and thus mask a potential additional contribution by lineage instruction. To prove the existence also of lineage instruction, it was therefore necessary to demonstrate the absence of cell death during the production of only one from multipotent progenitors depending on cytokine treatment. The problem here is the days-long delay between lineage decision making and the subsequent maturation leading to the cellular phenotypes required to detect their commitment to their lineage. During this time, hematopoietic progenitor cells proliferate quickly, producing dozens to hundreds of differentiated progenies. Excluding the possible death of one

individual cell during that time – which could have cell-intrinsically committed to another lineage and was then killed due to the lack of its required lineage specific cytokine – was impossible with previous technologies.

We therefore used continuous long-term observation of all individual cells produced from individual granulocyte-monocyte progenitor (GMP) cells over many days until their lineage commitment could be reliably detected. GMPs were cultured under chemically defined conditions with the presence of either cytokine G-CSF or cytokine M-CSF, leading to the production of only granulocytic or monocytic cells at the end of the cultures from the same starting GMP population, respectively. This allowed us to quantify the frequency of cell death and division events for all of the progeny of the initial cells. Between these culture conditions, we could not find relevant differences in cell proliferation, or in cell death. Importantly, the frequency of cell death events was not sufficient to explain the lack of production of granulocytic cells under M-CSF conditions or monocytic cells under G-CSF conditions. Thus, it was not the selective killing of the "other lineage" cells under lineage specific cytokine culture conditions leading to a uni-lineage differentiation output. Lineage choice must therefore have been directly instructed by signaling activity.

Signaling pathways activated by cytokine receptors therefore must change the molecular programs controlling lineage choice. This insight not only clarifies a long-standing dispute about a core mechanism of multipotent progenitor cell fate control. It is important also because it offers an excellent experimental system to now identify the pathways and relevant molecular mechanisms underlying lineage choice.

This should be easy. One would think that a simple comparison of the intracellular signaling pathways activated by the opposing cytokine receptors would identify the pathway(s) responsible for one or the other lineage choice. However, it turns out to be surprisingly difficult. Despite their opposing effects on lineage choice, the receptors for G-CSF and M-CSF both activate many signaling pathways, and most of them overlapping and highly interconnected. Add the shared confounding effects of both cytokines on cell survival, proliferation, maturation, adhesion and activation, it becomes very demanding to disentangle the effect of these in-

dividual pathways on the different cell fates, and to identify those influencing the molecular control of lineage choice. We used a molecular loss of function approach combined with long-term single-cell quantification of GMP lineage choice to identify the relevant pathway (combination) mediating the monocytic lineage instructive effect of M-CSF. M-CSF receptor deficient GMPs were rescued with mutants of the receptor which activate only one or a subset of the many pathways activated from the eight intracellular Tyrosine residues of the M-CSF receptor. We could finally show that src family kinases are sufficient to instruct monocytic lineage choice. However, they were also not strictly required since the other signaling pathways activated by the M-CSF receptor could apparently compensate for their absence (Endele et al., Blood 2017). Overall, it remains obscure how signaling from activated receptors exert their specific effects on cell fate choices.

The above experiments were based on the assumption that different combinations of intracellular signaling pathways activated by cytokine receptors are responsible for their specific effects. However, there is another possibility to encode specificity – dynamics of signaling pathway activity. The idea would be that different dynamics of pathway activity can activate different molecular target programs (Fig. 14). This would allow different cytokine receptors which activate the same intracellular pathway(s) to still have specific effects.

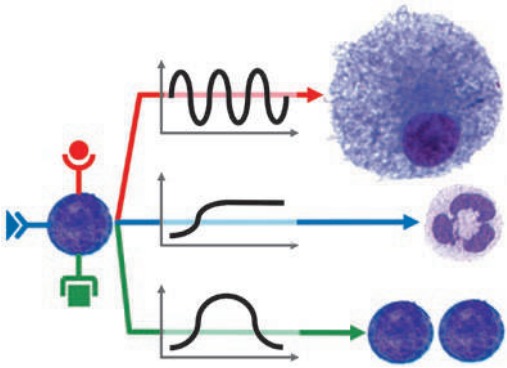

***Figure 14: Different activity dynamics of the same pathway induced by different cytokine receptors could explain cytokine-specific effects on cell fate choices.***

There is beautiful precedence for this concept from cell lines. However, due to the technological demands, the concept has never been tested for primary hematopoietic stem and progenitor cells. It, again, requires the demanding combination of non-invasive high-frequency quantification of signaling pathway activity over hours with the long-term quantification of future cell fate choices over days – in living single cells and all of their progeny. We have therefore developed approaches for the high-frequency live quantification of transgenic biosensors for signaling pathway activity – simultaneously for many different pathways in primary mouse and human stem and progenitor cells. Indeed, we find highly heterogeneous signaling pathway dynamics in individual cells of purified progenitor populations, despite stimulation with the same cytokine. It will now be interesting to link these specific dynamics to the future cell fate choices of individual cells to add another layer of molecular fate control to our understanding of hematopoietic cell fates.

*Asymmetric cell division of hematopoietic stem and progenitor cells*

Are hematopoietic stem and progenitor cell fates controlled by asymmetric cell division? This question has been under dispute for many years. In asymmetric cell division, the future asymmetric fates of two sister cells are fixed during the division of their mother cell. This could be due to e.g. the asymmetric inheritance of intracellular cell fate determinants into the two daughters, or the orientation of the division plane leading to unequal niche access of the two sisters after division (Fig. 15). It would be an attractive explanation how the number of stem cells could be kept constant throughout the body without the need of complex and potentially vulnerable systemic feedback mechanisms. Each stem cell would, under homeostatic conditions, give rise to one daughter which would go on to differentiate and produce the different cell types of the blood system, and one daughter which replaces its mother as a stem cell, thus keeping the stem cell pool size constant. While it has beautifully been shown to exists in other cell types, and some textbooks include asymmetric division even in the definition of hematopoietic stem cells, many researchers do not believe it exists in these cells. Again, the reason for this long-standing dispute is the lack of adequate technology. Observation of either asymmetric fates or asymmetric inheritance of intracellular molecules or niche

access alone is not sufficient to prove the existence of asymmetric cell division. To prove the existence of this peculiar, beautiful and therapeutically attractive mechanism, asymmetric events during division and asymmetric future daughter cell fates have to be quantitatively detected, and shown to correlate in the same cells (Schroeder, Cell Stem Cell 2007). Again, this requires continuous live molecular single-cell imaging in combination with long-term single-cell fate quantification of rare and difficult to purify and culture hematopoietic stem cells.

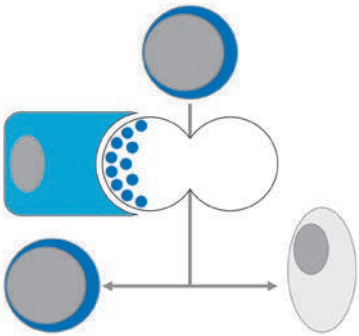

***Figure 15: Asymmetric cell division.***

Asymmetric fates of hematopoietic stem cell daughters had long been described. After the first years of imaging the potential asymmetric inheritance of intracellular molecules and organelles, we had also found some which are asymmetrically segregating during divisions. However, it took us almost a decade to be able to link those two together. For years, different combinations of asymmetries in inheritance during mitosis and in future fates did not correlate with each other, thus not allowing the conclusion that asymmetric molecular inheritance has any functional relevance. Recently, however, we were finally able to find clear correlations between the inheritance of specific molecules and organelles during hematopoietic stem cell divisions, and their future metabolic activation and differentiation (Loeffler et al., in revision). This only happens in a low but reproducible percentage of divisions, and the differences in inheritance between sisters are usually less than two-fold, thus requiring precise quan-

titation. However, the reproducible frequency, and the clear correlations found now finally allow us to conclude that asymmetric cell division exists in hematopoietic stem cells. It is an orthogonal and high-level regulatory mechanism controlling hematopoietic stem cell fates with a lot of potential for novel insights. It will now be exciting to unravel the molecular mechanisms, target effector programs and possibilities for molecular manipulation of this process for therapeutic intervention.

*Where to next?*

After 1.5 decades of my own independent research group, we have established important and unique technologies for long-term single-cell quantifications. These approaches work, both in my own, and in other groups. But they still need expert knowledge and further optimizations. They have contributed to answering diverse long-standing questions in different cell types and molecular systems. After slow and sometimes tedious development of technology, we are now getting faster and faster in successfully applying it to novel biological questions. In addition to quantitative observations, the precise molecular manipulation, both through fine control of fluidics, and increasingly through fast optogenetic approaches, will become important for unravelling the functional role of specific molecules in regulatory networks. With increasing numbers of well-defined culture systems for different cell and tissue types, more and more biological questions become available for long-term single-cell imaging and quantification. The advent of organoid cultures of many solid tissues in combination with light-sheet imaging will lead to a surge of imaging data to be analyzed for the same concepts, but will also require novel custom software components. Long-term in vivo single-cell imaging with sufficient throughput and duration remains a crucial goal in the field, but will likely require novel imaging modalities for many of the biological questions at hand. Improved automation, algorithms and software remain a crucial requirement, and a lot of work still has to be done in this area. After mostly working on murine systems for the first many years due to their better experimental accessibility, reproducibility and the availability of transgenic reporter systems, we have begun to increasingly build on the gathered experience for the analysis also of human cells. Finally, I am convinced that quantification of cellular and molecu-

lar dynamics will enable the next level of insights in high-throughput screening approaches in e.g. for new drugs in the pharmaceutical industries, and importantly also in clinical diagnosis, patient stratification, and the development of novel therapies.

I am very much looking forward to contributing to these and other areas of research. After many years of establishing required technologies, I feel that we can now finally tackle many biological and medical questions much more efficiently. We are ready to really get started. I will likely feel the same again in another 1.5 decades.

*Acknowledgements*

I would like to express my deep gratitude to the Professor Max Cloëtta Foundation for recognizing and honoring my work by this prestigious award. I would like to thank my mentors and teachers Ursula Just, Georg Bornkamm and Shinichi Nishikawa for my education and their generous support. I thank my past and current group members who did and do most of the work for their enthusiasm and dedication – I am expecting great things from you in the future. I am grateful to the ETH Zürich, the Helmholtz Centre Munich, the German Research Council and the Swiss National Science Foundation as my biggest funding sources for trusting me with research funding. Finally, I thank my wife and partner in life for putting up with the priorities and lifestyle that come with marrying a scientist.

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# JOHANNA A. JOYCE

BORN IN 1972 IN LONDON, ENGLAND
LUDWIG INSTITUTE FOR CANCER RESEARCH
DEPARTMENT OF ONCOLOGY
UNIVERSITY OF LAUSANNE

FOR HER GROUND-BREAKING CONTRIBUTIONS
TO THE UNDERSTANDING OF THE TUMOR ENVIRONMENT
AND THE FACTORS THAT INFLUENCE TUMOR PROGRESSION
AND THERAPEUTIC RESISTANCE

LAUSANNE, 9TH NOVEMBER 2018

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| 2011 | Boyer Young Investigator Award |
| 2007 | Geoffrey Beene Junior Faculty Chair |
| 2005 | Rita Allen Foundation Scholar |
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| 1998 | Cambridge Philosophical Society Fellow |
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| 2017–present | Editorial Board of *Genes and Development* |
| 2015–present | Editorial Board of *Trends in Cancer* |
| 2011–present | Editorial Board of *Cell Reports* |
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| | |
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Swiss Bridge Award (1/1/2018–31/12/2019)
*Investigating and Therapeutically Targeting Neutrophils in Brain Metastasis*

Swiss Cancer League (1/1/2017–31/12/2019)
*Targeting Tumor-Associated Macrophages to Enhance Therapeutic Efficacy in Gliomas*

Cancer Research UK Grand Challenge Team Award (1/5/2017–30/4/2023)
*IMAXT: Imaging and Molecular Annotation of Xenografts and Tumors*

Breast Cancer Research Foundation (1/10/2009–present)
*Microenvironmental Regulation of Breast Cancer Metastasis and Therapeutic Response*

National Cancer Institute, R01 grant (1/7/2014–30/6/2019)
*Investigating and Targeting TAMs in the Glioma Microenvironment*

Roche Strategic Alliance (1/11/2016–31/10/2018)
*Combinatorial Strategies for Therapeutically Targeting the Glioma Microenvironment*

Alan and Sandra Gerry Foundation (1/8/2014–7/31/2016)
*Investigation of the Microenvironmental Transcriptome in Breast to Brain Metastasis*

ONO Pharmaceuticals (1/7/2015–30/6/2016)
*Small Molecule Inhibitor Screen for M2 Macrophage Reprogramming*

American Cancer Society (1/1/2012–31/12/2015)
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Rosenkranz Foundation (1/1/2014–31/12/2015)
*Investigating Tumor-Stromal Interactions in Pancreatic Neuroendocrine Tumors*

Health Research Science Board of New York (1/9/2013–31/8/2015)
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National Cancer Institute, R01 grant (1/7/2007–31/5/2012)
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National Cancer Institute, U54 team grant (1/10/2006–30/9/2011)
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Rita Allen Foundation, Scholar Award (1/9/2005–31/8/2008)
*Molecular Dissection of the Tumor Microenvironment*

The V Foundation for Cancer Research, Scholar Award (1/9/2005–31/8/2007)
*Dissecting the Role of Cysteine Cathepsins in Cancer*

Sidney Kimmel Foundation for Cancer Research, Scholar Award (1/7/2005–30/6/2007)
*Genetic Analysis of Cathepsins in Cancer Development*

## SELECTED PUBLICATIONS


1. Quail DF*, Olson OC*, Bhardwaj P, Walsh LA, Akkari L, Quick M, Chen IC, Wendel N, Ben-Chetrit N, Walker J, Holt PR, Dannenberg AJ, Joyce JA (2017). Obesity alters the lung myeloid cell landscape to enhance breast cancer metastasis via IL5 and GM-CSF. *Nature Cell Biology* 19: 974–987.

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8. Quail DF and Joyce JA (2013). Microenvironmental regulation of tumor progression and metastasis. *Nature Medicine* 19: 1423–1437.

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# EXPLORING AND THERAPEUTICALLY EXPLOITING THE TUMOR MICROENVIRONMENT

*Johanna Joyce*

*Summary*

**Cancers do not arise within a vacuum; rather they develop and grow within complex organs and tissue environments that critically regulate the fate of tumor cells at each sequential step of malignant progression. The tumor microenvironment (TME) can be viewed as an intricate ecosystem populated by diverse innate and adaptive immune cell types, stromal cells, extracellular matrix, blood and lymphatic vessel networks that are embedded along with the cancer cells. While bidirectional communication between cells and their microenvironment is critical for normal tissue homeostasis, this active dialog can become subverted in cancer leading to tumor initiation and progression. Through their exposure to tumor-derived molecules, normal cells can become "educated" to actually promote cancer development. As a consequence of this tumor-mediated education, TME cells produce a plethora of growth factors, chemokines, and matrix-degrading enzymes that together enhance the proliferation and invasion of the tumor. Moreover, these conscripted normal cells also provide a support system for cancer cells to fall back on following traditional therapies such as chemotherapy and radiation, and additionally contribute to a general immune-suppressive state, thus limiting the efficacy of immunotherapies. Consequently, multi-targeted approaches in which co-opted cells in the microenvironment are "re-educated" to actively fight the cancer represent a promising strategy for the effective long-term treatment of this devastating disease.**

*Introduction*

Tumors contain diverse cell types and inflammatory mediators within their TME, including endothelial cells, fibroblasts, tissue-resident and peripherally-derived immune cells, among others (Joyce, 2005; Quail and Joyce, 2013; Quail and Joyce, 2017c) (Fig. 1). Depending on the organ, there are also unique compositions of tissue-specific resident cell types and extracellular matrix molecules, which can affect tumor development in different ways (Joyce and Pollard, 2009; Quail and Joyce, 2017a). Indeed, tumor progression is not only dictated by genetic alterations within the cancer cells, but also by whether the surrounding niche is permissive to growth at each stage of disease. Thus, a full mechanistic understanding of both tumor cell-intrinsic and -extrinsic mediators of malignant progression is critical to optimize therapeutic strategies against cancer.

Interactions between tumor cells and the associated stroma and cells of the immune system profoundly influences cancer initiation, progression and patient prognosis. The link between chronic inflammation and tumorigenesis was first proposed by Rudolf Virchow in 1863 following his seminal observation that infiltrating leukocytes are a hallmark of tumors (Virchow, 1863). Another clinical investigator of that era, Stephen Paget, specifically recognized the importance of the microenvironment in determining the organ-tropism of metastasis, leading to his seminal "seed and soil" hypothesis published in 1889 (Paget, 1889). Paget stated that "when a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall in congenial soil". However, despite these and other key findings dating back to the late 19th century, for many subsequent decades the TME was overlooked, as researchers predominantly focused on identifying the genetic drivers of cancer.

In recent years the TME field has exploded, with a plethora of studies contributing to a molecular and cellular understanding of the importance and complexity of the TME, further complicating the already challenging task of understanding and treating cancer. Thus, while cancer was long viewed as a heterogeneous disease driven by DNA mutations and genomic alterations in tumor cells, it is now evident that tumors are similarly diverse by nature of their microenvironmental composition. Moreover, in response to evolving environmental conditions and oncogenic

signals from growing tumors, the TME continually changes over the course of cancer progression and in the context of therapeutic intervention. This underscores the need to investigate the influences of the TME as a dynamic process and to understand how cancer cells drive the construction and evolution of their own niche.

In contrast to cancer cells, immune and stromal cell types within the TME are genetically stable and thus represent an attractive therapeutic target with reduced risk of resistance and tumor recurrence (Joyce, 2005; Quail and Joyce, 2013). However, specifically disrupting the pro-tumorigenic TME is a challenging task, as the TME has diverse capacities to induce either beneficial or adverse consequences for tumorigenesis, in a context- and stage-dependent manner. Indeed, the microenvironment is capable of normalizing cancer cell behavior, leading to the notion that re-education of immune and stromal cells, rather than their targeted ablation *per se,* could be a more effective strategy for effectively treating cancer (Quail and Joyce, 2013; Bowman and Joyce, 2014).

In this review, I will discuss the importance of the TME as a potent regulator of cancer development, metastasis, and therapeutic response. In the general overviews of each of these processes I have referred to several reviews I have written on these topics with different members of my lab and other colleagues over the years. I have included examples of my lab's contributions to understanding these different processes during the past decade and more, following the tradition of the Max Cloëtta Series.

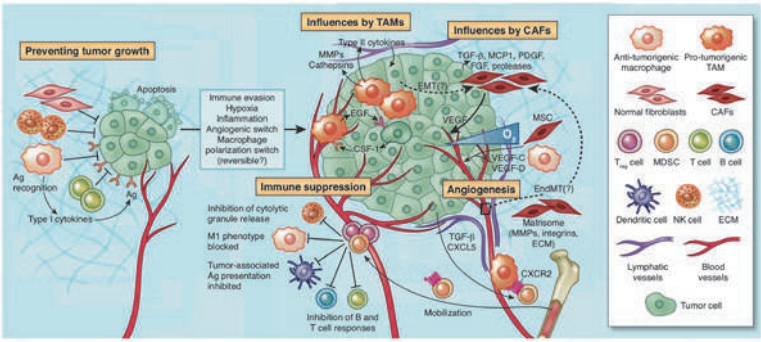


***Figure 1:*** *Multiple stromal cell types converge to support a tumorigenic primary niche. After circumventing cell-intrinsic mechanisms of apoptosis, tumor cells are subject to elimination pressures by the immune system. Tumor cell-specific antigens have a role during this process, which are recognized by cytotoxic immune cells, leading to their destruction. Fibroblasts and macrophages within the tumor microenvironment (TME) also contribute to a growth-suppressive state; however, these cells may later become educated by the tumor to acquire pro-tumorigenic functions. For instance, tumor-associated macrophages (TAMs) support diverse phenotypes within the primary tumor, including growth, angiogenesis and invasion, by secreting a plethora of pro-tumorigenic proteases, cytokines and growth factors. As tumors grow, immune-suppressor cells, including myeloid-derived suppressor cells (MDSCs) and regulatory T cells are mobilized into the circulation in response to activated cytokine axes that are induced by tumorigenesis, and infiltrate the growing tumor to disrupt immune surveillance through multiple mechanisms, including, but not limited to, disruption of antigen presentation by dendritic cells, inhibition of T and B cell proliferation and activation, or inhibition of natural killer (NK) cell cytotoxicity. Cancer-associated fibroblasts (CAFs), which become activated by tumor-derived factors, secrete extracellular matrix (ECM) proteins and basement membrane components, regulate differentiation, modulate immune responses and contribute to deregulated homeostasis. In addition to cellular contributions, several extracellular properties contribute to tumor progression, including low oxygen tension, high interstitial fluid pressure and changes in specific components of the ECM. From Quail and Joyce, Nature Medicine (2013).*

*Tumor-associated macrophages are key regulators of cancer initiation and progression*

One of the critical regulatory cell types in the TME are tumor-associated macrophages (TAMs) (Noy and Pollard, 2014), which can constitute up to 30-50 % of the tumor mass in some cancers. Analysis of clinical samples has shown that in the vast majority of malignancies, high TAM numbers are associated with more aggressive disease and poor patient prognosis, indicating tumor-promoting functions for these cells (Bingle et al., 2002; Zhang et al., 2012; Fridman et al., 2017). At the time of initiating our research program on TAMs over a decade ago, there was a limited understanding of precisely how TAMs regulate tumorigenesis. This led us to ask several critical questions: How are normal macrophages converted or "educated" to TAMs? What are the molecular and cellular changes that characterize TAMs? What are the mechanisms by which TAMs then regulate tumor progression, and can TAMs be therapeutically targeted? Do TAMs modulate the response to traditional or molecularly targeted anti-cancer agents, and if so, will combinatorial targeting approaches enhance therapeutic efficacy?

To answer these questions, we have investigated distinct TMEs and devised complementary experimental strategies by integrating the analysis of patient samples, diverse mouse models, *in vivo* cell lineage tracing, *ex vivo* tissue and cell culture systems, and a comprehensive panel of computational analyses. We have focused on primary tumors in the brain, breast, and pancreas, in addition to investigating metastases that disseminate to the brain, lung, or bone. Through these diverse and illuminating methodologies, we have been fortunate to make a number of key conceptual advances in the TME field as summarised below (see Figure 2 for schematic).

We have identified key molecular mechanisms driving the education of tumor-promoting macrophages in the pancreas and breast (Gocheva et al., 2010b; Yan et al., 2016), and uncovered the epigenetic and transcriptomic regulatory machinery underlying differential ontogeny and tumor-mediated education between distinct macrophage populations in the brain (Bowman et al., 2016). We found that a critical molecular difference between normal macrophages and TAMs is the increased activity

of matrix-degrading enzymes, specifically cathepsin proteases and heparanase (Gocheva et al., 2010b; Hunter et al., 2014), and identified the upstream cytokines responsible for their induction (Yan et al., 2016) (Figure 2, and discussed further below).

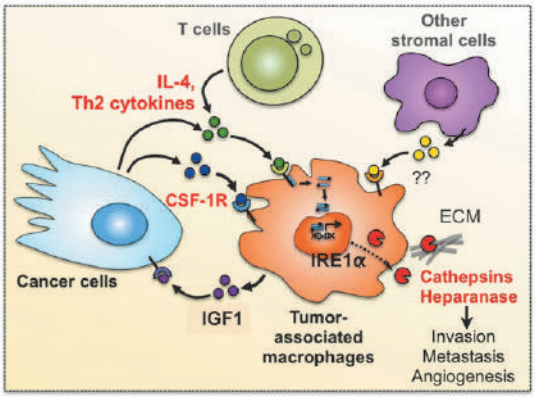


***Figure 2:*** *Model of reciprocal interactions between cancer cells, tumor-associated macrophages and additional cells in the tumor microenvironment that enhance malignant progression. IL-4 and other Th2 cytokines are produced by cancer cells and T cells in the TME, leading to an increase in protease activity in tumor-associated macrophages (TAMs) that promotes several hallmarks of cancer, including angiogenesis, invasion and metastasis. Schematic depicts data compiled from: Gocheva, Wang et al, Genes Dev (2010); Pyonteck, Akkari, Schuhmacher et al, Nat Med (2013); Akkari et al, Genes Dev (2014); Hunter et al, Oncogene (2014); Sevenich et al, Nat Cell Biol (2014); Yan, Wang, Bowman and Joyce, Cell Reports (2016); Quail et al, Science (2016).*

Through our exploration of the molecular differences between TAMs and their normal counterparts, we also became intrigued as to whether tissue-resident macrophages, such as microglia in the brain, differ from peripherally-recruited macrophages in terms of gene expression, epigenetic regulation and tumorigenic functions. To address this question, Robert Bowman, a graduate student in my lab, used complementary cell lineage-tracing genetic models to selectively distinguish resident microglia (MG) from bone marrow-derived macrophages (BMDMs) recruited from the periphery (Bowman et al., 2016). Using this strategy, he investigated the epigenetic and transcriptomic regulatory machinery underlying dif-

ferential ontogeny and tumor-mediated education between MG and BMDMs in multiple brain malignancies, including gliomas and breast-to-brain metastasis. Interestingly, we found there are distinct transcriptional networks in MG and BMDMs associated with tumor-mediated education, which are also influenced by differential chromatin landscapes that are established before tumor initiation (Figure 3). We showed that microglia specifically repress the integrin subunit Itga4 (CD49D), enabling its utility as a discriminatory marker between BMDMs and MG in primary and metastatic disease in mouse models and patient samples. We concluded that while macrophages have been shown to acquire tissue-resident traits upon entry into an organ (Lavin et al., 2014; Lavin et al., 2015), an inflammatory microenvironment, such as in the context of cancer or neuroinflammation, can further amplify differences between cell populations leading to diverse functional outcomes for tissue-resident and peripherally-derived macrophage populations. These results (Bowman et al., 2016) collectively have important implications for targeting TAMs in brain malignancies, and other cancers.

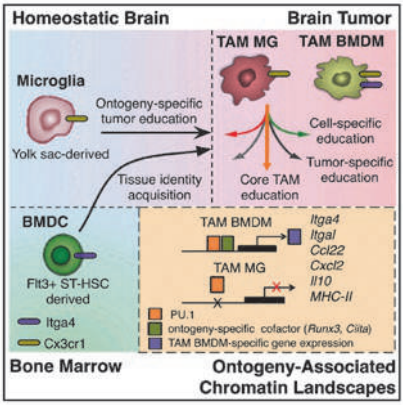


***Figure 3:*** *Macrophage ontogeny underlies differences in tumor-specific education in brain malignancies. Genetic lineage tracing models were used to interrogate the ontogeny of tumor-associated macrophages in brain malignancy. We found that bone-marrow-derived macrophages (BMDMs) and tissue-resident microglia (MG) are present in glioma and brain metastases, and show distinct transcriptional and chromatin states. We identified a number of differentially-expressed genes, as indicated in this schematic, which clearly distinguish these cell populations. From Bowman et al, Cell Reports (2016).*

*Therapeutic targeting of TAMs in cancer*

To evaluate the therapeutic potential of targeting TAMs, we have taken a number of different approaches, including pharmacological inhibition or genetic ablation of colony stimulating factor-1 (CSF-1) signaling (Quail and Joyce, 2017b), which is a key mediator of macrophage survival and differentiation (Noy and Pollard, 2014). We began by investigating the consequences of CSF-1 deletion using a pancreatic neuroendocrine cancer mouse model (RIP1-Tag2), as we had simultaneously revealed key functions for TAM-supplied cathepsins in RIP1-Tag2 mice (see below). Stephanie Pyonteck, a graduate student in my lab, found that CSF-1 deletion led to TAM depletion in the pancreas and a substantial reduction in cumulative tumor burden (Pyonteck et al., 2012). Interestingly, she determined that this resulted from a significant decrease in the initial angiogenic switching of progenitor lesions and subsequent development of tumors, rather than an evident effect on tumor growth. This study thus revealed important functions for TAMs at the earliest stages of tumor initiation, thereby expanding the repertoire of TAM functions beyond the promotion of advanced malignancy and metastasis. In collaboration with our colleague Laura Tang, at MSKCC in New York, we also analyzed a cohort of tissue samples from human pancreatic neuroendocrine tumors (PanNETs). We found that elevated TAM number in the pancreas increased with tumor grade, as in the mouse model, and importantly showed that this can be prognostic for PanNET patients that develop liver metastases (Pyonteck et al., 2012) (Figure 4).

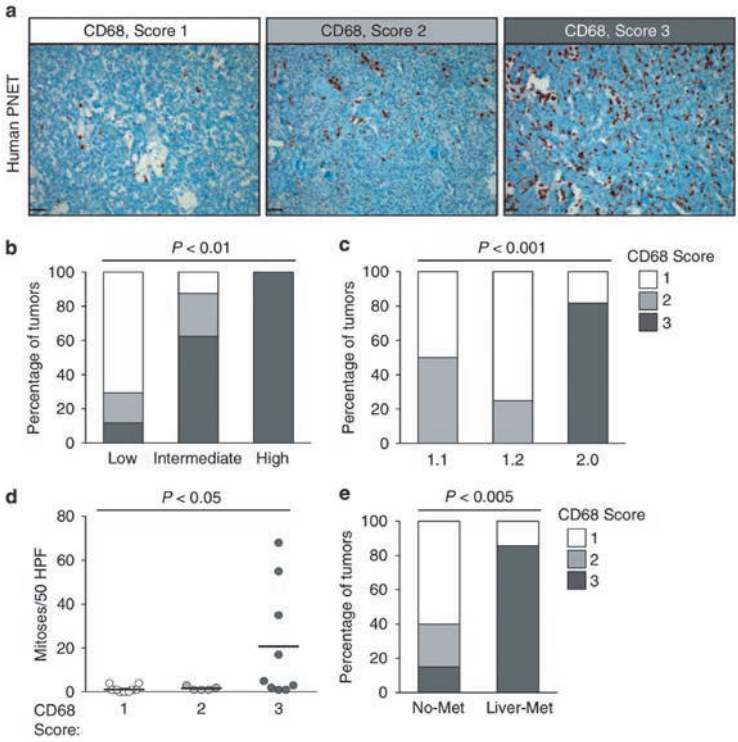


***Figure 4:*** *Macrophage infiltration positively correlates with aggressiveness of human pancreatic neuroendocrine tumors (PanNETs). (a) PanNET patient tissue sections were immunohistochemically stained for macrophages with a CD68 antibody (brown color). Stained tissue sections were then blindly scored by two independent investigators for the CD68+ macrophage density and classified into low (CD68 score 1), medium (score 2) or high (score 3). Representative images are depicted in (a). Scale bar, 50 um. (b–e) CD68 scores for each tumor were then de-coded and matched with their corresponding clinicopathological data: (b) histological tumor grade; (c) WHO tumor stage; (d) number of mitoses per 50 high-powered fields (HPF); (e) the absence and presence of distant metastasis to the liver. Fisher's exact test was used for statistical analyses. From Pyonteck et al., Oncogene (2012).*

Another tumor type in which TAMs are highly abundant and associated with aggressive disease are gliomas that arise in the brain. We and others have found that tumor-associated macrophages and microglia together can comprise up to 30% of the total tumor mass in glioblastomas, thus representing the most abundant non-cancerous cell type in this high-grade malignancy (Hussain et al., 2006; Komohara et al., 2008; Bowman et al., 2016; Quail and Joyce, 2017a). Most therapeutic approaches directly targeting tumor cells in glioblastoma have failed. We therefore proposed an alternative strategy: to target TAMs in the brain TME. Stephanie Pyonteck, Leila Akkari, Alberto Schuhmacher and several other key lab members teamed up to work on this exciting project. We used an inhibitor of the CSF-1 receptor, CSF-1R, to target TAMs in mouse glioblastoma models developed by our collaborator Eric Holland, who was also then at MSKCC. Treatment with this selective inhibitor (BLZ945 from Novartis) regressed established high-grade tumors, even after just one week of treatment (Pyonteck et al., 2013). We found that CSF-1R inhibition markedly increased tumor cell apoptosis and phagocytosis *in vivo,* while decreasing proliferation and glioma malignancy, and significantly extending survival (Figure 5).

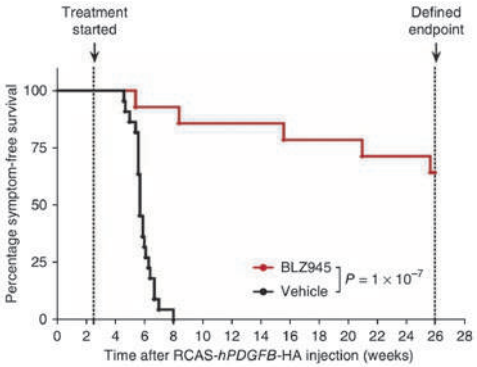


***Figure 5:*** *CSF-1R inhibition specifically targets macrophages, improves survival and decreases glioma malignancy in the transgenic PDGF-driven glioma (PDG) mouse model. Symptom-free survival curves are shown for PDG mice treated in an early intervention trial with a CSF-1R inhibitor BLZ945 (red) or vehicle (black), demonstrating a dramatic increase in survival following CSF-1R inhibition. From Pyonteck, Akkari, Schuhmacher et al, Nature Medicine (2013).*

Surprisingly, we found that while microglia in the normal brain were depleted, as expected, TAM numbers were not reduced in gliomas of the treated mice. Instead, we identified glioma-secreted factors, including GM-CSF and IFN-$\gamma$, which facilitated TAM survival in the face of CSF-1R inhibition (Pyonteck et al., 2013). Gene expression analysis of these surviving TAMs revealed a decrease in alternatively activated/ M2-like macrophage markers, consistent with their impaired tumor-promoting functions and enhanced capacity to phagocytose glioma cells. CSF-1R blockade additionally slowed intracranial tumor growth of multiple patient-derived glioma xenografts. Subsequent preclinical trials by Dongyao Yan, a postdoc in my lab, using a chemically-distinct CSF-1R inhibitor (PLX3397 from Plexxikon) showed a similar therapeutic efficacy and macrophage reprogramming (Yan et al., 2017).

Together, our results revealed a new therapeutic strategy for targeting the TME. Rather than depleting TME cells, as had been the goal with many microenvironment-targeted therapies up to that point, we proposed that "re-educating" these cells has the potential to not only abolish their tumor-promoting functions but also actively enlist them as suppressors of tumorigenesis (Quail and Joyce, 2013; Bowman and Joyce, 2014). This body of research has had an important impact in the TME field, and on the therapeutic evaluation of CSF-1R inhibitors in glioma patients.

This representative study (Pyonteck et al., 2013) and many others from colleagues in the TME field indicate that therapies targeted against the TME offer a promising approach for targeting cancer (Quail and Joyce, 2013; Binnewies et al., 2018). However, it remained unclear whether resistance may develop to TME therapies over time. Given that TME-targeted agents are increasingly being evaluated in the clinic, it was critical to mechanistically define how resistance may evolve in response to these therapies in order to provide long-term disease management for patients. We therefore addressed this important question by further investigating the case of CSF-1R inhibition of TAMs in gliomas, and extended the original preclinical trial design to treat mice over many months following the development of high-grade bulky glioblastoma. In this case, Daniela Quail, the postdoc in my lab who led this study, found that while overall survival was dramatically prolonged following CSF-1R inhibition, tumors eventually recurred in ~50 % of mice (Quail et al., 2016), allowing us

to explore the underlying mechanisms. Interestingly, upon isolation and transplantation of tumor cells from recurrent gliomas into naïve animals, Daniela found that sensitivity to CSF-1R inhibition was re-established, indicating that the resistance was in fact driven by the microenvironment.

Through RNA-sequencing of glioma cells and TAMs purified from treated tumors, and *ex vivo* cell culture assays, we discovered an elevation in PI3K pathway activity in recurrent glioblastoma following CSF-1R inhibition, which was driven by macrophage-derived IGF-1 and tumor cell IGF-1R (Quail et al., 2016) (Figure 6). Consequently, combining IGF-1R or PI3K blockade with continuous CSF-1R inhibition in recurrent tumors dramatically extended overall survival. By contrast, monotherapy with IGF-1R or PI3K inhibitors in rebound or treatment-naïve tumors was minimally effective, indicating the necessity of combination therapy to expose PI3K signaling dependency in recurrent disease. Mechanistically, Daniela found that T cell-derived IL4 led to macrophage activation in recurrent tumors, and elevated STAT6 and NFAT signaling upstream of IGF-1 induction. Similarly, inhibition of any of these pathways *in vivo* was also sufficient to significantly extend survival when combined with CSF-1R inhibition (Quail et al., 2016). Given that PI3K signaling is aberrantly activated in a substantial proportion of glioma patients, including through mutations in PTEN and other PI3K pathway components, it is possible that this pathway could also contribute to intrinsic resistance to CSF-1R inhibition. Our findings thus revealed the importance of continuous bidirectional feedback between cancer cells and the TME, and support the notion that although immune and stromal cells are less susceptible to genetic mutation than are cancer cells, a tumor can nonetheless acquire a resistant phenotype by exploiting its extracellular environment (Quail et al., 2016; Quail and Joyce, 2017b).

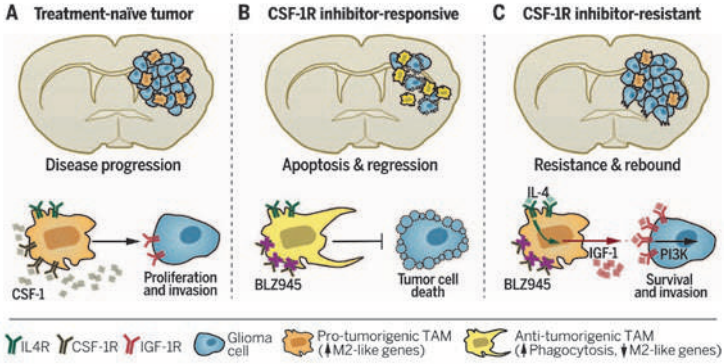


***Figure 6:*** *Resistance to CSF-1R inhibition in gliomas. (A) Macrophages contribute to glioblastoma progression by creating a protumorigenic niche associated with M2-like gene expression. CSF-1R is a critical receptor for macrophage biology and is under clinical evaluation as a therapeutic target in glioma. (B) Targeting CSF-1R early in gliomagenesis significantly prolongs survival in mouse models (using CSF-1R inhibitors e.g. BLZ945). CSF-1R inhibition reprograms macrophages to become antitumorigenic by down-regulating M2-like genes and enhancing phagocytosis. Tumor-derived survival factors sustain macrophage viability despite CSF-1R blockade (Pyonteck et al., Nat Med 2013). (C) After prolonged treatment, a subset of glioblastomas acquire resistance to CSF-1R inhibition, and tumors recur. This is driven by elevated macrophage-derived IGF-1 and high IGF-1R on tumor cells, resulting in PI3K pathway activation and enhanced glioma cell survival and invasion. Blocking this pathway in combination with CSF-1R in preclinical trials resulted in a pronounced survival benefit. Adapted from Quail et al., Science (2016).*

### *Matrix-degrading enzymes in the TME: cathepsin proteases and heparanase*

All tissues require extracellular matrix (ECM) to provide structural support and to facilitate the continuous intercellular communication that maintains tissue homeostasis (Mouw et al., 2014; Vogel, 2018). The ECM comprises secreted macromolecules including collagens, fibronectin, laminin, etc., and the precise composition can vary considerably in a cell type- and organ-dependent manner. In cancer, ECM production, composition and turnover are often aberrantly regulated by comparison to the normal tissue, contributing to enhanced invasion and proliferation of cancer cells (Pickup et al., 2014). Interestingly, through our investigation of the molecular differences between normal macrophages and TAMs, we found that upregulation of key matrix-degrading enzymes, specifically

cysteine cathepsin proteases and heparanase, was a prominent feature of TAMs in multiple TMEs (Gocheva et al., 2010b; Hunter et al., 2014).

In earlier experiments, dating back to my postdoc in Doug Hanahan's lab at UCSF, we had found that expression of a subset of 6 of 11 cathepsin family members were progressively upregulated during pancreatic cancer progression (Joyce et al., 2004) in the RIP1-Tag2 mouse model introduced above. Cathepsins are typically lysosomal enzymes, which are critical for terminal protein degradation (Olson and Joyce, 2015). To explore whether they might have extra-lysosomal functions in cancer, we used activity-based probes developed by Matthew Bogyo, a chemical biologist and long-standing collaborator now at Stanford University. We successfully imaged global cathepsin activity *in vivo* in several mouse models of cancer and found a specific increase in TAMs within the TME of PanNETs, breast cancer, and lung metastases (Joyce et al., 2004; Gocheva et al., 2010b) (Figure 7).

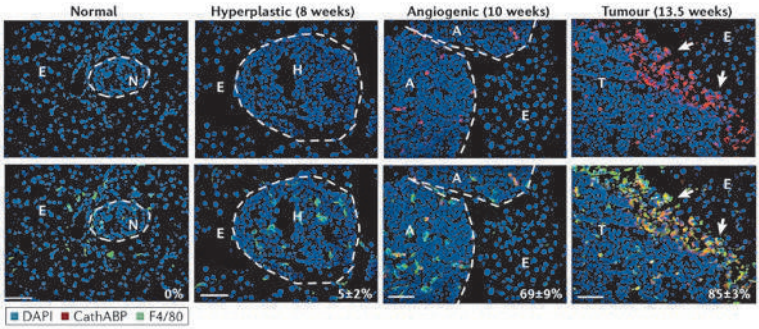


***Figure 7:*** *Increase in cathepsin activity in TAMs during pancreatic islet tumor development in the RIP1-Tag2 mouse model. Cathepsins are highly activated in infiltrating macrophages during tumor progression at the angiogenic islet and tumor stages of RT2 tumorigenesis. Mice were injected with the cathepsin activity-based probe (Cath-ABP), and the resulting tissues stained with a F4/80 antibody to visualize macrophages. Normal (N) Tag+ islets were analyzed at 4–7 wks of age, hyperplastic (H) islets at 8 wks, angiogenic (A) islets at 10 wks, and tumors (T) at 13.5 wks of age. The percentage of F4/80+ cells that were Cath-ABP+ was determined by image analysis and is indicated in the representative image for each stage. Macrophages present in the normal adjacent exocrine (E) pancreas did not show high levels of cathepsin activity. Arrows represent the invasive tumor front. Adapted from Gocheva, Wang et al., Genes and Development (2010).*

This led Leny Gocheva and Hao-Wei Wang, two graduate students in my lab, along with Bedrick Gadea and several other key lab members (Gocheva et al., 2010b), to explore how cathepsin activity is elevated in TAMs, and investigate the mechanisms by which cathepsin proteases supplied by TAMs contribute to tumorigenesis. To discover the factors that upregulate cathepsin activity in macrophages, Hao-Wei developed a novel cell-based assay to initially focus on cancer cell-secreted proteins, and identified interleukin (IL)-4 as a critical inducer of cathepsin activity. Consistently, he found that deletion of IL-4 *in vivo* resulted in a significant reduction in cathepsin-positive TAMs in tumors. In parallel, Leny asked whether the increase in active cathepsins in TAMs contributed to tumor progression by performing a series of reciprocal bone marrow transplantation (BMT) experiments using different cathepsin knockout mice (as either donors or recipients). She found that removal of BM-derived cathepsin B or S, but not C or L, significantly reduced pancreatic tumor growth and invasion. We employed co-culture assays to show that macrophage-supplied cathepsins B and S significantly promote the invasive behavior of tumor cells. Together, these results established IL-4 as an important regulator, and specific cathepsin proteases as critical mediators, of the cancer-promoting functions of TAMs (Gocheva et al., 2010b; Wang and Joyce, 2010).

We subsequently sought to identify the precise molecular mechanisms by which cathepsins are secreted from TAMs, and address whether this new extracellular location was critical for their tumor-promoting functions. Dongyao Yan, Hao-Wei Wang and Bobby Bowman in the lab teamed up and began by asking whether other Th2 cytokines in addition to IL-4 could increase cathepsin secretion. Whole-genome expression analyses of macrophages revealed that IL-4 synergizes with the Th2 cytokines IL-6 or IL-10 to activate the unfolded protein response (UPR) via STAT6 and STAT3, which resulted in a potent upregulation of cathepsin secretion (Yan et al., 2016). We found that pharmacological inhibition of IRE1-alpha, a UPR sensor, blocked cathepsin secretion and consequently blunted macrophage-mediated cancer cell invasion. Critically, genetic deletion of STAT3 and STAT6 signaling components also impaired tumor development and invasion *in vivo*. Together, these findings revealed that cytokine-activated STAT3 and STAT6 cooperate to promote a secretory phenotype in macrophages that leads to enhanced tumor progression and invasion in a cathepsin-dependent manner (Yan et al., 2016) (Figure 8).

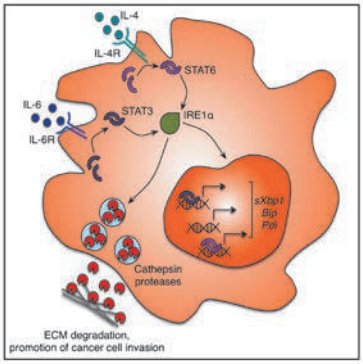


***Figure 8:*** *STAT3 and STAT6 signaling pathways synergize to promote cathepsin secretion from macrophages via IRE1-alpha activation. We found that the Th2 cytokine IL-4 synergizes with IL-6 and IL-10 in macrophages to promote pancreatic neuroendocrine tumor growth and invasion. This synergy depends on STAT3 and STAT6 interaction to activate IRE1-alpha, leading to a pronounced secretion of cathepsin proteases and induction of unfolded protein response-related genes. From Yan, Wang, Bowman and Joyce, Cell Reports (2016).*

### *Cathepsin proteases are potent regulators of multiple hallmarks of cancer*

To gain insights into the mechanisms by which cathepsins regulate different hallmark capabilities of cancer, we devised a comprehensive genetic strategy to delete individual cathepsins (alone and in combination) and determine the consequences for tumor proliferation, angiogenesis, and invasion. Using the RIP1-Tag2 model we first sought to identify the key tumor-promoting family members from the six that we found upregulated (B, C, H, L, S, and Z) from whole genome expression analyses (Joyce et al., 2004). There were several compelling reasons for undertaking this genetic analysis. First, to fully understand how cathepsins promote tumorigenesis it was critical to determine how each family member individually regulates tumor growth, invasion and angiogenesis. Second, from a translational perspective, when using pan-family inhibitors, there is the possibility of undesirable effects if some family members are actually tumor suppressors (Lopez-Otin and Matrisian, 2007). Thus, identifying the tumor-promoting proteases, and developing selective inhibitors that only target these enzymes is critical; as we have also addressed pharmacologically (Sadaghiani et al., 2007; Elie et al., 2010; Sevenich et al., 2014).

Our comprehensive analysis of multiple tumorigenic processes in the individual mutants, led initially by Leny Gocheva and subsequently by Leila Akkari, revealed specialized functions, in addition to phenotypes that were regulated by several cathepsins, as summarized in Table 1 (Gocheva et al., 2006; Gocheva and Joyce, 2007; Gocheva et al., 2010a; Akkari et al., 2014; Prudova et al., 2016). Importantly, cathepsin C, which was similarly upregulated during tumorigenesis (Joyce et al., 2004) had no impact when deleted (Gocheva et al., 2006), underlying the importance of rigorous genetic analyses for functional validation of whole genome expression data. We also made compound mutants of cathepsins B, S and Z, uncovering both additive and overlapping roles in tumorigenesis (Akkari et al., 2016). This body of work was critically enabled by the generosity of Thomas Reinheckel, Christoph Peters and other collaborators in sharing cathepsin mutants, and represented the first comprehensive genetic analysis of a family of proteases in cancer (Olson and Joyce, 2015). As a result, we successfully identified the key tumor-promoting family members, elucidated their different tumorigenic roles, and revealed that many of their tumorigenic functions are mediated via TAMs, rather than cancer cells (Gocheva et al., 2010b; Akkari et al., 2014).

| | $B^{-/-}$ RT2 | $H^{-/-}$ RT2 | $L^{-/-}$ RT2 | $S^{-/-}$ RT2 | $Z^{-/-}$ RT2 | $B^{-/-}S^{-/-}$ RT2 | $B^{-/-}S^{-/-}Z^{-/-}$ RT2 | $C^{-/-}$ RT2 |
|---|---|---|---|---|---|---|---|---|
| Angiogenic switching | 24% ↓ | 32% ↓ | No change | 24% ↓ | 53% ↓ | 60% ↓ | *Not evaluated* | No change |
| Tumor volume | 72% ↓ | 40% ↓ | 88% ↓ | 47% ↓ | 63% ↓ | 51% ↓ | 58% ↓ | No change |
| Proliferation | 44% ↓ | No change | 58% ↓ | No change | 86% ↓ | 85% ↓ | 53% ↓ | No change |
| Apoptosis | 2.3 fold ↑ | 2.0 fold ↑ | 3.4 fold ↑ | 1.6 fold ↑ | 1.8 fold ↑ | No change | 3.8 fold ↑ | No change |
| Tumor vascularization | 56% ↓ | 32% ↓ | No change | 48% ↓ | No change | No change | *Not evaluated* | No change |
| Invasion | Significant reduction | No change | Significant reduction | Significant reduction | Significant reduction | No change | Significant reduction | No change |

***Table 1:*** *Summary of the effects of cathepsin deletion on multiple tumorigenic processes. Each of the six cathepsin knockout RIP1-Tag2 lines (single and compound mutants) was compared to wild-type RIP1-Tag2 littermates. Significant changes for each tumorigenic process are indicated in black, with no change indicated in grey. Data compiled from Gocheva et al, Genes Dev (2006); Gocheva et al, Biol Chem (2010); Akkari et al, Genes Dev (2014); Akkari, Gocheva et al, Genes Dev (2016).*

To unravel the molecular mechanisms by which cathepsins promote the different hallmarks of cancer summarized above we employed both candidate-based strategies and unbiased proteomic screens to reveal their substrates within the TME (Figure 9). For example, Leny Gocheva identified cleavage of the cell adhesion protein E-cadherin by the pro-invasive cathepsins B, L and S as a key mechanism that contributes to tumor invasion (Gocheva et al., 2006). Lisa Sevenich discovered a brain metastasis-promoting function for cathepsin S via shedding of the junctional adhesion molecule, JAM-B, which facilitates extravasation of tumor cells into the brain across the blood-brain barrier (BBB) (Sevenich et al., 2014). Leila Akkari, with Leny Gocheva and other lab members, found that cathepsin Z promotes cancer cell invasion and proliferation through a unique RGD-binding motif in its pro-domain, which promotes attachment via integrins to different ECM components, in a FAK/Src-dependent manner (Akkari et al., 2014). Moreover, we demonstrated that for this cathepsin family member, its tumor-promoting functions are actually independent of its enzymatic activity, and instead rely on ECM-mediated signaling. In collaboration with the Vlodavsky lab in Israel, cathepsin L was identified as the major protease responsible for activation of the key matrix-degrading enzyme, heparanase (Abboud-Jarrous et al., 2008). In an interesting convergence, we had previously shown that inhibition of heparanase (Joyce et al., 2005) disrupts several of the same tumorigenic pathways as pan-cathepsin inhibitors (Joyce et al., 2004; Bell-McGuinn et al., 2007; Elie et al., 2010). Karen Hunter, a graduate student in my lab, thus investigated how genetic modulation of heparanase levels regulates tumor progression using heparanase knockout and heparanase-overexpressing mice in the RIP1-Tag2 model, and thereby identified critical roles for heparanase in promoting lymphangiogenesis and tumor invasion (Hunter et al., 2014).

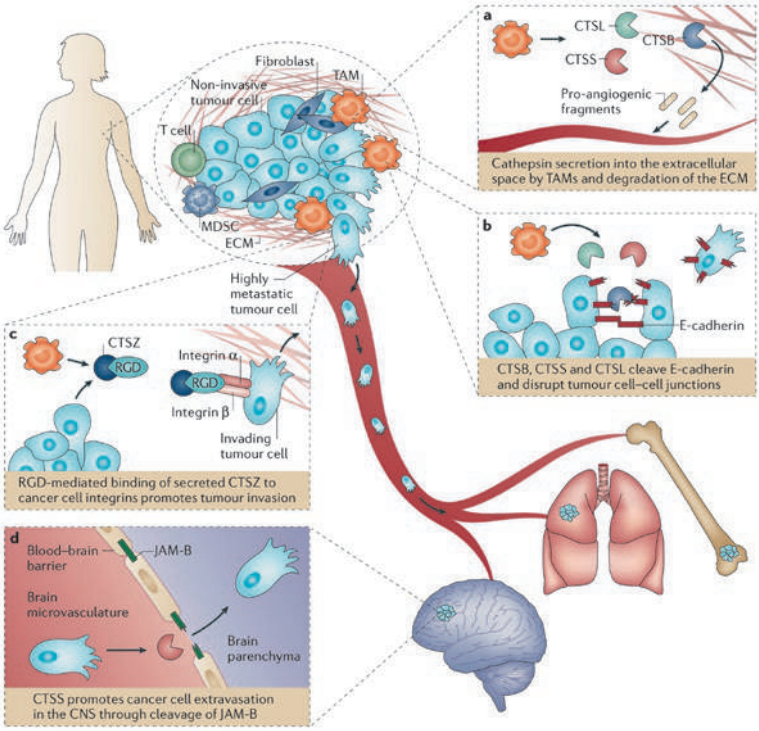


***Figure 9:*** *Cathepsin proteases in tumor progression and the metastatic cascade. (a) Cathepsins can be supplied from multiple cellular sources within the tumor microenvironment, including cancer cells and infiltrating immune cells such as TAMs. Cathepsins have crucial roles both intracellularly and extracellularly in the promotion of tumor progression, for example, by ECM degradation. (b) Secreted cathepsin B (CTSB), CTSL and CTSS can cleave the cell adhesion molecule E-cadherin, promoting cancer cell invasion into the surrounding tissue. (c) The pro-form of CTSZ, secreted by either TAMs or cancer cells, binds to cancer cell integrins through the Arg-Gly-Asp (RGD) domain to promote invasion. (d) Secretion of CTSS by circulating breast cancer cells has been shown to be crucial for their ability to cross the blood-brain barrier (BBB) and metastasize to the central nervous system. Cancer cells use this proteolytic activity to cleave junctional adhesion molecules, specifically JAM-B, in order to disrupt the integrity of the BBB and allow for their extravasation. From Olson and Joyce, Nat Rev Cancer (2015), depicting data compiled from Gocheva et al, Genes Dev (2006); Akkari et al, Genes Dev (2014); Sevenich et al, Nat Cell Biol (2014).*

In addition to these targeted candidate approaches, which were each very fruitful in identifying *bona fide* cathepsin substrates, we also collaborated with the lab of Chris Overall in Vancouver to perform unbiased proteomics screens *in vivo* (Prudova et al., 2016). By applying 8-plex iTRAQ terminal amine isotopic labeling of substrates (TAILS), a systems-level N-terminome degradomics approach, we identified cleavage sites for *in vivo* substrates of cathepsins B, H, L, S, and Z within the TME by taking advantage of the different cathepsin knockouts we had generated in the RIP1-Tag2 background (Table 1). We validated several of the substrates using independent experimental approaches, including the glycolytic enzyme pyruvate kinase M2 associated with the Warburg effect, the ER chaperone GRP78, and the oncoprotein prothymosin-alpha.

Collectively, our studies over the past decade have revealed novel, unexpected roles for cathepsin proteases as critical processing and activation enzymes, functioning as "master regulators" at the apex of multiple protease networks, thereby greatly expanding their functions in cancer beyond simple matrix degradation (Mason and Joyce, 2011; Sevenich and Joyce, 2014; Olson and Joyce, 2015).

In parallel with the findings discussed here, we have also had many successful collaborations with colleagues exploring the roles of tumor-associated macrophages and myeloid cells in numerous diverse contexts. This includes investigating TAM metabolism within the TME with Carlos-Carmona Fontaine and Joao Xavier; exploring how the senescence-associated secretory program in the liver TME impacts macrophage polarization with Amaia Lujambio, Leila Akkari and Scott Lowe; targeting TAMs in thyroid cancer with Mabel Ryder and Jim Fagin; working with Matej Krajcovic and Mike Overholtzer on phagocytosis, lysosome fission and nutrient uptake; Rich Bakst and Rich Wong on the promotion of perineural cancer invasion by inflammatory monocytes; imaging of TAMs in breast cancer with Avigdor Leftin, Nir Ben-Chetrit and Jason Koutcher, and lipid flux in macrophages with Prakrit Jena and Dan Heller; and injury-related brain inflammation with Nduka Amankulor and Eric Holland (Amankulor et al., 2009; Carmona-Fontaine et al., 2013; Krajcovic et al., 2013; Lujambio et al., 2013; Ryder et al., 2013; Bakst et al., 2017; Carmona-Fontaine et al., 2017; Jena et al., 2017; Leftin et al., 2017), among other studies.

*Microenvironmental regulation of therapeutic efficacy*

While the TME is now recognized to critically modulate cancer progression, our understanding of its potential role in regulating treatment response is still in its infancy (Klemm and Joyce, 2015). Solid tumors respond to conventional anti-cancer therapies, including chemotherapy and radiation, with many acute changes. Unfortunately, tumors frequently recover from these assaults and re-establish growth. Several years ago, we postulated that there are specific needs for stromal cells and TME-supplied factors under these conditions to enhance tumor cell survival and drive ECM remodeling and revascularization, thus re-establishing a favorable environment for growth. Similar processes at work during different stages of tumor progression have been shown to require the trophic functions of TAMs (Noy and Pollard, 2014). We therefore reasoned that TAMs and their associated products are ideal candidate modulators of response to therapy.

Indeed, Tanaya Shree and Oakley Olson, two graduate students in my lab, found increased TAM accumulation and cathepsin protease levels in breast tumors from patients and mouse models following Taxol chemotherapy (Shree et al., 2011). Cathepsin-expressing macrophages protected against Taxol-induced tumor cell death in co-culture, an effect fully reversed by cathepsin inhibition and mediated partially by cathepsins B and S. They also found that macrophages protected against tumor cell death induced by additional chemotherapies from a broader panel that they investigated, specifically etoposide and doxorubicin. Critically, combining cathepsin inhibition with chemotherapy *in vivo* significantly enhanced efficacy against primary and metastatic tumors (Shree et al., 2011), supporting the therapeutic relevance of this effect.

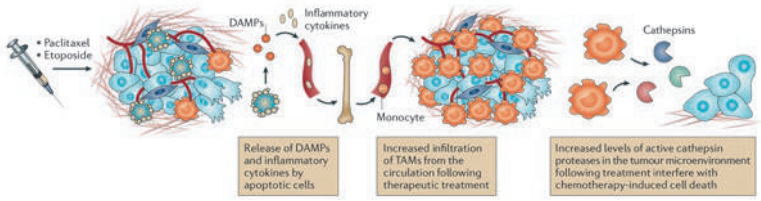


***Figure 10:*** *Cathepsin proteases and therapeutic resistance. Adaptive upregulation of cathepsins can occur through the increased recruitment of cathepsin-high TAMs in response to chemotherapeutic agents such as paclitaxel or etoposide. These adaptive increases in intratumoural cathepsin activity levels blunt therapeutic efficacy, which can accordingly be improved by cathepsin inhibition. Schematic from Olson and Joyce, Nat Rev Cancer (2015), depicting data from Shree, Olson et al, Genes Dev (2011).*

We recently extended this initial finding (Figure 10) by incorporating live cell imaging to investigate precisely how TAMs impact Taxol-induced alterations in the mitotic arrest of cancer cells, through a collaboration with Emily Foley at MSKCC, that was led by Oakley Olson in my lab. Oakley found that macrophages suppress the duration of Taxol-induced mitotic arrest in breast cancer cells and promote earlier mitotic slippage (Olson et al., 2017a). This correlated with a decrease in the phosphorylated form of histone H2AX ($\gamma$H2AX), decreased p53 activation, and reduced cancer cell death in interphase. He found that acute and specific depletion of major histocompatibility complex class II (MHCII)-low TAMs increased Taxol-induced DNA damage and apoptosis in cancer cells, leading to greater efficacy in preclinical intervention trials. Oakley's mechanistic investigations also revealed the importance of the MAPK/ERK kinase (MEK) pathway in this protective effect (Figure 11), and MEK inhibition blocked the protective capacity of TAMs and phenocopied the effects of TAM depletion on Taxol treatment *in vivo* (Olson et al., 2017a). Thus, we found that TAMs suppress the cytotoxic effects of Taxol, in part through cell non-autonomous modulation of mitotic arrest in cancer cells, and consequently targeting TAM-cancer cell interactions potentiates Taxol efficacy (Shree et al., 2011; Olson et al., 2017a).

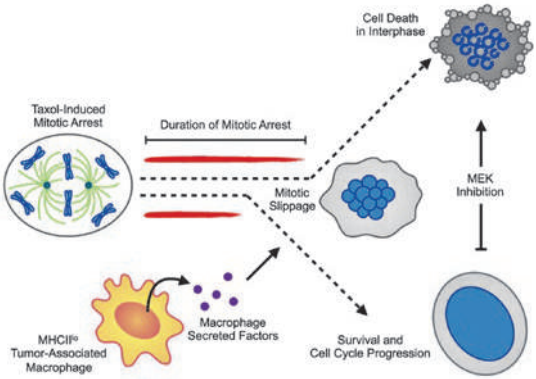


***Figure 11:*** *Tumor-associated macrophages (TAMs) suppress the cytotoxic activity of anti-mitotic agents. We investigated how TAMs suppress the duration of Taxol-induced mitotic arrest in breast cancer cells using live cell imaging. We found that TAMs promote cancer cell viability following mitotic slippage through a mechanism that is sensitive to MEK inhibition. Acute depletion of MHCII-low TAMs in a preclinical breast cancer model increased the ability of Taxol to induce apoptosis and improved therapeutic response. From Olson et al, Cell Reports (2017).*

In addition to our investigation of TAMs in breast cancer, we are actively exploring how the TME changes dynamically in response to therapeutic intervention in brain cancers, and consequently determining which TME components to target for combination therapies. One recent example of this analysis relates to gliomas, where we have shown that TAMs interfere with the efficacy of molecularly-targeted tyrosine kinase inhibitors (TKIs) *in vivo* (Yan et al., 2017). Dongayo Yan in my lab found that while these inhibitors effectively killed glioma cells in culture, they showed minimal effects in mice; indicating that a TME-medicated resistance mechanism may be involved. Indeed, we showed that the CSF-1R inhibitor PLX3397 restored the sensitivity of glioma cells to TKIs *in vivo* in preclinical drug combination trials. Together, these representative studies highlight the importance of TAMs and the microenvironment in modulating therapeutic response, a concept that has been demonstrated in additional cancers by a number of other groups (reviewed in Klemm and Joyce, 2015; Ruffell and Coussens, 2015), and which may have important translational relevance for patients.

*Microenvironmental regulation of metastasis*

Cancer cells in an aggressive primary tumor are adept at exploiting their local tissue environment. By contrast, when metastatic cells leave these favorable surroundings, they must possess or acquire traits that will allow them to survive and colonize foreign, potentially hostile tissue environments (Figure 12). The obstacles that metastasizing tumor cells encounter vary from organ to organ, and are highly influenced by cells of the TME (Joyce and Pollard, 2009; Quail and Joyce, 2013). Indeed, dissemination can occur to multiple organs, yet metastatic tumors typically grow in only one or a few sites, indicating critical roles for the microenvironment in this process, as already appreciated by Paget in the late 19th century (Paget, 1889).

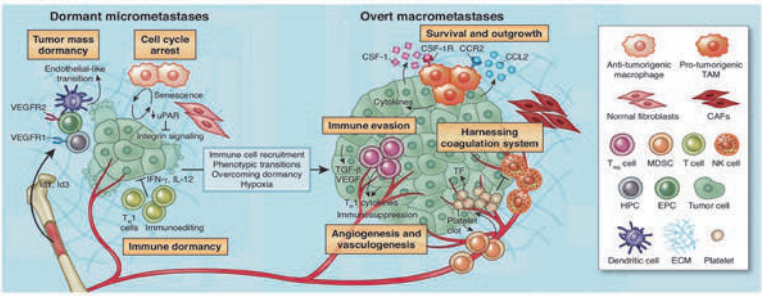


***Figure 12:*** *Initiation of secondary outgrowth in metastatic niches. Dormant micrometastases are held in check by several mechanisms including tumor mass dormancy, or angiogenic dormancy, when proliferation is balanced by apoptosis because of a lack of vasculature and limited supply of nutrients and oxygen. Multiple TME cell types contribute to the re-establishment of vascularity at the secondary site, including myeloid and endothelial cell progenitors and TAMs. In addition, tumor cells can enter immune-induced dormancy whereby immunogenic cells are cleared, and cells that are able to survive enter a state of equilibrium. Immune suppressor cells are recruited to tumors in response to this process and contribute to the establishment of an immunosuppressive state within secondary tissues. Once micrometastases overcome dormancy, they become receptive to signals and cell types within the TME to further support their expansion. For example, TAMs are abundant in metastases of multiple cancer types and support different tumorigenic processes to allow for outgrowth, including vascularization, impaired immunogenicity and enhanced survival in overt metastases. Platelets, and components of the coagulation system are also important mediators of metastatic outgrowth, as they interfere with the ability of natural killer (NK) cells to destroy micrometastases and support clot formation, which in turn causes the recruitment of myeloid suppressor cells. From Quail and Joyce, Nature Medicine (2013).*

To gain insights into how different tissue environments influence metastasis we analyzed tumor–microenvironment interactions that modulate organ tropism of brain, bone and lung metastasis. We took advantage of organ-specific models of breast cancer metastasis to these sites which had been previously developed by our collaborator Joan Massagué at MSKCC, and investigated gene expression in a tissue- and stage-dependent manner (Sevenich et al., 2014). The "HuMu" screens we performed focused on analysis of proteases and their endogenous inhibitors, that we and others had shown to be important in the primary cancer TME (reviewed in Mason and Joyce, 2011; Sevenich and Joyce, 2014), but which were relatively understudied in metastasis.

We identified numerous differentially expressed proteases and inhibitors that were regulated in either a stage- or tissue-specific manner in different metastatic TMEs (Sevenich et al., 2014). By querying whether expression of these genes in primary breast cancer patients was associated with metastasis-free survival in brain, bone or lung, we were able to apply an additional filter that allowed restriction of the gene lists to only those that showed a significant correlation with survival. One such protease was cathepsin S in which high expression in breast cancer patients correlated with decreased brain metastasis-free survival. Lisa Sevenich, a postdoc in my lab who led this study along with Bobby Bowman and Steve Mason (Sevenich et al., 2014), found that both TAMs and tumor cells produce cathepsin S, and only their combined depletion significantly reduced brain metastasis *in vivo*. Lisa discovered that cathepsin S specifically mediates blood-brain barrier penetration through proteolytic processing of the junctional adhesion molecule, JAM-B, thereby enabling endothelial cell transmigration (Sevenich et al., 2014) (Figure 9d). Interestingly, cathepsin S is typically predominantly produced by immune cells during homeostasis (Olson and Joyce, 2015). In brain metastasis, we therefore proposed that the induction of cathepsin S expression in cancer cells of epithelial origin may indicate a type of "leukocytic mimicry" whereby metastatic tumor cells could implement immune-cell-like expression programs that enhance mobilization and cell motility (Sevenich et al., 2014); a hypothesis that may extend to other components of the brain TME (Quail and Joyce, 2017a).

Beyond the local TME, an inflammatory systemic environment can also affect disease outcome, by perturbing homeostasis within multiple tissues throughout the body. This becomes particularly important during metastasis, where systemic alterations can modify the tissue landscape of distant organs and support tumor cell colonization by establishing a pre-metastatic niche (McAllister and Weinberg, 2014). Indeed, chronic inflammation can significantly increase cancer risk and disease progression (Quail and Joyce, 2013). Investigation into how the systemic environment affects tumor biology is therefore critical for an integrated understanding of cancer. As such, we have begun to explore how the systemic microenvironment modulates tumorigenesis and metastasis. We first chose to assess the clinically relevant case of obesity-associated chronic inflammation, as it can disrupt homeostasis within tissue microenvironments (Olson et al., 2017b). Given the correlation between obesity and increased relative risk of death from breast cancer, we focused on determining whether obesity-associated inflammation promotes metastatic progression (Quail et al., 2017).

In this study, Daniela Quail and Oakley Olson together showed that obesity causes lung neutrophilia in otherwise-normal individuals (Quail et al., 2017). They found this occurred independently of diet content; rather it was directly related to increased adiposity and the production of IL5 by adipose tissue. They found that IL5 increases *Csf2* (GM-CSF) expression by IL5R+ monocytes, and enhances neutrophil trafficking to lung (Figure 13). Furthermore, elevated serum GM-CSF promotes myelopoiesis, leading to an expansion of peripheral neutrophils. In mouse models, obesity-associated lung neutrophilia enhanced breast cancer metastasis to this organ, and depletion of Gr1+ neutrophils in obese animals reversed this effect (Olson et al., 2017b; Quail et al., 2017). We also found that GM-CSF is predominantly expressed in the lungs of obese mice, and that GM-CSF blockade *in vivo* reverses the pro-metastatic effects of obesity. Interestingly, weight loss was equally effective at reversing all these phenomena in mice, including breast-to-lung metastasis.

In collaboration with Andrew Dannenberg, Peter Holt and colleagues in their labs in New York, we had the opportunity to analyze human serum from morbidly obese individuals who had undergone a 10 % weight loss following diet restriction. This weight loss was associated with reduced

serum IL5 and GM-CSF, concomitant with decreased circulating neutrophils. Collectively, our findings also have implications for the long-term management of obese breast cancer patients, as lung inflammation may have prognostic value. Clinical studies are thus needed to appropriately manage the obese cancer patient population, and to uncouple the comorbidity of obesity and cancer (Olson et al., 2017b).

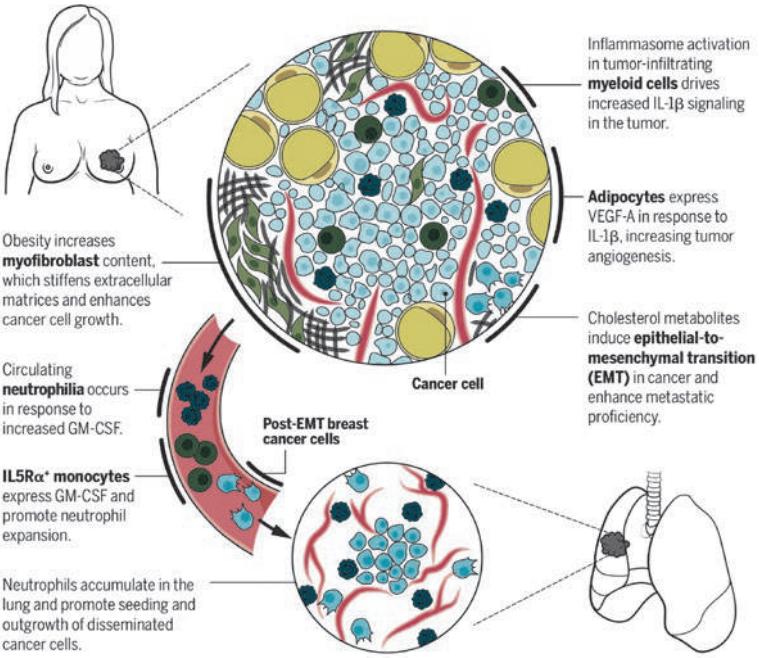


***Figure 13:*** *Obesity drives alterations in the local tumor microenvironment, and systemic changes, which enhance cancer progression and metastasis. The effects of obesity on cancer progression are depicted using breast cancer as a representative example, based on studies from mice and humans, including our own research (Quail, Olson et al, Nat Cell Biol 2017). Obesity promotes both primary tumor growth and metastatic progression through systemic alterations that affect tissue homeostasis. From Olson, Quail and Joyce, Science (2017).*

*Conclusions and perspectives*

We have been fortunate to gain important insights into many of the questions posed when I initiated my lab's research program over a decade ago, as highlighted by the representative studies discussed here. We have identified several mechanisms of TAM education, elucidated processes by which TAMs, neutrophils and other immune cells promote tumorigenesis, discovered that TAMs have potent protective functions in blocking therapeutic efficacy, identified the TME as a major mediator of resistance to TAM therapies, and have helped to illuminate the interplay between the TME and cancer cells during different stages of the metastatic process.

Going forward, we are focusing much of our efforts in my lab on understanding and therapeutically targeting brain malignancies and metastatic disease, both from the perspective of the TME (Figure 14). Glioblastomas and brain metastases are among the most lethal of cancers, with an average lifespan of a year or less following diagnosis. Given this dismal patient prognosis, we became very interested in studying these particular brain malignancies several years ago, and in investigating both the similarities and differences between primary and metastatic brain cancers, which may have important implications for understanding differential immunotherapy efficacy, for example. While we have been able to make several important insights into the brain TME from our recent studies (Pyonteck et al., 2013; Bowman and Joyce, 2014; Sevenich et al., 2014; Bowman et al., 2016; Quail et al., 2016; Yan et al., 2017), as a field we have much to discover and understand about the unique and particularly challenging microenvironment of brain cancers (Quail and Joyce, 2017a).

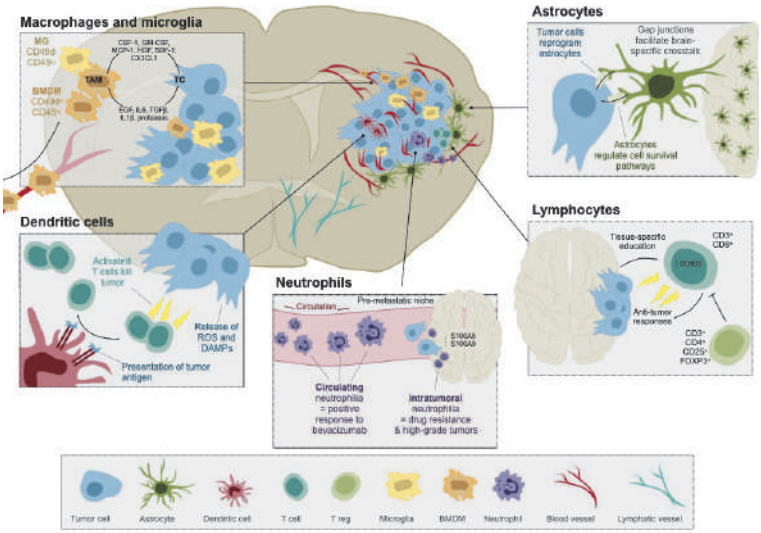


***Figure 14:*** *The microenvironmental landscape of brain cancers. Brain tumors are composed of diverse cellular players, ranging from peripherally-derived immune cells to various specialized organ-resident cell types, such as astrocytes. Each of these cell types contributes to brain tumor biology in unique ways. For example, tumor-associated macrophages and microglia (TAMs) arise from two distinct sources, including the periphery (bone marrow-derived macrophages, BMDMs; CD49d+) or the yolk sac (microglia, MG; CD49d-). TAMs engage in significant bidirectional crosstalk with tumor cells (TC) in the brain, whereby brain tumor cells release cytokines and chemoattractants to recruit TAMs to the microenvironment, and TAMs in turn supply pro-tumorigenic, pro-survival factors. Adapted from Quail and Joyce, Cancer Cell (2017).*

It will be essential to advance our current knowledge of individual brain TME components into a more complex microenvironmental landscape in which we analyze these cellular and non-cellular components as part of an integrated whole. Moreover, investigating the evolution of the brain TME as a dynamic process, incorporating detailed timecourse analyses in patients and live imaging of TME cells and components in mice, will reveal critical information that single timepoints cannot capture. Similarly, major insights can be expected from a detailed comparison of how distinct molecular sub-types or genetic drivers in cancer cells may differentially sculpt their microenvironment during the course of cancer pro-

gression. Although as a field it is widely recognized that there are cancer cell-intrinsic differences in tumor evolution and response to therapy by virtue of different molecular subtypes, appropriate dissection of the different TME determinants of therapeutic response is still in its infancy, and largely untapped clinically. Going forward, it will therefore be critical to determine the many differences in microenvironmental composition between distinct tumor subtypes in order to achieve a comprehensive understanding of tumor biology, including consideration of matrix stiffness, tumor-stromal interactions, and immune cell landscapes.

Moreover, it will be important to globally address how all aspects of the TME are affected by both standard of care therapy and new investigational therapies across all brain tumors and their respective molecular subtypes. From a practical perspective, we need to engage actively with medicinal chemists to improve drug delivery into the brain; a perennial challenge for all brain-targeted therapies, including those directed against the TME. We need to understand how the generally immunosuppressive environment of the brain is further exacerbated in the context of brain cancers in order to devise therapies to overcome this. Finally, if we cannot take a "one size fits all" approach for targeting the TME in different brain malignancies, we will need to determine where the vulnerable points are to attack at a more personalized level. Given the current advances being made in the immunotherapy and TME fields, however, we can also expect an exciting and illuminating time ahead for basic research and clinical translation in brain cancers, and for microenvironment biology as a whole.

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## Die Stiftung Prof. Dr. Max Cloëtta

Die Stiftung Prof. Dr. Max Cloëtta wurde am 27. September 1973 in Zürich von Dr. Antoine Cloëtta zu Ehren seines Vaters Prof. Dr. Max Cloëtta errichtet.

In Absatz 1 von Art. 3 der Stiftungsurkunde wird der Zweck der Stiftung wie folgt umschrieben:

«Die Stiftung bezweckt:
a) die Unterstützung und Förderung der medizinischen Forschung sowie der damit verbundenen naturwissenschaftlichen Hilfsdisziplinen in der Schweiz;
b) die Schaffung und jährliche Verleihung eines

Cloëtta-Preises

zur Auszeichnung schweizerischer und ausländischer Persönlichkeiten, die sich in besonderer Weise um bestimmte Gebiete der medizinischen Forschung verdient gemacht haben.»

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