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STIFTUNG PROFESSOR DR. MAX CLOËTTA

Heft Nr. 48

Prof. Dr. Mohamed Bentires-Alj

«Breast tumor heterogeneity, metastasis, and therapy resistance in the era of personalized medicine»

Prof. Dr. Nadia Mercader Huber

«Heart development and regeneration in the zebrafish»

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INHALT

VORWORT

Prof. Dr. Fritjof Helmchen

Das Jahr 2020 ist durch die Covid-19 Pandemie geprägt, welche uns deutlich vor Augen führt, wie wichtig wissenschaftlicher Fortschritt, medizinische Forschung und die Entwicklung neuer klinischer Behandlungsmethoden sind. Alle drei Bereiche greifen ineinander: Erstens die Grundlagenforschung, welche durch Neugierde angetrieben neues Wissen schafft, teils planmässig, teils unerwartet und zufällig; zweitens die gezielte Erforschung spezifischer medizinischer Probleme, mit dem Ziel, durch ein verbessertes Verständnis von Krankheitsmechanismen die Basis für neue Behandlungsmethoden zu legen; und drittens die Therapieentwicklung und klinische Anwendung, welche die neuen wissenschaftlichen Erkenntnisse zum Wohle der Patientinnen und Patienten umsetzen. Auch dieses Jahr freuen wir uns sehr, zwei Forscherpersönlichkeiten mit dem Cloëtta-Preis zu ehren, die herausragende Beiträge zum Brückenschlag zwischen diesen verschiedenen Aspekten der medizinischen Forschung geleistet haben und leisten.

Prof. Dr. Nadia Mercader Huber hat grundlegende Erkenntnisse zur Regenerationsfähigkeit von Gewebe gewonnen. Am Modell des Herzmuskels von Zebrafischen untersucht ihr Team die molekularen und zellulären Mechanismen, welche beeinflussen, inwieweit sich Herzmuskelgewebe nach Verletzungen erholen kann. Ein vertieftes Verständnis dieser Mechanismen kann zu neuen Ansätzen zur Unterstützung von Geweberegeneration auch im Säugetier führen.

Als zweiter diesjähriger Preisträger wird **Prof. Dr. Mohamed Bentires-Alj** für seine grundlegenden Forschungsbeiträge zum Verständnis der Tumorvielfalt bei Brustkrebs ausgezeichnet. Sein Team erforscht die genetischen und zellulären Ursachen der Tumorheterogenität sowie die Konsequenzen für Metastasenbildung und Therapieresistenz. Die Ergebnisse können auch hier neue Wege für Therapien bahnen.

Mit der Verleihung des Cloëtta-Preises wird die beeindruckende wissenschaftliche Leistung von Prof. Dr. Nadia Mercader Huber und Prof. Dr. Mohamed Bentires-Alj gewürdigt. Die Stiftung Prof. Dr. Max Cloëtta

freut sich, dass trotz Covid-19-Pandemie die Feier zur Preisverleihung stattfinden kann – wenn auch in kleinerem Kreis als gewohnt – und dass sie die Preisträger und Gäste am 6. November 2020 in Bern begrüssen kann.

Zum Schluss noch ein ganz besonderer Dank an Brigitt Küttel, die seit 25 Jahren die Geschäfte der Stiftung Prof. Dr. Max Cloëtta geführt hat. In diesem Vierteljahrhundert hat sie sich mit grossem Einsatz für die Stiftung eingesetzt, und die Stiftung ist ihr sehr ans Herz gewachsen, wie wir alle immer sehr deutlich spüren konnten. Nach dieser langen und intensiven Zeit gibt sie nun die Geschäftsführung ab, und Anja Witte wird die Geschäftsführung ad interim übernehmen.

Liebe Brigitt, im Namen des gesamten Stiftungsrats danken wir Dir sehr herzlich für Dein grossartiges Engagement für die Stiftung und für Deine unschätzbaren und vielfältigen Beiträge. Wir wünschen Dir alles Gute für Deine Zukunft und freuen uns, wenn Du der Cloëtta Stiftung auch weiterhin verbunden bleibst.

Brigitt Küttel

Geschäftsführerin

Stiftungsrat

Nach Drucklegung des Heftes Nr. 47/2019 erreichte uns die traurige Nachricht vom Hinschied unseres langjährigen Stiftungsratsmitglieds Prof. Dr. Max M. Burger (Mitglied 1995–2000, Präsident 2000–2008). Während 13 Jahren hatte Professor Burger – neben seinem grossen beruflichen Engagement – die Geschicke der Stiftung Prof. Dr. Max Cloëtta massgeblich geformt. Preisträger, Stipendiaten, Stiftungsrat und Geschäftsstelle durften miterleben, wie er sich unentwegt für den wissenschaftlichen Nachwuchs in der medizinischen Forschung eingesetzt hat. Unsere Zusammenarbeit war geprägt von seinem immensen Fachwissen, unerschöpflichen Engagement, Humor, Interesse für den einzelnen Menschen und von grosser persönlicher Wertschätzung. Es war eine Zeit, die wir in bester Erinnerung behalten und für welche wir ihm von Herzen dankbar sind.

Die Zusammensetzung des Stiftungsrates aus sechs hochkarätigen Medizinprofessoren und drei anerkannten Experten auf dem Gebiet der Finanzen und des Rechts hat sich auch im sehr speziellen Pandemiejahr 2020, das von allen Beteiligten Flexibilität und Innovation verlangt, wieder bewährt.

An dieser Stelle möchten wir uns – wie jedes Jahr – ausdrücklich bedanken bei den Mitgliedern des Stiftungsrates, die ihr Fachwissen und ihre Erfahrung einbringen, und bei den Expertinnen und Experten, deren Gutachten die Entscheidungsfindung auch bei der Auswahl der Cloëtta-Preisträger unterstützen. Erst diese breit abgestützte Kompetenz ermöglicht es der Stiftung, ihren Zweck wirkungsvoll umzusetzen.

Cloëtta-Preis

Der Stiftungsrat und die Geschäftsstelle freuen sich, 2020 zwei hochkarätige Preisträger aus der medizinischen Grundlagenforschung mit dem Cloëtta-Preis auszuzeichnen: Der erste Preis geht an Prof. Dr. Nadia Mercader Huber, Co-Direktorin des Instituts für Anatomie und ordentliche

Professorin an der Universität Bern. Mit Herrn Prof. Dr. Mohamed Bentires-Alj wird ein herausragender Forschungsgruppenleiter und ordentlicher Professor des Departement Biomedizin der Universität Basel und des Universitätsspitals Basel geehrt. Unser herzlicher Dank gilt den Verantwortlichen der Universität Bern, wo wir dieses Jahr zu Gast sein dürfen, und ihrem Vertreter in unserem Stiftungsrat, Prof. Dr. Hugues Abriel, für seine grosse Unterstützung.

Forschungsstellen

Die Forschungsstellen 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 Berufung auf eine ordentliche Professur zu überbrücken. Die Stipendien werden alle zwei Jahre vergeben, die Evaluation der Bewerbungen auf die Ausschreibung 2020 läuft derzeit.

2020 finanzierte die Stiftung Prof. Dr. Max Cloëtta folgende Forschende an Schweizer Universitäten mit dreieinhalb- bis fünfjähriger Unterstützungsperiode:

Dr. András Jakab, 1985, Universitäts-Kinderspital Zürich, Center for MR-Research. Projekt: «From axons to therapy: Characterizing the connectivity of the human thalamus with 3D multi-scale imaging». Unterstützungsdauer: 1.10.2020 – 31.5.2024

Dr. Mathias Hauri-Hohl, 1975, Universitäts-Kinderspital Zürich, Abt. Stammzellentransplantation. Projekt: «Improving T-cell reconstitution and enhancing central tolerance mechanism in hema-topoietic stem cell transplantation». Unterstützungsdauer: 1.1.2016 – 31.5.2021 (Sistierung 1.4.2018 – 31.8.2018)

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

Dr. Paula Nunes-Hasler, 1980, Universität Genf, Institut für Pathologie und Immunologie. Projekt: «Exploring the ER-phagosome connection during antigen cross-presentation». Unterstützungsdauer: 1.10.2019 – 30.9.2024

Dr. Aiman Saab, 1982, Universität Zürich, Institut für Pharmakologie und Toxikologie. Projekt: «Impact of neuron-glia metabolic coupling on brain function, plasticity and aging». Unterstützungsdauer: 1.6.2019 – 30.9.2020 (ursprünglich bis 31.5.2024, ab 1.10.2020 Wechsel auf SNSF Eccellenza Professorial Fellowship)

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 – 30.9.2021 (Sistierung 1.9.2019 – 31.12.2020)

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. 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

Klinische Medizin Plus

Seit 2010 vergibt die Stiftung Prof. Dr. Max Cloëtta Stipendien «Klinische Medizin Plus». Medizinerinnen und Medizinern werden während oder unmittelbar nach Abschluss ihrer Facharztausbildung Stipendien von drei bis maximal zwölf Monaten für die Absolvierung einer Spezialausbildung an einer renommierten, vornehmlich ausländischen Institution ausgerichtet.

2020 kommen folgende Medizinerinnen und Mediziner in den Genuss eines Stipendiums:

Dr. med. Aurélien Lathuilière, 1983, Resident, Neurology division am HUG-Hôpital de Bellerive, Genf. Projekt: Personalized medicine in Alzheimer's disease. Guest Institution: Massachusetts Alzheimer's Disease Research Center, Massachusetts General Hospital and Harvard Medical School, Boston, USA, 1.8.2019 – 31.7.2020

Dr. med. Thomas Nestelberger, 1986, Resident, Klinische Kardiologie am Universitätsspital Basel. Projekt: Clinical Research Fellowship: Incidence, Predictors, Biochemical Signatures and Prognostic Value of Spontaneous Coronary Artery Dissection. Guest Institution: Vancouver General Hospital and University of British Columbia in Vancouver, Kanada, 1.7.2020 – 30.06.2021

Dr. med. Pascale Tinguely, 1983, Staff surgeon, Universitätsklinik für Viszerale Chirurgie und Medizin am Inselspital Bern. Projekt: Advanced studies of clinical science, epidemiology, and biostatistics. Local ablation versus surgical resection for colorectal liver metastases – Population-based analyses on survival and local recurrence prediction. Guest Institution: Department of Clinical Science at Danderyd University Hospital, Karolinska Institute, Stockholm, Schweden, 1.1.2020 – 31.12.2020

Dr. med. Julia Velz, 1983, Resident, Klinik für Neurochirurgie am UniversitätsSpital Zürich. Projekt: 1. Specialized training to gain knowledge and expertise in the field of Pediatric Neurosurgery. 2. Investigation of the underlying genetical and immunological mechanisms in medulloblastoma, the most common malignant pediatric brain tumor. Guest Institution: Department of Pediatric Neurosurgery at Hôpital Necker-Enfants Malades, Paris, Frankreich, 1.7.2020 – 30.6.2021

Wechsel in der Geschäftsführung

Ende 1995, im 22. Jahr der Stiftung, durfte ich die Geschäftsführung der Stiftung Prof. Dr. Max Cloëtta übernehmen. Während dieser 25 Jahre wurde die Stiftung stets durch einen engagierten Stiftungsrat und von fünf Präsidenten geleitet. Mit jedem einzelnen von Ihnen war die Zusammenarbeit stets erfreulich und von gegenseitiger Wertschätzung geprägt. 2008 wurde mit Prof. Susanne Suter die erste Frau in den Stiftungsrat gewählt, seit 2014 ist Prof. Daniela Finke die zweite weibliche Vertretung einer der medizinischen Fakultäten. Darüber habe ich mich besonders gefreut.

Die Arbeit für die Stiftung durchlief im letzten Vierteljahrhundert eine starke Veränderung. Bekamen wir jahrelang von ausländischen Laudatoren per Kurier ihre Beiträge auf Papier und verbrachten Stunden mit der genauen Überprüfung der Druckfahnen, sind diese Abläufe durch die Digitalisierung heute viel einfacher geworden. Stets aber blieb das Ziel im Fokus: junge, talentierte Forscherinnen und Forscher zu fördern. Als privatrechtliche Stiftung hat die Stiftung Prof. Dr. Max Cloëtta die Möglichkeit, dort aktiv zu werden, wo den Universitäten und Kliniken Grenzen gesetzt sind, und flexibel und bedürfnisgerecht neue Programme zu lancieren. Die Forschungsstellen unterstützen, wie oben erwähnt, den akademischen Mittelbau und tragen dazu bei, dass hoch qualifizierte Forscherinnen und Forscher in der Schweiz bleiben, statt ins Ausland zu wechseln. Die vor zehn Jahren geschaffenen Stipendien Klinische Medizin Plus ermöglichen klinisch tätigen Medizinerinnen und Medizinern eine Spezialausbildung an renommierten Institutionen im Ausland, das dort erworbene Fachwissen bringen sie anschliessend zurück in die Schweiz.

2023 wird die Stiftung ihr 50-jähriges Bestehen feiern dürfen. 50 Jahre erfolgreiches Wirken für die Förderung der medizinischen Forschung in der Schweiz. Die «Cloëtta-Family» von aktuellen und ehemaligen Preisträgerinnen und Preisträgern, Stipendiatinnen und Stipendiaten und alle Mitglieder des Stiftungsrates dürfen stolz sein auf das Erreichte, und ich bin sicher, dass sie dies in würdigem Rahmen feiern werden.

Für mich persönlich ist es nach 25 Jahren Zeit geworden, Abschied zu nehmen. Die Stiftung ist mir ans Herz gewachsen, auch ich bin mit Stolz

erfüllt, sie so lange Zeit mit geprägt haben zu dürfen. Ich freue mich, dass mit Anja Witte eine wunderbare, qualifizierte und sehr engagierte Frau meine Nachfolge zunächst ad interim übernimmt. Liebe Anja, ich wünsche dir viel Freude und Erfolg als neue Geschäftsführerin (ad interim)!

Meine guten Gedanken begleiten die Stiftung, der ich mich immer verbunden fühlen werde, in ihre Zukunft. Dem Stiftungsrat danke ich für das grosse Vertrauen. Ihr werdet mir fehlen!

THE CLOËTTA PRIZE 2020
IS AWARDED TO

PROFESSOR

MOHAMED BENTIRES-ALJ

BORN IN 1972 IN CASABLANCA, MOROCCO
DEPARTMENT OF BIOMEDICINE
UNIVERSITY OF BASEL AND UNIVERSITY
HOSPITAL BASEL

FOR HIS GROUND-BREAKING CONTRIBUTIONS TO
BREAST CANCER RESEARCH AND HIS
EXTRAORDINARY COMMITMENT TO NETWORKING
BETWEEN BASIC AND CLINICAL RESEARCH

BERN, 6TH NOVEMBER 2020

IN THE NAME OF THE FOUNDATION BOARD:

THE PRESIDENT

THE VICE PRESIDENT

A MEMBER

MOHAMED BENTIRES-ALJ

CURRICULUM VITAE

Family name: Bentires-Alj
First name: Mohamed
Date of birth 18.07.1972
Place of birth: Casablanca (Morocco)
Citizenships: Belgian and Moroccan

Professor of Experimental Surgical Oncology
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E-mail: m.bentires-alj@unibas.ch; +41 (0) 61 26 53 313
URL for web site: https://bentireslab.org/

Education

1996	Pharmaceutical Sciences, University of Liège, Belgium
2001	Ph.D. *(summa cum laude)* in Pharmaceutical Sciences, University of Liège, Belgium

Current Positions

2017–	Chair of the Swiss Personalized Oncology Chair of the Basel personalized health "cancer cluster"
2016–	Professor of experimental surgical oncology, University of Basel

Previous Positions

2013–2016	Senior staff scientist at the Friedrich Miescher Institute, Basel, Switzerland
2006–2013	Junior group leader at the Friedrich Miescher Institute, Basel, Switzerland
2004–2006	Research Assistant, National Fund for Scientific Research (FNRS), Belgium
2001–2006	Postdoctoral fellow: Harvard Medical School, Beth Israel Deaconess Medical Center, Boston, USA

Approved research projects (since 2015)

2021–2024:	MSCA ITN project EVOMET: Horizon 2020
2020–present	Board member of IABCR: International Association for Breast Cancer Research
2019–2023:	Swiss National Foundation (SNF)
2019–2021:	Krebsliga Beider Basel
2019–2022:	OncoSuisse grant. Swiss Cancer League
2018–2021:	Swiss Personalized Health Network (SPHN) driver project
2016–2021:	European Research Council (ERC) advanced investigator grant
2015–2018:	Swiss Initiative in Systems Biology: SystemsX

Prizes, fellowships, distinguished memberships (since 2010)

2016	Elected European Molecular Biology Organization (EMBO) member
	European Research Council (ERC) Advanced grant
2015	American Association for Cancer Research (AACR): Outstanding Investigator in Breast Cancer Research Award
2014	Robert Wenner Award of the Swiss cancer league
	S. G. Komen for the Cure, European Association for Cancer Research (EACR) Award
	Proffered Paper Award, EACR23
	Novartis Select Award
	Chair of the Mammary Gland Biology Gordon Research Conference
2013	Novartis Select Award
2012	Dora-Seif Prize for Cancer Research, University of Basel, Switzerland
2010	European Research Council (ERC) young investigator starting grant

Board memberships

- Elected board member of the Metastasis Research Society (www.metastasis-research.org) (since 2020).

- Board member of IABCR: International Association for Breast Cancer Research (since 2020).
- Journal of Mammary Gland Biology and Neoplasia (since 2012) – *Editorial board*
- Breast Cancer Research (since 2008) – Associate *Editor*
- Cancer Research (2013-2019) – *Editorial board*
- Krebsliga Beider Basel (since 2010) – *Scientific board*
- European Network for Breast Development and Cancer (www.enbdc.org) (since 2008) – *Founder and President*
- Basel Breast Consortium (BBC) (www.BaselBC.org) (since 2014) – *Co-founder and Coordinator*
- Translational working group EU-Life (2013–2017) – *Committee Member*
- Medalis University of Strasbourg, France (since 2015) – *Scientific advisory board*
- F.R.S.-FNRS, Belgium (2015-2019) – *Scientific Commission*
- Breast Cancer Now Toby Robins Research Centre at the ICR, London – *Scientific advisory board*
- Scientific Committee of the Dora-Seif Stiftung (since 2020)

Organization of conferences (since 2014)

2019	Personalized oncology 2019, Basel, Switzerland
2018	Co-chair of the EuroPDX meeting, Weggis, Switzerland
2018	International PhD course on Frontiers in Metastasis, Basel, Switzerland
2016 – 2020	Basel Breast consortium annual meeting on personalized breast cancer treatment, Basel, Switzerland
2016	Scientific committee of the EuroPDX meeting, Weggis, Switzerland
	EU-LIFE Tumour Microenvironment and Metastasis PhD course, Copenhagen, Denmark
2015	Member of the scientific committee of the 2015 LS2 meeting, Zurich, Switzerland
2014	Chair of the Mammary Gland Biology Gordon Research Conference, Tuscany, Italy
	Organizing committee of the Targeting the kinome III meeting, Basel, Switzerland

Patents

1 Combination of a phosphoinositide 3-kinase inhibitor and a modulator of the Janus Kinase 2 – Signal Transducer and Activator of Transcription 5 pathway, FMI-087/00EP
2 Interleukin-8 and breast cancer, FMI-090/00EP
3 PTPN11 and tumor-initiating cells, FMI-077/00WO
4 Culture medium suitable for the culture of undifferentiated cells, FMI-082/00WO
5 CDCP1 and breast cancer, FMI-088/00EP
6 PTPN11 and triple-negative breast cancer, FMI-083/00WO
7 Roles of RHAU in cancer (with Yoshi Nagamine), FMI-061/00WO
8 LATS and breast cancer, FMI-EP14186104.7
9 Treating cancer by modulating RNA helicases, US Patent App. 13/120,353

SELECTED PUBLICATIONS

Glucocorticoids promote breast cancer metastasis.
Obradović MMS, Hamelin B, Manevski N, Couto JP, Sethi A, Coissieux A, Münst S, Okamoto R, Kohler H, Schmidt A, **Bentires-Alj M**
Nature, 567(7749):540-54 (2019)

The Hippo kinases LATS1/2 control human breast cell fate via crosstalk with ERα.
Britschgi A, Duss S, Kim S, Couto JP, Brinkhaus H, De Silva D, Mertz KD, Kaup D, Varga Z, Voshol H, Vissieres A, Leroy C, Roloff T, Stadler M, Koren S, Scheel C, Miraglia L., Orth P.A., Bonamy G.M.C., Reddy V, **Bentires-Alj M**
Nature, 541(7638):541-545 (2017)

PIK3CA^{H1047R} induces multipotency and multi-lineage mammary tumors.
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Nature, 525(7567):114-8 (2015)

Tyrosine phosphatase SHP2 increases cell motility in triple negative breast cancer via activation of SRC-family kinases.
Sausgruber N, Coissieux MM, Britschgi A, Wyckoff J, Aceto N, Leroy C, Voshol H, Bonenfant D, **Bentires-Alj M**
Oncogene, 34(17):2272-8 (2015)

Cessation of CCL2 inhibition accelerates breast cancer metastasis by promoting angiogenesis.
Bonapace L, Coissieux MM, Wyckoff J, Mertz K, Varga Z, Junt T, and **Bentires-Alj M**
Nature, 515(7525):130-3 (2014)

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Meier-Abt F, Milani E, Roloff T, Brinkhaus H, Duss S, Meyer DS, Klebba I, Balwierz P, van Nimwegen E, **Bentires-Alj M**
Breast Cancer Research, 15(2): R36 (2013).

The calcium activated chloride channel ANO1 promotes breast cancer progression by activating EGFR- and CAMK-signaling.
Britschgi A, Bill A, Brinkhaus H, Rothwell C, Clay I, Duss S, Rebhan M, Raman P, Guy C, Wetzel K, George E, Oana Popa M, Lilley S, Choudhury H, Gosling M, Wang L, Fitzgerald S, Borawski J, Baffoe J, Labow M, Gaither LA, **Bentires-Alj M**
PNAS plus, 110 (11) 1026-34 (2013).

JAK2/STAT5 inhibition circumvents resistance to PI3K/mTOR blockade, providing a rationale for co-targeting these pathways in metastatic breast cancer.
Britschgi A, Andraos R, Brinkhaus H, Klebba I, Romanet V, Müller U, Murakami M, Radimerski T, **Bentires-Alj M**
Cancer Cell, 22(6):796-811 (2012)

Tyrosine phosphatase SHP2 promotes breast cancer progression and maintains tumor-initiating cells via activation of key transcription factors and a positive feedback signaling loop.
Aceto N, Sausgruber N, Brinkhaus H, Gaidatzis D, Martiny-Baron G, Mazzarol G, Confalonieri S, Hu G, Balwierz P, Pachkov M, Elledge SJ, van Nimwegen E, Stadler MB, **Bentires-Alj M**
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Luminal expression of mutant *PIK3CA* in the Mammary Gland Induces Heterogeneous Tumors.
Meyer D, Brinkhaus H, Muller U, Muller M, Cardiff RD, **Bentires-Alj M**
Cancer Research 71(13):4344-51 (2011)

BREAST TUMOR HETEROGENEITY, METASTASIS, AND THERAPY RESISTANCE IN THE ERA OF PERSONALIZED MEDICINE

Mohamed Bentires-Alj [1]

Summary

Breast cancer is the second leading cause of cancer death in women and 2.1 million new patients are diagnosed with breast cancer annually. While 98% of patients survive 5 years or more after diagnosis of a localized (confined to the primary site) breast cancer, this number drops to 15–25% if the cancer has metastasized to distant organs. Thus, curing metastatic breast cancer is clearly an unmet medical need. The cellular and biochemical mechanisms that lead to drug-resistant metastases remain largely unknown and their identification has been my primary goal for the last 20 years. New therapies are likely to result from a more thorough understanding of cancer as a systemic disease involving both genomic alteration of cancer cells and dynamic crosstalk between cancer cells and the tumor microenvironment (e.g., immune cells). The thread connecting the research topics in my lab is tumor heterogeneity. We assess fundamental mechanisms that influence normal and neoplastic breast stem cells, metastasis, and resistance to targeted therapies at the molecular, cellular, and whole organism levels. These interdisciplinary projects seek to leverage a mechanistic insight into personalized therapy, which is a recent focus of the translational research that we pursue in close collaboration with clinicians from the University Hospital Basel (USB) (Figure 1) (www.bentireslab.org). In this review, I summarize a selection of our basic and translational research findings, discuss some of our ongoing projects, and highlight our efforts in personalized medicine in Basel, in Switzerland, and worldwide.

[1] Department of Biomedicine, Department of Surgery, University Hospital Basel, University of Basel, Switzerland

Introduction

"If I have seen further it is by standing on the shoulders of Giants", wrote Isaac Newton.

Observation and research in mammary gland biology and cancer over the previous centuries have laid the foundation for our current understanding of this fascinating organ. But, despite the spectacular breakthroughs in our understanding of its pathophysiology and the corresponding clinical advances made by several key figures in our field (for an oral history of our field, see https://enbdc.org/interviews/), breast cancer is still a source of worry and distress for patients.

The mammary gland. The mammary gland is an epidermal appendage that evolved with mammals around 300 million years ago, plausibly from apocrine sweat glands[1]. The branched ductal-alveolar tree making up the

Figure 1. *Research areas within the Bentires-Alj lab*

mammary gland is surrounded by a basement membrane and stromal cells, and is composed of hierarchically organized cell types that contribute to tissue homeostasis. Two major cell lineages, organized in a bi-layered structure, constitute the mammary gland epithelium. The luminal layer lining the ducts and the alveoli is composed of cells that express keratin 8/18 (K8/18) and/or estrogen and/or progesterone receptor (ER/PR). The myoepithelial layer with a basal location is composed of cells that express K5/14 and/or smooth muscle actin (SMA) and/or p63[2,3]. Distinct mammary epithelial cell subpopulations can be isolated from mouse mammary glands by fluorescent-activated cell sorting (FACS) using specific cell-surface markers[4–7]. Inducible genetic lineage tracing, which permits targeted expression of a fluorescent reporter in a given cell and its progeny, has identified unipotent luminal K8/18-positive and basal K5/14- and Lgr5- (leucine-rich-repeat-containing G-protein-coupled receptor 5) positive stem cells after birth[8] (Figure 2). Multipotent cells that generate both the luminal and basal lineages are present in the mouse embryonic mammary gland[8,9]. Breast cancer originates from mammary epithelial cells and a key issue in breast cancer biology is the effect of genomic lesions in specific mammary cell lineages on tumor subtype, heterogeneity, and progression.

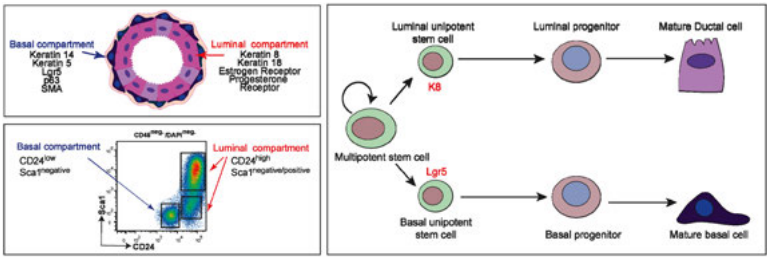

Figure 2. Upper left: *Schematic of a cross-section of a mammary gland duct showing the two major cell lineages that constitute the mammary gland epithelium.* Lower left: *FACS strategy for sorting different mammary subpopulations.* Right: *Mammary gland hierarchy. K8: keratin8. Lgr5: leucine-rich-repeat-containing G-protein-coupled receptor 5*

Breast cancer. Worldwide, nearly 650,000 lives are lost to breast cancer annually, the vast majority due to drug-resistant metastases[10–12]. Breast cancer is a heterogeneous disease that progresses to metastases of lung,

bone, liver, and/or brain, with fatal complications[13–16]. Molecular profiling of primary tumors has identified six intrinsic breast cancer subtypes: normal-like, luminal A, luminal B, HER2-enriched, claudin-low, and basal-like breast cancer[17–19]. Each subtype has a characteristic disease progression and clinical outcome[18,20]. Integrated genome-wide analyses of DNA copy number, RNA expression, and exome sequencing of human breast tumors has revealed a multitude of alterations within cancer cells[21–25].

Although such findings in the last decades have improved our understanding of molecular mechanisms underlying the disease, we still lack effective targeted therapies for many aggressive breast cancer subtypes. In the clinic, three main biomarkers are used to define pharmacological treatment: estrogen receptor α (ERα), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Expression of ERα and/or PR is typically associated with luminal A and B breast cancers, which are frequently responsive to endocrine therapy. Targeted therapies are available (e.g., Trastuzumab) for HER2-positive breast cancer. Tumors lacking expression of all three biomarkers are commonly referred to as triple-negative breast cancer (TNBC) and patients are treated with chemotherapy[26,27].

Tumor heterogeneity. Long before the era of molecular biology, pathologists observed breast cancer heterogeneity in tumors from different patients (intertumoral) and within the same tumor (intratumoral)[13,28–30]. Several factors are thought to contribute to breast tumor heterogeneity: the differentiation state of the cell in which the cancer originates, genetic and epigenetic oncogenic alterations, stochastic events, the tumor microenvironment, and/or a therapy. Notably, a single tumor genotype can have multiple phenotypic manifestations, indicating that cancer phenotype may also result from non-genetic determinants[13,31,32]. Non-genetic mechanisms have been shown to influence normal and neoplastic tissue stem-cell hierarchy, raising the possibility that they may also generate hierarchically organized breast tumors with a self-renewing cancer stem-cell subpopulation. The genetic evolution and the cancer stem-cell models are not necessarily mutually exclusive and a unifying model has been proposed[33].

Clinical implications. Altogether these observations indicate the existence of cancer cells with different biological properties (e.g., self-renewal, proliferation, survival, metastatic capability, response to therapy) within the same tumor. Cancer progression seems to follow a Darwinian evolution model and the genetic and epigenetic alterations in cancer cells result in subclones with different phenotypes that are subjects of selective evolution. The clinical implications of tumor heterogeneity and selective evolution are paramount. Because of region-to-region and cell-to-cell heterogeneity, biopsy of a small tumor region may confuse prognostic and predictive biomarkers and result in therapy failure.

1. Examples from previous studies from our laboratory:

1.1. Breast tumor heterogeneity: the importance of the cell-of-origin of breast cancer

Breast cancer is a heterogeneous disease and, besides the nature and number of genomic-transforming events and microenvironmental factors, the differentiation state of the cell-of-origin of cancer also determines the phenotype, tumorigenicity, and metastatic potential of this malignancy.

First, we addressed the impact of an activated phosphoinositide 3-kinase (PI3K) pathway on fate conversion in different cancer cells-of-origin and thus their contribution to tumor heterogeneity. The PI3K pathway is a central regulator of diverse normal cellular functions. It is one of the most essential pathways producing hallmarks of cancer[34]. PI3Ks are lipid kinases that phosphorylate phosphoinositides, leading to the activation of downstream kinases that influence key physiological processes such as metabolism, proliferation, cell growth, survival, and motility. It is estimated that up to 70% of breast cancers feature a hyperactive PI3K cascade[35,36]. Given the key effects of the PI3K pathway in solid cancers, important drug discovery programs have yielded a variety of compounds that efficiently target this pathway and are currently being evaluated in clinical trials. Notably, Alpelisib (BYL719), an alpha-specific PI3K inhibitor, was approved by the FDA for use in combination with the endocrine therapy fulvestrant for treatment of hormone receptor-positive and HER2-negative breast cancer. The gene *PIK3CA* encodes the PI3K catalytic subunit p110α and its amplification and/or mutation is associated with several kinds of human solid tumors. Activating somatic mutations

in *PIK3CA* are present in ~30% of human breast cancers at all stages. In 47% of these cases, mutations occur in the kinase domain, the most frequent being H1047R in exon 20. A hyperactive PI3K pathway results in cancer cells with a competitive advantage because of a decrease in cell death and increases in cell proliferation, migration, invasion, metabolism, angiogenesis, and resistance to chemotherapy[37,38]. We and others have shown that inducible expression of *PIK3CA* mutants induces mammary tumors in mice[34,38–41].

Using *in situ* genetic lineage tracing and limiting dilution transplantation, as well as mouse models of *PIK3CA*^{H1047R} generated in our lab, we have unraveled the potential of *PIK3CA*^{H1047R} to induce multipotency during tumorigenesis in the mammary gland (Figure 3). Our results and those of others define a key effect of *PIK3CA*^{H1047R} on mammary cell fate in the pre-neoplastic mammary gland[42,43]. We show that the cell-of-origin of *PIK3CA*^{H1047R} tumors dictates their malignancy, thus revealing a mechanism underlying tumor heterogeneity and aggressiveness[42].

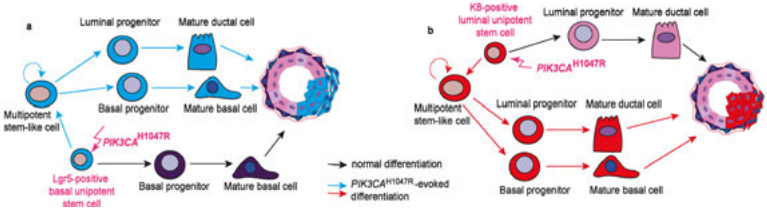

Figure 3. *The effect of* PIK3CA^{H1047R} *expression in basal Lgr5-* ***(a)*** *and luminal K8-positive* ***(b)*** *lineage-restricted mouse mammary cells. Mammary cells expressing* PIK3CA^{H1047R} *dedifferentiate into a multipotent stem-like state from which they further differentiate into the basal and luminal cell lineage. Expression of* PIK3CA^{H1047R} *in Lgr5-positive cells led mostly to benign but in K8-positive cells mostly to malignant mammary tumors.* Black arrows *indicate the differentiation potential of Lgr5- and K8-positive cells under physiological conditions*

Second, we used a high-content confocal image-based shRNA screen for tumor suppressors regulating human breast cell fate. By studying primary human breast epithelial cells, we have discovered that ablation of the Hippo kinases large tumor suppressors (LATS) 1 and 2 promotes luminal fate and increases the number of breast bipotent and luminal progen-

itors, the proposed cell-of-origin of most human breast cancers. Mechanistically, we discovered a crosstalk between Hippo and ERα signaling. In the presence of LATS, ERα was targeted for ubiquitination and Ddb1–cullin 4-associated-factor 1 (DCAF1)-dependent proteasomal degradation. Removal of LATS in ERα-positive cancer cells reduced their sensitivity to the widely used selective ER downregulator fulvestrant. Our findings reveal a non-canonical (i.e., YAP/TAZ-independent) effect of LATS in the regulation of human breast cell fate[44].

1.2. Breast tumor heterogeneity and progression to metastasis
Glucocorticoids promote breast cancer metastasis

A thorough understanding of the molecular and cellular mechanisms underlying both intra-patient breast tumor heterogeneity and metastasis is crucial for the success of personalized cancer therapy. Intra-patient tumor heterogeneity describes a poorly understood phenomenon during malignant progression by which cancer cells and patients themselves undergo genetic and epigenetic as well as hormonal and immunological changes. Phenotypic changes in cancer cells are a consequence of selection and adaptation that may result in cancer growth at distant sites years after primary tumor diagnosis and removal. Tumor heterogeneity is an obstacle to treatment, spawning divergence in diagnostic markers between primary tumors and matched metastases that may lead to inadequate treatment. We have recently shown cancer site-specific phenotypes and increased glucocorticoid receptor (GR) activity in distant metastases using transcriptional profiling of triple-negative breast tumors and matched metastases. GR mediates the effects of the stress hormones and synthetic derivatives (i.e., dexamethasone) used widely in the clinic as anti-inflammatory and immunosuppressive agents. We show that increase in stress hormones during breast cancer progression results in GR activation at distant metastatic sites, increased colonization, and ultimately reduced survival. To address the molecular mechanism underlying these observations, we performed transcriptome profiling, proteomics, and phosphoproteomics studies. The results implicated GR in the activation of multiple processes in metastasis and in increased expression of kinase ROR1, which correlates with shorter patient survival.

We also find that the stress hormone pathway is an effective inducer of colonization and the death of the animals, and that ROR1 knockdown counteracts this deleterious effect of GR activation and prolonged survival in preclinical models. The data also reveal that GR activation decreases the efficacy of the widely used chemotherapy paclitaxel. Corticosteroids such as dexamethasone are widely used in the treatment of breast cancer to combat side-effects of chemotherapy and to treat symptoms related to advanced cancer. Given that cancer cell dissemination has already occurred by the time of primary tumor surgical resection in a substantial number of breast cancer patients[14,29], and that GR activation fosters colonization at the distant sites, our results call for caution when administering corticosteroids to patients with cancer-related complications[45].

Targeting SHP2 in breast cancer

The first *bona fide* protein tyrosine phosphatase proto-oncogene is the Src-homology 2 domain-containing phosphatase SHP2. A ubiquitously expressed protein, SHP2 transduces mitogenic, pro-survival, cell fate, and/or pro-migratory signals from almost all growth factor, cytokine and extracellular matrix receptors[46]. SHP2 is required for full activation of the ERK/MAPK pathway downstream of most of these receptors. In cancer, SHP2 is hyperactivated either by mutations or downstream of oncogenes. We and others have shown that these mutations occur at various incidences in myeloid malignancies but rarely in solid cancers[47–49]. SHP2 is also activated downstream of oncogenes upon binding to phosphorylated proteins[50]. Whereas we found no mutations of SHP2 in human breast cancer samples[49], we initially discovered that SHP2 is required for GAB2-evoked increased proliferation and invasiveness in breast cancer models[51]. We have demonstrated a fundamental effect of SHP2 on breast tumor maintenance and progression. SHP2 knockdown eradicated breast tumor-initiating cells *in vitro* and in xenografts. Serial limiting dilution transplantation experiments over three passages revealed that SHP2 knockdown decreases tumor seeding and propagation. SHP2 activated c-Myc and ZEB1, which resulted in repression of let-7 microRNA and the expression of a set of "SHP2 signature" genes found to be co-activated in human primary breast tumors. Using phosphoproteomics and intravital imaging, we found that SHP2 also activates c-SRC, leading to an

increase in cancer cell motility. Our studies provided new insights into signaling cascades that regulate neoplastic breast stem cells and a rationale for targeting SHP2 in breast cancer. SHP2 inhibitors are currently being evaluated in clinical trials[52–54].

1.3. Resistance to therapy, therapy for resistance
Inhibition of PI3K and tumor heterogeneity

Selection of specific tumor clones or activation of a bypass pathway upon exposure of cancer cells to treatment also results in tumor heterogeneity. We discovered a JAK2/STAT5-evoked positive feedback loop that dampens the efficacy of dual PI3K/mTOR inhibition in triple-negative breast cancer. Mechanistically, PI3K/mTOR inhibition increased IRS1-dependent activation of JAK2/STAT5 and secretion of IL8. Genetic or pharmacological inhibition of JAK2 abrogated this feedback loop, and combined PI3K/mTOR and JAK2 inhibition synergistically reduced cancer cell number, decreased tumor seeding and metastasis, and increased overall survival of the animals. Our results provide a rationale for combined targeting of the PI3K/mTOR and IL8/JAK2/STAT5 pathways in triple-negative breast cancer[55].

In luminal breast cancer cells, we found an increase in IGF1R, IRS1/IRS2 and p85 phosphorylation in cancer cells resistant to the p110α isoform-selective inhibitor BYL719. Co-immunoprecipitation experiments identified an IGF1R/IRS/p85/p110β complex that causes the activation of AKT/mTOR/S6K and stifles the effects of BYL719. Pharmacological inhibition of members of this complex reduced mTOR/S6K activation and restored sensitivity to BYL719. Our study demonstrates that p110β confers resistance to BYL719 in *PIK3CA* mutant breast cancers. This provides a rationale for the combined targeting of p110α with IGF1R or p110β in patients with breast tumors harboring PIK3CA mutations[56,57].

Halting blockade of the innate immune system results in cancer heterogeneity

We have discovered a paradoxical effect of the CC chemokine ligand 2 (CCL2) in metastatic breast cancer. Secretion of CCL2 by mammary tumors recruits CCR2-expressing inflammatory monocytes to primary tu-

mors and metastatic sites, and CCL2 neutralization in mice inhibits metastasis by retaining monocytes in the bone marrow. Surprisingly, interruption of CCL2 inhibition leads to an overshoot of metastases and accelerates death. This is the result of monocyte release from the bone marrow, enhancement of cancer cell mobilization from the primary tumor, as well as blood vessel formation and increased proliferation of metastatic cells in the lungs in an IL-6/VEGF-A-dependent manner. Our results emphasize the need for long-term follow-up of patients with metastatic disease after treatments that interfere with the tumor microenvironment, such as tumor immunotherapy[58].

2. Examples from current studies from our laboratory:
2.1. Swiss Personalized Oncology

"I have been impressed with the urgency of doing. Knowing is not enough; we must apply. Being willing is not enough; we must do". (Leonardo da Vinci).

The Swiss Personalized Oncology (SPO) driver project, part of the Swiss Personalized Health Network (SPHN), is chaired by myself and Prof. Olivier Michielin (CHUV, Lausanne). SPO is a Switzerland-wide effort that aims at integrating clinical and molecular information from cancer patients, which should ultimately enable more precise diagnoses and thus treatments tailored to individual patients. SPO's main goal is to achieve interoperability of the clinical and laboratory data from cancer patients in Switzerland. We have already made major progress in this challenging but urgently needed endeavor – thanks to the great work of all the SPO centers and their very productive meetings and networking activities, as well as to the tight collaboration with the SOCIBP SPHN driver project led by Prof. Mark Rubin (University of Berne). First, we identified a minimal dataset that specifies the critical data to be harmonized and captured from digital medical records within the routine clinical flow in university hospitals. Furthermore, we composed a digital clinical reporting form to capture these data from non-university cancer clinics (e.g., Swiss Association for Clinical Cancer Research, SAKK). Second, a strong alignment between the SPO Driver project and SAKK was further consolidated, both at the technical and the governance level. Third, we set up the infrastructure of the Swiss Molecular Tumor Board (SMTB), which brings

together experts from the five Swiss university hospitals to discuss complex oncology cases. The originality of the SMTB lies not only in its nationwide format but also in the fact that both clinicians and translational research scientists participate in these meetings. The scope of the SMTB could now be extended from a purely educational board to one delivering clinically relevant input; it will also be expanded to more institutions. Finally, we have assembled retrospective, archived breast cancer and melanoma specimens for broader analysis (e.g., tissue microarrays). Prospectively, we have established and disseminated protocols for live tumor-cell biobanking that have been collated and distributed in coordination with the SAKK. These nationwide efforts have initiated the integration of clinical and molecular information from cancer patients and fostered numerous interactions and fruitful collaborations between clinicians and researchers all over Switzerland.

2.2. Personalized breast cancer treatment: ongoing studies

While the SPO is a nationwide effort, we have founded, together with Prof. Walter Weber (USB), the Basel Breast Consortium (https://baselbc.org), an interdisciplinary organization committed to the development of basic, clinical and translational research projects by supporting interdisciplinary communication and mutual education in Switzerland and neighbouring cities. We have also assembled, a local group of USB colleagues (Surgery, Gynecology, Pathology, Radiology, and Oncology) to make up a breast cancer personalized medicine team that should ultimately improve treatment of patients. Our goal is to collect patient samples and to use multiomics, combined with drug response profiling and computational analysis, in the assessment and modeling of cancer and tumor microenvironment heterogeneity in a longitudinal way. We apply a personalized systems medicine interdisciplinary approach to discover predictive biomarkers and mechanisms of resistance, to identify novel targets, and to rationally design combination therapy. We have already succeeded in establishing many of these approaches. The flagship project focuses on ERα-positive breast cancer and aims to identify mechanisms of resistance to endocrine therapy and CDK4/6 inhibitors using patient material.

ERα-positive breast cancers, which make up the majority of breast cancers (70% of cases), are frequently responsive to endocrine therapy that interferes with estrogen synthesis or signaling. Unfortunately, in 25% of cases, endocrine therapy-resistant metastases develop that initiate an inexorable downhill course. Mechanisms of resistance often culminate in the activation of the Cyclin D1-CDK4/6 complex[59].

Proliferation of cancer cells is often deregulated and sustained chronic proliferation is a fundamental hallmark of cancer[60]. The cell cycle is usually a tightly controlled process and both the serine/threonine cyclin dependent kinases (CDK), their associated regulatory subunits (the cyclins), and their inhibitors (e.g., p16, p21, p27) are important for progression from one phase of the cycle to the next. For example, extracellular signals (e.g., estrogen, growth factors) increase the levels of D-type cyclins during the G1 phase, and the CDK4/6-cyclin D complex triggers transition of cells from early to late G1 phase, progressing through the restriction point gate. The tumor suppressor RB (retinoblastoma related) binds the transcription factor E2F and arrests cells in G1. Both estrogen and growth factors increase D1 expression. The resulting RB phosphorylation and inactivation by the CDK4/6-D complex during the G1 phase allows cells to pass the restriction point. The CDK2-Cyclin E complex induces hyperphosphorylation of RB, thus completing its inactivation and triggering the transition from G1 to S phase. Mechanisms that enhance these transitions are significant in breast cancer initiation and maintenance and include activation of D and E cyclins (e.g., amplification, translocation) and loss of RB or CDK inhibitors[61]. Not surprisingly, inhibition of CDK4/6 has been proposed as a means of treating ERα-positive breast cancers. Several selective CDK4/6 inhibitors (CDK4/6i) have been developed and tested, including Palbociclib/PD0332991, Abemaciclib/LY5219, and Ribociclib/LEE011. Notably, preclinical studies and recent clinical trials (e.g., PALOMA1, 2, MONALEESA2, PALOMA3, MONARCH-1, MONARCH-2, MONARCH-3) have shown the efficacy of combined endocrine therapy and CDK4/6 inhibition in metastatic ERα-positive breast cancers. Ribociclib, Abemaciclib and Palbociclib have been FDA approved in combination with Letrozole for use as first-line therapy in patients with metastatic breast cancer (MBC), and in combination with fulvestrant for patients with MBC who progressed on prior

endocrine therapies[62–70]. While these treatments show high efficacy compared to single endocrine agents, some patients do not respond to such treatment or they develop resistance. The basis of resistance in the clinic remains ill-defined. Possible resistance mutations have been identified, mainly mutations in RB1, PIK3CA and ESR1[71]. Preclinical studies in model systems suggest that loss of RB, overexpression of cyclin E or PDK1, amplification of CDK6, or activation of the D1-CDK2 pathway may account for resistance to CDK4/6i as a single agent[72–75].

To capitalize on the early clinical success of CDK4/6i, it is very important to assess mechanisms of pre-existing and acquired resistance to such inhibitors. We are using different patient-derived *ex vivo* and *in vivo* model systems, including patient-derived organoids (PDOs) and patient-derived xenografts (PDXs), before treatment and after tumor progression. By continuous exposure of PDXs to endocrine therapy and/or CDK4/6i, we are also generating models that are resistant to such treatments (Figure 4). First, we apply a combination of unbiased genomic and proteomic analyses to identify the underlying mechanisms of resistance.

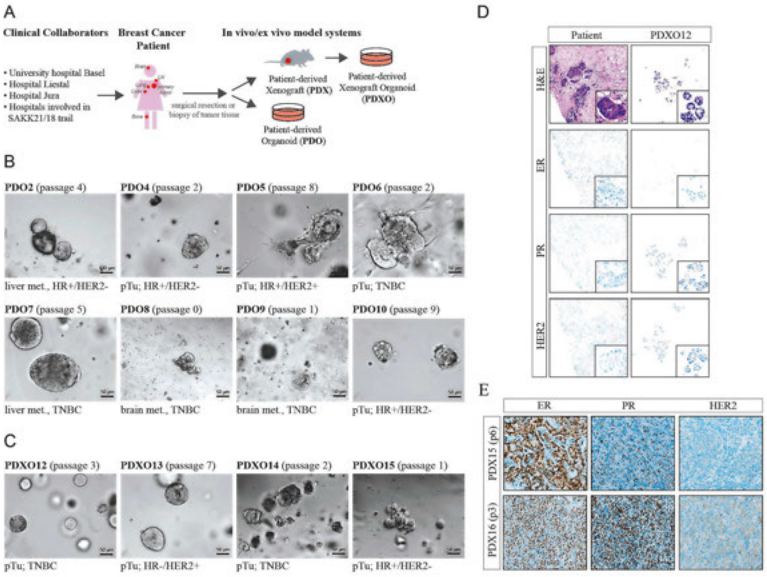

Figure 4*. Patient-derived* ex vivo *and* in vivo *model systems from breast cancer patients.* ***A.*** *Scheme displays our clinical collaborators and patient-derived organoids (PDO), patient-derived xenografts (PDX), and patient-derived xenografts organoids (PDXO) models from different human primary breast tumors (pTu) and metastases (met).* ***B-C.*** *Representative bright field images of established PDO (B) and PDXO (C) cultures.* ***D.*** *Images of sections of a primary tumor and corresponding PDXO models.* ***E.*** *Images of sections of PDXs from ER+ breast tumors. Expression of ER, PR and HER2 was analyzed by IHC.*

Second, we use high-throughput fluorescent microscopy and high-end single-cell imaging in drug sensitivity functional profiling assays (i.e., pharmacoscopy) to discover means to circumvent and overcome CDK4/6i/endocrine therapy resistance, and to develop new mechanism-based personalized therapy for our patients (Figure 5). The future of cancer therapy relies on the diversity of target inhibitors, applied in combination[12,30,56,76,77]. Our studies should lead to the identification of novel personalized combination therapies.

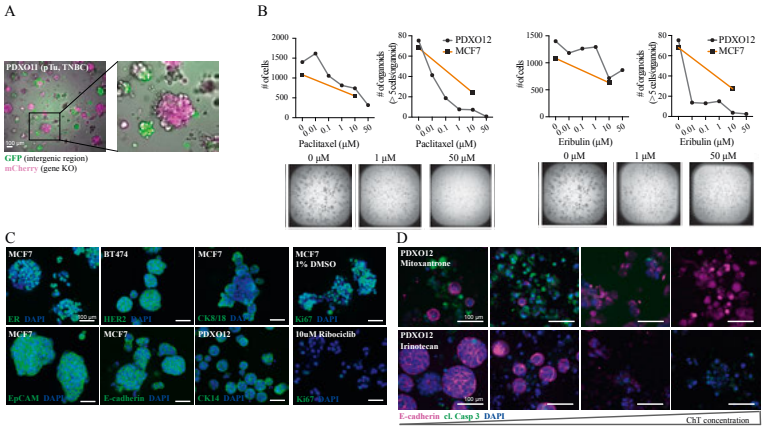

Figure 5. Ex vivo *chemosensitivity functional profiling.* ***A.*** *Bright field image of a PDXO (organoids derived from a TNBC PDX) transduced with vectors targeting an intergenic region (GFP) or a specific gene (mCherry)* (left). *Black box indicates enlarged region* (right). ***B.*** *Image-based drug sensitivity screen: shown are representative whole-well bright field images of organoids treated as indicated and the quantification of single cells and organoids cultured in Matrigel.* ***C-D.*** *Immunofluorescence images of PDXO12 or MCF7 treated and stained as indicated.*

2.3. Effects of mammary tumor heterogeneity on tumor initiation, metastasis, and resistance to therapy: ongoing studies

Transposon insertional mutagenesis: a genetic tool for generating heterogeneity

Transposon insertional mutagenesis is a powerful tool for the discovery of cancer-related genes in mice[78–81]. Indeed, the fact that transposons change their relative position within the genome and alter gene function in cells that express the transposase make these systems ideal for whole-genome screens. The *PiggyBac (PB)* transposon was engineered to be active in mammalian cells[82]: it has higher activity than other transposon systems (e.g., *Sleeping Beauty*)[83]; it moves larger DNA segments[54,56]; it leaves no footprint after transposition; it has a low tendency

for local hopping[78]; it has been used successfully for cancer gene discovery in mice[78–81]. Our lab has been using this genome-wide mutagenesis approach to identify genes and pathways that regulate normal and neoplastic mammary stem cells, the progression to metastasis, and the resistance to therapy. The *PiggyBac* transposon includes two splice acceptors (CβASA, Carp b-actin splice acceptor; En2-SA, Engrailed-2 exon-2 splice acceptor), two poly-A signals (bidirectional SV40 polyadenylation signal, pA), a cytomegalovirus enhancer, a chicken beta-actin promoter (CAG), and a splice donor (Foxf2 exon-1 splice donor, SD). The transposons are mobilized by the *PiggyBac* transposase in a cut-&-paste manner and can be inserted throughout the genome wherever there is a TTAA. This system allows the identification of both oncogenes and tumor suppressor genes, depending on the site of insertion and orientation of the transposon (Figure 6).

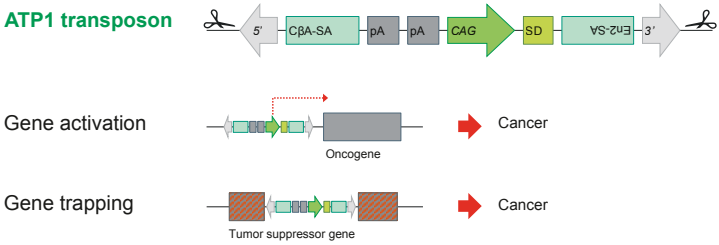

Figure 6. *Design of the transposable element ATP1 and dual mode of action at the integration site*

2.4. Cancer poses a global challenge that requires global efforts: ongoing studies

Together with several colleagues, we have created an international network of labs working on breast biology and cancer (www.enbdc.org) with the goal to foster scientific exchange and collaboration, as well as mutual training and education worldwide.

To develop more fidelitous ex vivo and in vivo models for studying breast cancer, we have teamed up with the labs of Profs. Alana Welm, Brian Welm (Huntsman Cancer Institute, Salt Lake City) and Mike Lewis (Bay-

lor College of Medicine, Houston) to harmonize our respective collections of patient-derived organoids (PDOs) and primary-derived xenografts (PDXs). By combining efforts locally, nationally and internationally, we aim to create synergies that will lead to a better understanding of breast cancer biology and thus more relevant treatments.
Didn't Aristotle say that *"The whole is greater than the sum of its parts"*?

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THE CLOËTTA PRIZE 2020
IS AWARDED TO

PROFESSOR

NADIA MERCADER HUBER

BORN IN 1974 IN ZURICH, SWITZERLAND

INSTITUTE OF ANATOMY
UNIVERSITY OF BERN

FOR HER GROUND-BREAKING CONTRIBUTIONS
TO RESEARCH IN CARDIAC REGENERATION
WITH THE ZEBRAFISH MODEL AND ITS RELEVANCE
TO MEDICINE

BERN, 6TH NOVEMBER 2020

IN THE NAME OF THE FOUNDATION BOARD:

THE PRESIDENT

THE VICE PRESIDENT

A MEMBER

Nadia Mercader Huber

CURRICULUM VITAE

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Education

2003	PhD in Biology, Universidad Autónoma de Madrid, Madrid, Spain
1998	Degree in Biology, Swiss Federal Institute of Technology (ETH) - Zürich, Switzerland

Employment History

Since 2020	Full Professor in Anatomy, Developmental Biology and Regeneration Visiting Professor at CNIC, Madrid, Spain
2015–2020	Associate Professor in Anatomy, Developmental Biology and Regeneration Visiting Professor at CNIC, Madrid, Spain
2014–2015	Assistant Professor Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid, Spain
2007–2014	Junior Scientist – Department of Cardiovascular Development and Repair – CNIC, Madrid, Spain
2004–2007	Postdoctoral fellow – EMBL Heidelberg, Germany
2003–2004	Postdoctoral fellow – Centro Nacional de Biotecnología CNB-CSIC, Madrid, Spain
1998–2003	PhD Student – CNB-CSIC-Group Miguel Torres, Madrid, Spain

Institutional responsibilities

2020	Vicedean of research Medical faculty, University of Bern (4 year mandate)
Since 2015	Co-director of the Institute of Anatomy, University of Bern, Head of Section Developmental Biology and Regeneration

Current research grants

- H2020-SC1-2019-Single-Stage-RTD REANIMA-874764. New-generation cardiac therapeutic strategies directed to the activation of endogenous regenerative mechanisms. (2020–2024). Co-PI
- ERC Consolidator grant 819717 – TransReg (2019–2023). PI
- SNF Project ForceInRegeneration. 310030L_182575. Deciphering the influence of biomechanics and mechanotransduction during cardiac regeneration in normal and pathological contexts (2019–2021). PI
- HSFP RGP0016/2018 Handling OXPHOS structural heterogeneity and metabolic plasticity (2019–2021). Co-PI
- European Industrial Doctorate Program H2020-MSCA-ITN-2016 4DHeart 722427 (2017–2021). Scientific Coordinator.
- Scherbarth Foundation. The zebrafish as tool for personalized diagnosis of human cancer cell behaviour (2019–2021). Co-PI.

Reviewing activities and participation on evaluation boards

Since 2019	Head of grant committee from Bern Centre of Precision Medicine BCPM (2 year mandate)
Since 2018	Member of the SNSF Commission University of Bern

Evaluation of research projects and fellowships for the Spanish Agency ANEP, the French Agency ANR, The British Heart Foundation, the European Research Council. N. Mercader acts as reviewer for several journals including Circulation, Cell Death Diff, Current Biology, eLife, Development, Developmental Cell, PNAS, PlosOne, Science, Stem Cell Reports, Cell Reports.

Organization of conferences

As main organizer: 10th International Workshop on Cardiomyocyte Biology, Ascona 2021, 10th Swiss Zebrafish Conference 2017, UNIA Workshop 2014 “Cardiovascular Extracellular Matrix in health and disease”, CNIC conference 2013 “Cardiovascular Development, Homeostasis and Repair”.

As Co-Organizer: CNIC conference on Heart regeneration 2020 (postponed); 9th International Workshop on Cardiomyocyte Biology, Ascona 2018; CNIC conference 2016 "Mechanical forces in physiology and disease"; EMBO conference 2016 "The molecular and cellular basis of Regeneration and Tissue Repair", MIC-Symposium Cells in Motion 2016; Weinstein Conference on Cardiovascular Development 2013.

Teaching activities
Coordinator of the subject Embryology and Genetics at Medical Faculty University of Bern and giving Lectures and Tutorials, Lecturer in practical Histology Courses, Professor responsible for the Cutting Edge Microscopy PhD Program at University of Bern (2017–2020).

Awarded prizes and fellowships
Pexeider Prize 2011 for the best oral presentation, ESC Working Group of Anatomy and Embryology Meeting, Liblice.
Ramón y Cajal fellow (2007–2011)
Postdoctoral fellowship Ministerio de Educardion y Ciencia (2006–2007)
EMBO Long Term Postdoctoral fellowship (2004–2006)
Marie Curie PhD fellowship-SNF Internationale Austauschprogramme (1999–2002)

Mentoring
Current members: 5 postdoctoral researchers (Inês Marques, Indre Piragyte-Langa, Myra Chávez, Alexander Ernst, Carolina García-Poyatos), 4 PhD Students (Eleonora Lupi, Benedetta Coppe, Joao Carvalho, Marius Botos).

Past PhD and Postdoctoral fellows: Marina Peralta (PhD in 2013, received Marie Curie fellowship to join IGBMC), Juan Manuel González-Rosa (PhD in 2014, received EMBO fellowship, now PI at Harvard Medical School), Héctor Sánchez-Iranzo (PhD in 2015, received EMBO fellowship, starting own research group in Januray 2021 at KIT, Germany), Laura Andrés-Delgado (Postdoctoral fellowship from Spanish Ministry of Science and Competitiveness from 2013 to 2015, now lecturer at Universidad Autónoma de Madrid), Marcos Sande (PhD in 2019), Andrés-Sanz Morejón (MSc in 2015, obtaining Spanish national Arquímedes Prize for Best Master Thesis, PhD in 2020, obtained SNSF early postdoc mobility grant to work at EMBL Heidelberg).

SELECTED PUBLICATIONS

Sande-Melón M, Marques IJ, Galardi-Castilla M, Langa X, Pérez-López M, Botos MA, Sánchez-Iranzo H, Guzmán-Martínez G, Ferreira Francisco DM, Pavlinic D, Benes V, Bruggmann R, **Mercader N.** Adult sox10(+) Cardiomyocytes Contribute to Myocardial Regeneration in the Zebrafish. ***Cell Rep.*** 2019 Oct 22;29(4):1041-1054.e5. doi: 10.1016/j.celrep.2019.09.041. PubMed PMID: 31644901; PubMed Central PMCID: PMC6856760.

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HEART DEVELOPMENT AND REGENERATION IN THE ZEBRAFISH

Nadia Mercader Huber

Abstract

In humans, myocardial infarction results in ventricular remodeling, progressing ultimately to cardiac failure, one of the leading causes of death worldwide. In contrast to the adult mammalian heart, the zebrafish model organism has a remarkable regenerative capacity, offering the possibility to research the bases of natural regeneration. In our group, we have investigated the cellular and molecular mechanism of heart regeneration in the zebrafish. We further also take advantage of this model organism to study embryonic development of the heart. Understanding the developmental processes of heart formation might help to unravel the causes of congenital heart disease. Here, I will summarize some of our contributions to this research field.

Introduction

The heart is among the first organs to acquire its function. Long before its development is completed, it starts beating, and puts in motion the blood flow, controlling in this manner the overall progression of the organism's development. Blood flow is important not only because it promotes oxygenation of the embryonic tissues. Blood flow forces themselves act as biomechanical signals sensed by endothelial cells of the vasculature and the endocardium, the inner lining of the heart. In this manner, the heartbeat induces, for example, the development of the cardiac valves. Congenital heart defects are among the most common type of birth defects and can have both environmental and genetic underlying causes. They can have early phenotypic consequences, but they can also manifest only later in the adult. A good understanding of cardiac development constitutes therefore an essential asset to promote and preserve health.

Apart from inherited predispositions, environmental stressors and nutritional habits can impact cardiovascular health during adulthood. Heart failure is among the leading causes of death worldwide. Coronary artery occlusion, for example as a consequence of atherosclerosis, can lead to myocardial infarction (MI) and millions of cardiac muscle cells can die as a result of interrupted blood flow. Fortunately, rapid intervention protocols are currently significantly reducing the mortality after MI. Nonetheless, reperfusion injury after ischemia still poses an important stress to cardiomyocytes, leading to their elimination. As a reaction to the lost myocardium, cardiac fibroblast start proliferating and producing extracellular matrix (ECM). This fibrotic response avoids cardiac wall rupture and is therefore lifesaving. However, the surplus of fibroblasts has a long-term detrimental effect. The cardiac muscle loss affects cardiac contractility and fibrotic tissue disrupts electrical propagation. As a response, remaining cardiomyocytes that now need to work more undergo hypertrophy, and secondary interstitial fibrosis occurs throughout the heart. These events, altogether defined as ventricular remodeling, lead to arrhythmias and cardiac dysfunctions ultimately leading to heart failure.

The zebrafish is an excellent model to study cardiovascular development (Figure 1). Zebrafish *(Danio rerio)* are small freshwater ray-finned fish that reach around 3 cm in length. They display characteristic horizontal blueish and white stripes along the trunk and fins. Their natural habitat is the Ganges basin in Northern India and Bangladesh. It became a popular animal model in research around 1980 thanks to the work of George Streisinger, a researcher who originally held zebrafish as a pet and then converted it into an experimental model. An important boost for the model occurred when geneticists such as Nobel Prize Winner Christiane Nüsslein-Vollhardt used the facts that large clutches of animals are easily available, that embryo development is rapid and that zebrafish embryos are transparent allowing to visualize development of all organ primordia, to perform mutagenesis screens in the zebrafish. This led to the identification of a multitude of new genes leading to developmental alterations and, in this way, allowed the study of gene function during the formation of body parts and organs. Importantly, more recently, the sequencing of the zebrafish genome confirmed that over 70% of genes are conserved between humans and zebrafish. As such, the zebrafish repre-

sents an excellent model to interrogate function of genes involved in human disease progression. Furthermore, imaging technologies and genome editing nowadays even allow for the study of gene function and organ development in vivo at the single cell level.

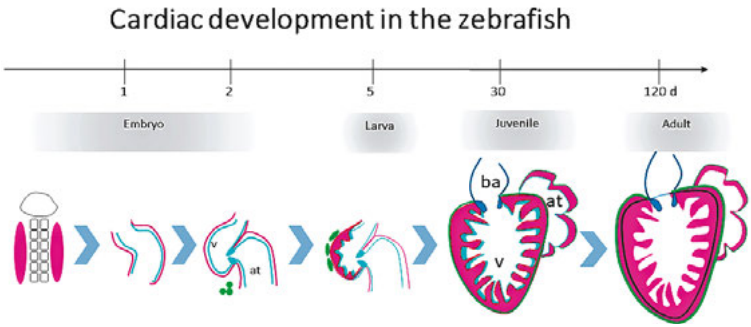

Figure 1. Cardiac development in the zebrafish. *Cardiac precursors derive from the lateral plate mesoderm that migrate rostrally and fuse to form a primordial heart tube during the first 24 hours after fertilization (24 hpf). The heart tube is formed by the cardiac muscle (myocardium, pink) and an inner endocardial layer (blue). Heartbeat starts at 25 hpf. Subsequently, the heart tube loops, forming the atrial and ventricular chamber, connected by the atrioventricular valve. At that time, a third layer is added to the heart, the epicardium. Epicardial precursors derive from the proepicardium (green cells) that detach from the pericardial wall and reattach to the myocardial surface. Around 5 days postfertilization (5 dpf), the heart becomes trabeculated. A bulboventricular valve starts forming. In juveniles a third outer myocardial layer is added by trabecular cardiomyocytes that breach the initial primordial layer and form the cortical layer.at, atrium; ba, bulbus arteriosus; v, ventrile.*

While initially the attention to the zebrafish was centered on its role as a model in developmental biology, the work from Ken Poss and others incorporated the zebrafish as a central vertebrate animal model to understand organ regeneration (Figure 2). While in humans, the loss of cardiomyocytes upon MI or other cardiovascular events leads to the irreversible loss of cardiomyocytes, the zebrafish has the extraordinary capacity to renew the lost myocardium in response to injury. Since this initial observation, research has advanced to understand the underlying mechanisms.

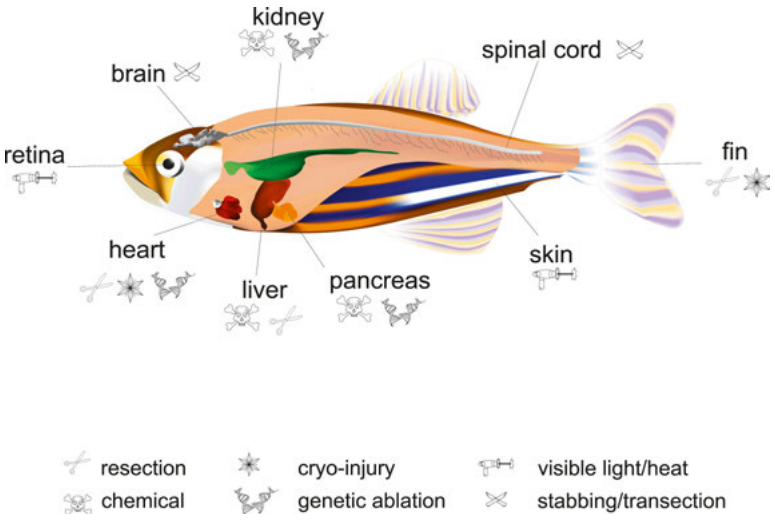

Figure 2. Organ regeneration in the zebrafish. *Highlight of some of the organs and tissues used for regeneration studies in zebrafish. Commonly used injury models are marked for each organ. Adapted from (Marques et al., 2019).*

Results

Zebrafish as a model to study epicardium formation

During heart development, the epicardium is the last layer to be added. The epicardium plays important roles in cardiac development and homeostasis, as a source of progenitor cells for the coronary vasculature and cardiac fibroblasts as well as a source for signalling molecules influencing myocardial growth (Quijada et al., 2020). Several hypotheses were put forward to understand the development of this outer mesothelial layer. Initially, it was thought that the outer myocardial cells transdifferentiate into epicardial cells. Examinations in several species revealed the presence of a cluster of cells close to the inflow tract of the heart that expressed epicardial marker genes. This structure was defined as the proepicardium. Two further hypotheses were discussed: (1) proepicardial cells are transferred to the myocardium to form the epicardium through a cellular and ECM based bridge or, (2) proepicardial cell clusters detach from

the proepicardium, are released into the pericardial cavity and subsequently attach to the heart. To prove any of the hypotheses, in vivo imaging is necessary. Therefore, we decided to make use of the zebrafish model to interrogate the mechanisms of epicardium formation (Figure 3). We first generated a transgenic reporter line in which green fluorescent protein (GFP) is expressed specifically in the proepicardium and epicardial cells. Next, we used this line to image proepicardium and epicardium formation in vivo. Anaesthetized zebrafish were imaged using high-speed confocal imaging between 48 and 60 hpf, the time point of proepicardium formation and the time when first epicardial cells start to be present. We found that proepicardial cells indeed detach from the dorsal pericardium and are released into the pericardial cavity. They are advected for several minutes in the pericardial cavity until finally adhering to the myocardial surface. This process continues until most of the myocardium is covered. Importantly, blocking the heart beat impaired advection of cells from the proepicardium to the myocardium (Peralta et al., 2013). In this manner, we found that the heartbeat promotes morphogenesis not only by putting in motion the blood flow, but also by promoting pericardial flow forces outside the heart. In a follow up work, we wanted to understand in more detail how proepicardial cells emerge from the pericardial mesothelium. We found that cells delaminate from the mesothelium through apical extrusion, a process that had been extensively studied in the context of epithelial homeostasis but not much in the context of embryonic development. Here we found that, rather than being eliminated, as happens during epithelial homeostasis, extruded proepicardial cells survive and form the epicardium upon attachment to the myocardium (Andres-Delgado et al., 2019). Again, *in vivo* imaging was crucial to make this discovery.

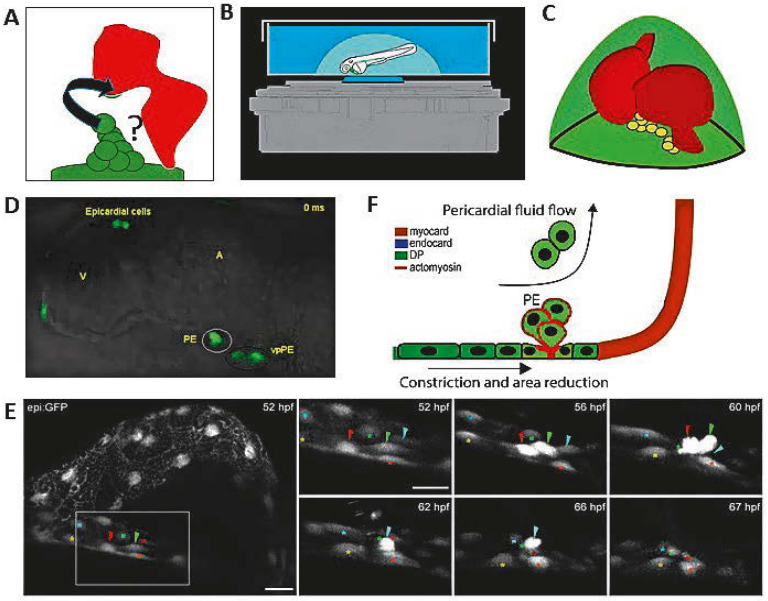

Figure 3. Cellular mechanism of epicardium formation. A, *Question to be answered: how are proepicardial cells transferred to the heart? Heart tube is shown in red, proepicardial cells in green.* ***B,*** *set up for in vivo imaging of epicardium formation using the zebrafish embryo.* ***C,*** *Schematic representation of the heart tube within the pericardial cavity. Proepicardial cells are shown in green. Shown is a frontal view.* ***D,*** *Frame of a video of the epi:GFP line used to image epicardium formation. epi:GFP positive cells are shown in green.* ***E,*** *epi:GFP in vivo time lapse. Individual cells are marked with dots of different colors. PE cells that extrude are marked with an arrowhead.* ***F,*** *Scheme of proepicardium delamination. PE cells are extruded apically into the pericardial cavity in a process that required actin and myosin II. Pericardial flow allows transfer of the cells to the myocardial surface. A, atrium; PE, proepicardium; vpPE, venous pole proepicardium, V, ventricle.*

How does a zebrafish regenerate the heart?

The zebrafish heart is built up similarly to the human heart, with some obvious difference (Figure 4). It is formed by a single atrium, a single ventricle and a prominent outflow tract named bulbus arteriosus. The chambers are separated by an atrioventricular as well as a bulboventricular valve. As in humans, zebrafish hearts are formed by myocardium –

the cardiac muscle – an inner lining of endocardium and an outer epicardium. The myocardium is highly trabecular and as in mammals, contains cardiac fibroblasts. The heart is irrigated by a poorly developed coronary

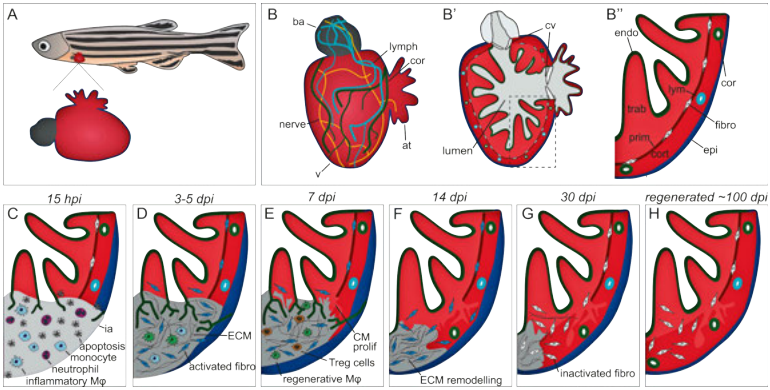

Figure 4. Representation of cardiac regeneration in the adult zebrafish. ***A,*** *Adult zebrafish heart anatomical position.* ***B,*** *Overview of the uninjured zebrafish heart, comprising the atrium, ventricle and bulbus arteriosus. The heart is covered and wired by the epicardium, lymphatic system, coronary arteries, and nerves.* ***B',*** *Section of the zebrafish heart. Cardiac valves separate the chambers.* ***B",*** *Zoomed region of B'. Three myocardial layers can be identified: trabecular, primordial, and cortical myocardium. The endocardium coats the lumen. The cortical layer is covered by the epicardium. Fibroblasts lie between the cortical and trabecular myocardium.* ***C–H,*** *Timeline of cardiac regeneration events upon cryoinjury.* ***C,*** *Fast freezing of the ventricular apex leads to the formation of the injury area. Necrotic and apoptotic cells trigger an inflammatory response characterized by the infiltration and activation of neutrophils, monocytes, and macrophages, among others. Endothelial and epicardial cells are activated and infiltrate the injury area.* ***D,*** *The acute inflammation regresses and activated fibroblasts elicit a fibrotic response by depositing extracellular matrix (ECM).* ***E,*** *Peak of cardiomyocyte proliferation followed by migration along epicardial and endocardial cells.* T_{reg} *cells home to the injured tissue.* ***F,*** *The ECM remodels, and cardiomyocyte proliferation continues.* ***G,*** *Fibroblasts undergo inactivation and the fibrotic scar regresses.* ***H,*** *Complete regression of the fibrotic scar and replenishment by functional myocardium. The cortical myocardial layer remains thickened and the primordial layer does not regenerate. Abbreviations: at, atrium; ba, bulbus arteriosus; CM prolif, cardiomyocyte proliferation; cor, coronary arteries; cv, cardiac valves; ECM, extracellular matrix; epi, epicardium; endo, cardiac endothelium; dpi, days post injury; fibro, fibroblast; ia, injury area; hpi, hours post injury; lymph, the lymphatic system; MΦ, macrophage; prim, primordial layer; trab, trabecular layer; v, ventricle. Picture from (Sanz-Morejon and Mercader, 2020).*

vasculature, as well as a lymphatic system and is innervated by sympathetic and parasympathetic nerve fibres.

As a first response to injury, the epicardium and endocardium start to re-express developmental genes and proliferate. The epicardium undergoes epithelial-to-mesenchymal transition and inflammatory cells home to the heart. The damaged muscle becomes replaced by fibrotic tissue. Simultaneously, CMs initiate proliferation and regenerate the injured myocardium, while the transiently deposited extracellular matrix (ECM) gets eliminated (Sanz-Morejon and Mercader, 2020).

The initial model to study heart regeneration in the zebrafish was based on resection of the ventricular apex. In this model, ¼ of the ventricle is amputated with dissection scissors in the anaesthetized animal (Poss et al., 2002). After the initial formation of a fibrin clot the heart heals within 30 days, including the regeneration of epicardial layer, the myocardium and endocardium. We wondered whether zebrafish would also be able to regenerate upon a different lesion, involving tissue damage rather than tissue loss. We reasoned that this could be to some extent more similar to a pathological situation. Therefore, we established ventricular cryoinjury as a protocol to damage the zebrafish heart (González-Rosa and Mercader, 2012). Injuring the cardiac ventricle by freezing was developed simultaneously in our group as well as the groups of Anna Jazwinska and Gilbert Weidinger (Chablais et al., 2011; González-Rosa et al., 2014; Schnabel et al., 2011). Using this model, we found that, similar to cardiac resection, zebrafish could also regenerate the heart upon tissue damage. However, we noted that regeneration took longer, around double time being complete at 130 days postinjury (dpi). An important second difference to resection was that a massive fibrotic response preceded myocardial regeneration (Figure 5). This simple observation was very important, because it revealed that fibrosis is not blocking regeneration, a common belief among scientists as well as clinicians at the time. Moreover, it showed that fibrosis is not irreversible in the zebrafish. That means that the capacity to regenerate in the zebrafish is not relying on not producing fibrosis but, at least among others, to get rid of the fibrotic tissue.

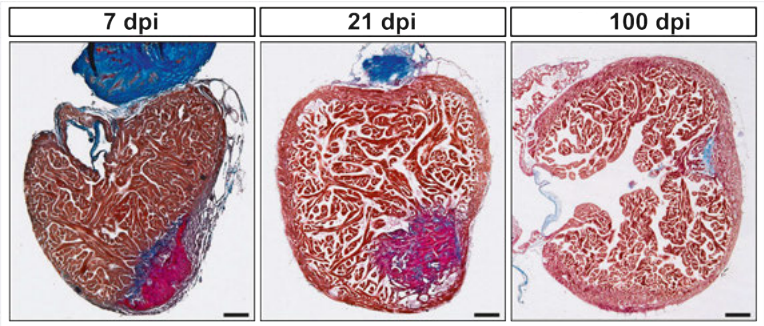

Figure 5. Cardiac regeneration and fibrosis regression upon cryoinjury of the cardiac ventricle in the zebrafish. *AFOG histological stainings on heart sections at 7, 21 and 100 days post cryoinjury (dpi). Myocardium is stained brown, collagen, blue and granular tissue and fibrin red. Note that at 7 dpi, the apex of the ventricle exhibits granular tissue and collagen staining. At 21 dpi, a new myocardial outer layer has been formed engulfing the injured area. At 100 dpi only small remnants of collagen deposits are visible. Pictures adapted from (Gonzales-Rosa et al., 2011).*

Fibrosis as a process compatible with regeneration

We next were interested in understanding where the fibrotic tissue was coming from as, at that time, there had not been any reports on the presence of fibroblasts in the zebrafish heart. So, which cells are generating ECM upon injury in the zebrafish? Are there yet to be identified fibroblasts in the zebrafish heart or are other cell types generating ECM in this species? I was extremely lucky to get funding from the European Research Council through an ERC Starting Grant which enabled us to tackle these questions (337703 "zebraHeart"). First, we set up to analyse the expression of some genes known to mark fibroblasts in humans and other animal models such as the mouse. One gene caught our attention, namely *periostinb (postnb).* It was strongly upregulated in response to injury. We regenerated a transgenic reporter line, to study the dynamics of *postnb* expression as well as the transcriptional profile of *postnb*-positive cells. We found that indeed, *postnb*-positive cells had a transcriptional profile reminiscent of fibroblasts. Interestingly, in the zebrafish, the cells were not only expressing genes related to ECM production and remodelling but also genes related to vasculogenesis and neurogenesis, also includ-

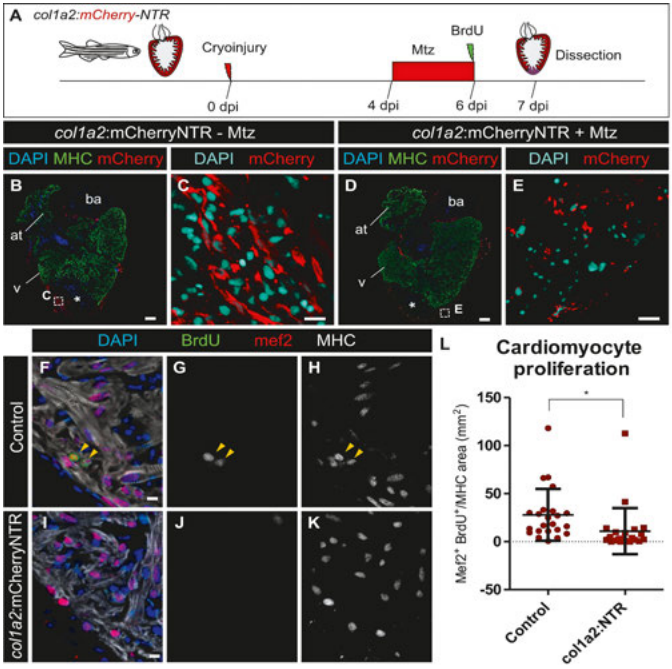

Figure 6. Genetic ablation of collagen 1a2 expressing cells impairs cardiomyocyte proliferation in the cryoinjured heart. A, *Schematic illustration of experimental set up. A transgenic line using a* col1a2 *regulatory sequences from a BAC to drive the expression of nitroreductase (NTR) fused to mCherry was used for this experiment. Adult animals were cryoinjured and treated with Metronidazol (Mtz) from 4 to 6 days postinjury (dpi). Mtz administration leads to cell death of NTR expressing cells. BrdU injection was performed one day prior to fixation to assess cardiomyocyte proliferation.* ***B–E,*** *Immunofluorescence on heart sections of* col1a2:mCherry-NTR *treated with Mtz (B,C) or untreated controls (D,E). C and E are zoomed views of panels B and D, respectively. mCherry is shown in red, myosin heavy chain (MHC) in green and nuclei (DAPI) in blue for B and D, and in cyan for C and E. Note that in Mtz-treated fish,* col1a2:mCherry-NTR *labels cells with fragmented nuclei and the homogeneous expression as shown in the WT heart is lost.* ***F–K,*** *Immunofluorescence using anti-mef2 (red) and anti-MHC (white) to mark cardiomyocytes and anti-BrdU (green) in* col1a2:loxP-tagBFP-loxP-mCherry-NTR *(control) and* col1a2:mCherry-NTR *treated with Mtz and BrdU as described in A. Nuclei are counterstained with DAPI (blue).* ***L,*** *Quantification of BrdU+ cardiomyocytes in* col1a2:mCherry-NTR *and control hearts. Shown are individual measurements as well as median±intercuartile range; *** P=0.0004 by Mann-Whitney test, n = 23 fish per condition, from 2 different experiments. For each point, 3 whole heart sections of a ventricle were quantified. Scale bars, 10 µm (**C,E,F,I**), 100 µm (**B,D**). Figure from (Sanchez-Iranzo et al., 2018b).*

ing several growth factors. This suggested, that not only were fibroblasts not interfering with regeneration, but that fibroblasts might be actively involved in the regeneration process. Indeed, we performed genetic ablation of collagen 1 alpha 2 producing cells and found that cardiomyocyte proliferation was impaired (Sanchez-Iranzo et al., 2018b) (Figure 6).

So where are fibroblasts coming from? We found that fibroblasts derived mainly from pre-existing fibroblasts, but also from the epicardium. The endocardium also contributed to ECM production, but in this case, we observed that cells were not fully undergoing epithelial-mesenchymal transition and often remained attached to each other as an endothelial layer. Later, macrophages were also shown to contribute to ECM deposition in the zebrafish (Simoes et al., 2020).

Further, we used Cre/lox based lineage tracing to study the fate of *postnb*-positive cells in the heart (Figure 7). Fibrotic tissue regresses concomitant with regeneration of the new myocardium. Therefore, we expected to gradually loose *postnb*-positive cells at later stages of regeneration. However, the overall number of *postnb*-positive fibroblasts that accumulated after the first week of injury decreased only slightly and even at late stages, in which regeneration should be complete, we still found a considerable amount of *postnb*-positive cells. Clearly, ECM deposits are removed by then, so, what are fibroblasts expressing at such a late time point? We compared the transcriptome of fibroblasts in uninjured hearts, hearts at 7 days postinjury (7dpi) and hearts at 60 dpi. After completion of regeneration, fibroblast returned to a transcriptional state similar to fibroblasts in uninjured hearts, suggesting that activated fibroblasts initially actively contribute to ECM production and later, rather than being eliminated, remained in a quiescent state. Noteworthy, there were few differences in the gene expression profile between fibroblasts at 60 dpi compared to fibroblasts from uninjured hearts, suggesting that even after complete regeneration, the hearts do not recover completely. Indeed, in line with this observation we had previously noticed that cardiac wall contraction is not fully recovered, maybe partly due to the accumulation of these extra fibroblasts (González-Rosa et al., 2014).

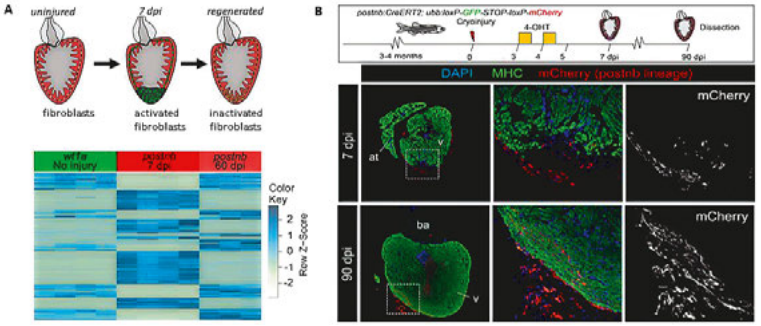

Figure 7. Fate of fibroblasts during heart regeneration. A, wt1a:*GFP allowed labelling of cardiac fibroblasts in the uninjured adult zebrafish heart.* Postnb *expression was used in genetic fate mapping studies of activated fibroblasts in the injured (red, 7dpi) and regenerating (pink, 60 dpi) heart. The transcriptome of* wt1a:*GFP cells was compared to the transcriptome of* postnb-*derived cells at 7 days postinjury (dpi) and 60 dpi. For this, double transgenics* postnCre:ERT2;ubb:Switch *were recombined 3 and 4 dpi and mCherry positive cells FAC-sorted at 7 and 60 dpi. Note that the heat map of* wt1a:*GFP-positive cells is more similar to the gene signature from 60 dpi* postnb-*derived cells than to those from 7 dpi* postnb-*derived cells.* ***B,*** *Lineage tracing of* postnb-*derived cells during heart regeneration. Upper panel shows a schematic representation of the experimental set up and transgenic lines used. Lower panels are immunofluorescence stainings of heart sections and zoomed views of injury area. Note that* postnb-*derived cells (red) are present at 7 as well as 90 dpi, a stage at which regeneration is nearly complete. MHC, myosin heavy chain, marker for myocardium. Image adapted from (Sanchez-Iranzo et al., 2018b).*

Inflammation as a further prerequisite of regrowth

Similar to fibrosis, inflammation has been classically associated with deleterious effects during regeneration, but inflammation is clearly required for wound healing. Indeed, a properly controlled inflammatory response, determines whether a damaged tissue undergoes fibrotic healing or proceeds to regeneration (Godwin et al., 2017b). In a first phase, damage-associated molecular patterns (DAMPS) and pathogen-associated molecular patterns (PAMPS) as well as release of cytokines lead to the accumulation of neutrophils and monocytes. Monocytes further differentiate into macrophages that can be polarized to more pro-inflammatory or more anti-inflammatory phenotypes. Furthermore, tissue-resident macrophages have also been shown to play an essential role in the control of

organ regeneration (Mescher, 2017; Pinto et al., 2014; Wynn and Vannella, 2016). Macrophage depletion by clodronate liposomes treatment leads to the blockage of several regenerative processes such as limb regeneration in the axolotl (Godwin et al., 2013) as well as heart regeneration in the zebrafish, axolotl and neonatal mice (Aurora et al., 2014; Godwin et al., 2017a; Lai et al., 2017).

There is still very little information on macrophage subtypes and macrophage polarization in the zebrafish. Given the accumulated evidence of macrophages in organ regeneration, and that the zebrafish poses such a good model to study regenerative capacity, we sought to investigate the role of macrophages in the cryoinjured heart (Figure 8). We made an initial interesting observation when imaging the epicardium. We found that labelling with the epicardial marker wilms' tumor 1 b (wt1b) also stained a subset of macrophages in the cryoinjured zebrafish heart. We decided to characterize this population more in detail and found that *wt1b*-positive macrophages were transcriptionally distinct from the rest of macrophages (Sanz-Morejon et al., 2019). They differed in the expression of genes related to leukocyte migration, TNF-alpha responsiveness as well as the expression of genes promoting vasculogenesis. Given that migration was one hallmark enriched in *wt1b*-positive macrophages we decided to analyze their migratory capacity in vivo. For this, we switched from the cardiac injury model to the larval fin amputation model, that readily allows in vivo imaging over long time periods at cellular resolution. We found that indeed, *wt1b*-positive macrophages migrated at a lower speed and accumulated more at the site of injury. We also found that *wt1b*-positive macrophages arrived only around 48 h after amputation, while the first macrophages arrive very fast, already within minutes following amputation. This observation is opposite to what has been described for pro-inflammatory *tnf-alpha*-positive macrophages (Nguyen-Chi et al., 2015), suggesting that *wt1b*-positive macrophages represent a population of anti-inflammatory more pro-regenerative macrophages, a hypothesis which is in line with the transcriptome analysis. Next, we were curious to understand if wt1b itself represents only a marker for this macrophage population or, if the gene plays a role in determining part of their phenotype. Firstly, we generated transgenic lines to specifically overexpress a wt1b dominant negative form in macrophages. We found that abrogation

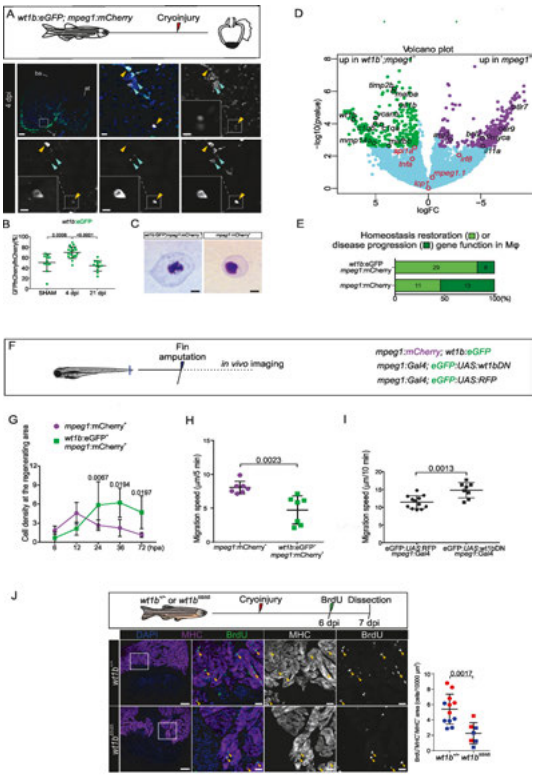

Figure 8. **wt1b-*positive macrophages are involved in heart regeneration in the zebrafish. A–C,*** *Detection by immunofluorescence staining and morphological characterization of* wt1b*:GFP;*mpeg1*:mCherry double positive cells in the uninjured and regenerating zebrafish heart.* ***D–E,*** *RNA-seq analysis of* wt1b*:GFP;*mpeg1*:mCherry compared to* mpeg1*:mCherry positive cells in the injured zebrafish hearts at 4 dpi. Shown is Volcano plot as well as literature search results.* ***F–H,*** *Macrophage migration assay in zebrafish amputated larval fin.* wt1b*:GFP;*mpeg1*:mCherry double positive cells migrate differently and accumulate at later time points at amputation site compared to compared* mpeg1*:mCherry positive cells.* ***I****, Macrophage migration in* wt1b *dominant negative gain of function model using Gal4/UAS system. Compared to a control line, macrophages migrate faster in the transgenic line impairing wt1b function.* ***J****, Analysis of cardiomyocyte proliferation upon cryoinjury of the cardiac ventricle. Shown are immunostaining of heart sections. Proliferation is assessed by BrdU incorporation in Myosin Heavy Chain (MHC)-positive cardiomyocytes. Figure adapted from (Sanz-Morejon et al., 2019).*

of wt1 function affected macrophages migration: They now migrated faster and did not accumulate any longer at the site of injury. Secondly, we studied heart regeneration in *wt1b* null mutant zebrafish. Indeed, we found reduced cardiomyocyte proliferation at 7 dpi compared to control siblings. The drop in proliferation was concomitant with a change in macrophage accumulation at the regeneration front, suggesting that changes in macrophage migration controlled by wt1b function influences proliferative capacity of cardiomyocytes. Overall, together with other recent studies (Bevan et al., 2020; Simoes et al., 2020), we contributed to better understand the role of macrophages during heart regeneration.

Cardiomyocyte subpopulations: can all do the same?

An essential difference between the adult human heart and the zebrafish heart is that the myocardium lost after damage can be replaced. Therefore, a central question is: Where are the new cardiomyocytes coming from? Cardiomyocytes are highly specialized cells, with their cytoplasm nearly completely filled with sarcomeres that allow cell contraction. To draw parallelisms to the skeletal muscle, there, new myofibres are formed, also in humans, through a stem cell pool of satellite cells. One hypothesis therefore was that myocardial regeneration could be contributed by a specific stem cell pool. So far, however, all accumulated evidence goes against this hypothesis. A seminal study was performed using Cre/lox fate mapping of cardiomyocytes using the *myosin light chain 7 (myl7)* promoter. Tracing of cells descendant of cardiomyocytes present in the uninjured heart revealed that the regenerated myocardium derived from pre-existent cardiomyocytes (Jopling et al., 2010; Kikuchi et al., 2010).

These results represented a convincing evidence that zebrafish regenerated the myocardium through re-entry into the cell cycle of differentiated cardiomyocytes upon injury. However, further questions remained. For example, can all cardiomyocytes contribute equally to the regenerated heart, or is there a subset of cardiomyocytes that preferentially proliferated in response to damage? Coincidentally, we observed that a subpopulation of *sox10*-derived cells accumulated at the injury border sites (Figure 9). We were investigating the fate of *sox10*-positive cells as we were interested in studying glia cells in the heart. However, the pattern ob-

served suggested that *sox10*-derived cells were indeed cardiomyocytes. We were able to confirm this by restricting the fate mapping to the myocardial lineage, using a lox reporter line with a promoter specific for ventricular cardiomyocytes. Again, in injured hearts, *sox10*-derived cardiomyocytes accumulated at the injury area. Interestingly, a large proportion of the regenerated myocardium was *sox10*-derived at 60 dpi. So, when are these *sox10*-positive cardiomyocytes emerging? Are they pre-existent in the adult zebrafish heart or do cardiomyocytes at the injury site upregulate *sox10* in response to injury and then contribute to regeneration? We found support for both hypotheses. Using the tamoxifen inducible CreERT2 system, we labelled *sox10*-derived cardiomyocytes well before injuring the heart. We found that in adult zebrafish heart, there was a very small population (less than 1%) of cardiomyocytes that were *sox10*-derived. Our results suggest that upon injury, this population expands 20-fold. We also characterized the transcriptional profile and found that *sox10*-derived cardiomyocytes revealed a distinct gene expression pro-

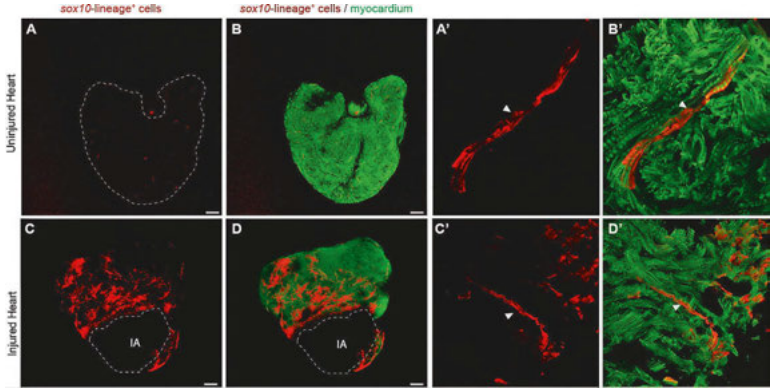

Figure 9. A subset of* sox10*-derived cardiomyocytes contributes to the regenerating myocardium. sox10:CreERT2 *lineage tracing was performed in* sox10:CreERT2; ubb:loxP-GFP-loxP-mCherry (ubi.Switch) *adult zebrafish by 4-Hydroytamoxifen administration 2 weeks before collection of control hearts and 2 weeks before cryoinjury.* ***A–D,*** *Whole mount views of an uninjured heart and a heart at 14 dpi. Note that the uninjured heart reveals few mCherry-positive cells, while upon injury, many mCherry-positive cells are close to the injury area (IA)* ***A'–D',*** *close up view of immunostainings in uninjured and injured heart showing that mCherry colocalizes with the myocardial marker Myosin Heavy Chain (green). Figure adapted from (Sande-Melon et al., 2019).*

file both in uninjured zebrafish heart, but in particular in response to injury, when compared with the rest of ventricular cardiomyocytes. To assess the importance of this population to heart regeneration we performed genetic ablation of *sox10*-derived cells and found that regeneration was impaired. Altogether, the data suggest that a subset of cardiomyocytes might be contributing preferentially to rebuild the injured heart (Sande-Melon et al., 2019). *Sox10* is a neural crest and neural crest derivative marker. Indeed, neural crest cells had been suggested to contribute to the heart tube during embryogenesis and also to contribute to regeneration of the adult heart (Abdul-Wajid et al., 2018; Tang et al., 2019). Whether neural crest progenitors actually contribute to the zebrafish myocardium or if a subset of cardiomyocytes upregulates a neural crest specific program will require further investigation.

The ventricular myocardium can be divided into three main layers, an inner trabecular layer, that fills most of the ventricular cavity, a single layered primordial layer, and an outer cortical layer. We learned from experiments listed above, that it seems that not all cardiomyocytes are equally capable to enter cell cycle and contribute to regenerate the lost myocardium. We also wondered whether cardiomyocytes from one layer are able to regenerate cardiomyocytes from other layers, or if they and their progeny are determined to a particular cardiomyocyte subtype. To answer this question, we first had to identify a reporter line that allows us to distinguish cardiomyocytes from particular myocardial layers. We identified the gene *tbx5a* to be expressed specifically in the trabecular layer and absent from the cortical layer (Figure 10). Therefore, we generated a *tbx5a:CreERT2* line as well as a *tbx5a:mCherry-P2A-CreERT2,* and used it in combination with loxP reporter lines to trace the fate of trabecular cardiomyocytes during regeneration. We found that indeed, when trabecular cardiomyocytes were recombined before injury, we could observe their descendants in the regenerated hearts not only in the trabecular layer, but also in the cortical layer. Interestingly, these *tbx5a*-derived cells were now not expressing trabecular markers any longer, but adopted not only a cortical position, but also expressed cortical marker genes. These results were showing that trabecular cardiomyocytes can undergo a phenotypic switch from trabecular to cortical myocardium in response to injury (Sanchez-Iranzo et al., 2018a). This cellular plasticity might be fundamental to allow rebuilding a heart in an efficient manner.

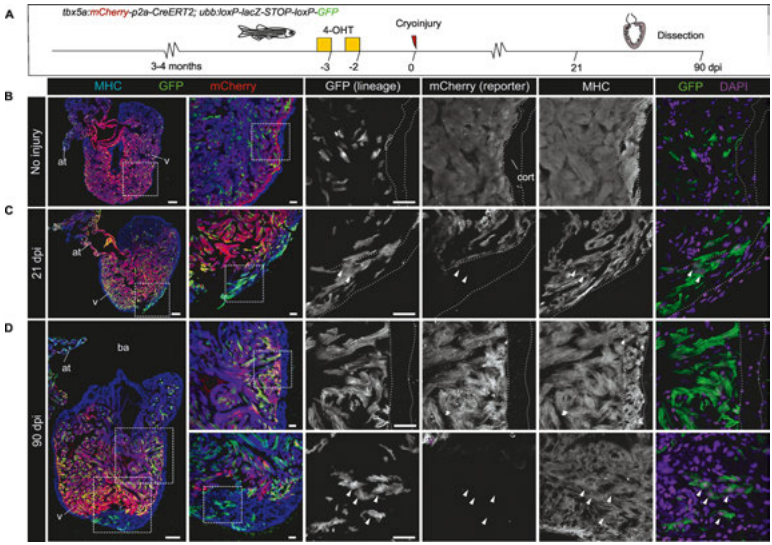

Figure 10. Contribution of trabecular cardiomyocytes to regeneration of the cortical myocardium. A, tbx5a:Cherry-p2A-CreER^{T2} *transgenic zebrafish were crossed into* ubb:loxP-lacZ-STOP-loxP-GFP. *4-OHT was added 2 and 3 days before cryoinjury to induce recombination of* loxP *sites. Hearts were fixed at 21 and 90 days postinjury (dpi) and sectioned for immunofluorescent detection of GFP*$^{+}$ tbx5a*-derived cells and mCherry*$^{+}$ tbx5a*-expressing cells. Nuclei were counterstained with DAPI.* ***B,*** *In the uninjured heart, mCherry expression was homogeneous in the trabecular myocardium and absent in the cortical layer. GFP+ cells were found in the trabecular layer. Single channels of boxed area are also shown.* ***C–D,*** *Section of a heart- at 21 and 90 dpi. Upon cryoinjury to the ventricular apex,* tbx5a^{+} *cardiomyocytes in general were restricted to the trabecular myocardium, but* tbx5a*-derived cardiomyocytes were present also in the cortical layer, particularly at the site of injury. Nuclear counterstaining revealed GFP*$^{+}$ *cell bodies in the cortical layer (arrowheads). at, atrium, v, ventricle. Scale bars, 100 µm (whole heart section), 25 µm (zoomed views). Figure adapted from (Sanchez-Iranzo et al., 2018a).*

While *tbx5a* expression was found throughout most of the trabecular myocardium of the ventricle, there was a region close to the outflow tract of the heart that remained *tbx5a*-negative. This region was first visible already in the embryonic heart. At 24 hours postfertilization, the whole heart tube is *tbx5a*-positive but after this, a *tbx5a*-negative domain starts to appear at the cranial pole of the heart. This is the region where new

progenitors from the second heart field (SHF) are entering the cardiac ventricle. Thus, the *tbx5a*-negative cardiomyocytes seemed to represent SHF-derived cardiomyocytes. It was well-known that, as in mammals, the zebrafish heart is build up from first heart field (FHF) precursors that make up the primordial heart tube and that cells from the SHF then are added to the venous and cranial pole of the embryonic heart to allow fur-

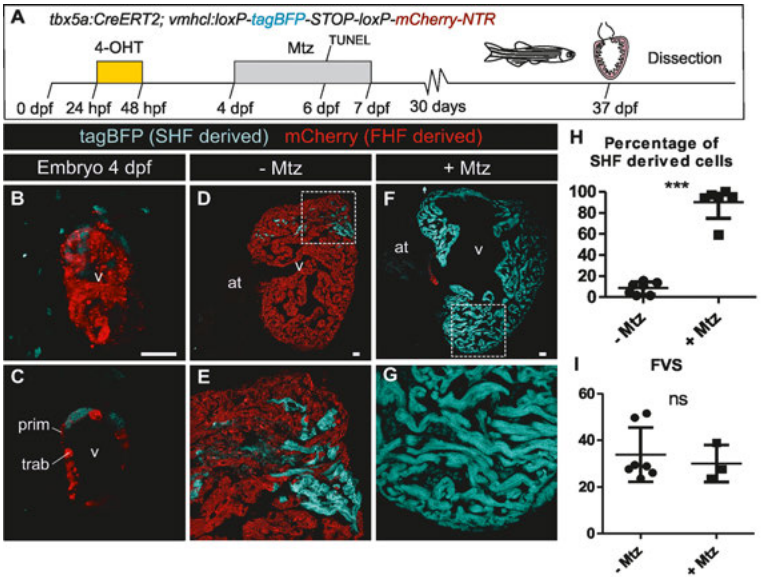

Figure 11. Cellular plasticity during development: second heart field progenitors can compensate for the loss of first heart field derived cardiomyocytes. A, tbx5a^{+} *ventricular cardiomyocytes were genetically ablated in* tbx5a:CreERT2;vmhcl:loxP-tagBFP-loxP-mCherry-NTR *double transgenic zebrafish. Recombination was induced by administration of 4-OHT. Cell ablation was induced by administration of Metronidazol (Mtz) from 4 to 7 dpf. Hearts were dissected 30 days later.* ***B–C,*** *Ventral views of larval hearts at 4 dpf. Anterior is to the top. Note that the proximal ventricle is completely mCherry^{+}, and that the distal ventricle is blue (tagBFP^{+}).* ***D–E,*** *Section of the ventricle of an adult recombined heart. Most cells are mCherry+. Only the* tbx5a- *region is tagBFP^{+}. (**F–G**) Sagittal section of an Mtz-treated fish. Most of the cardiomyocytes are BFP^{+}.* ***H,*** *Quantification of the percentage of myocardium that is tagBFP^{+} (SHF derived). I, Cardiac function is not affected by ablation of tbx5a-derived cells. FVS, fractional ventricular shortening (in %). mean±s.d; *** P<0.0001 by two-tailed unpaired t-test. at, atrium; prim, primordial; trab, trabecular; v, ventricle. Figure adapted from (Sanchez-Iranzo et al., 2018a).*

ther heart development (Knight and Yelon, 2016). Our genetic lines now allowed us to interrogate if FHF and SHF progenitors are interchangeable or in other words, if SHF progenitors can also give rise to FHF structures, if needed. To test this hypothesis, we ablated FHF derived ventricular cardiomyocytes using the nitroreductase (NTR) system (Figure 11). We crossed the line *tbx5a:CreERT2* into the line *vmhcl:loxP-BFP-loxP-mCherryp2A-NTR*. Upon recombination, these double transgenic animals express mCherry and NTR in *tbx5a*-derived FHF ventricular myocardium. Addition of the compound Metronidazol leads to cytotoxicity in NTR-expressing cells, and as such, eliminates the FHF-derived ventricle. With this experimental set up, we now could investigate if the FHF-derived myocardium can be regenerated from SHF-derived progenitor cells. Indeed, we found that while in control situation recombined animals have an mCherry-positive (red) and blue fluorescent protein (BFP)-positive (blue) ventricle, animals that underwent genetic ablation revealed some days later a fully BFP-positive ventricle. Interestingly, we did not observe neither major morphological alterations nor changes in cardiac function in these hearts, now comprised fully by SHF derived myocardium. In conclusion, while in a wildtype scenario the cardiac ventricle is built up from FHF and SHF precursors, SHF precursors can fully compensate the loss of FHF-derived myocardium (Sanchez-Iranzo et al., 2018a). This is a second example of the high plasticity that cardiomyocytes reveal during heart regeneration.

Conclusions and Outlook

Cardiac regeneration is a complex process during which several cell types interact and communicate with each other to promote heart regrowth, including the re-establishment of cardiac function. Our own studies combined with those from others show that a first injury response including inflammation and fibrosis are key steps towards myocardial regeneration. Our studies also show that a regenerated heart is not completely equal to an uninjured heart, to a low degree, cellular composition and function is altered. The zebrafish remains a key model organism in the research of the cellular and molecular mechanisms of heart regeneration. Further technological improvements such as intra vital imaging methods in the adult zebrafish and genome editing approaches allowing the generation

of spatial and temporal control of gene expression will strongly contribute to provide further knowledge on heart regeneration. A very important central question will be, in my opinion, to understand the epigenetic control mechanisms underlying heart regeneration. Which cells are prone to contribute to regeneration and how is this "readiness" encoded?

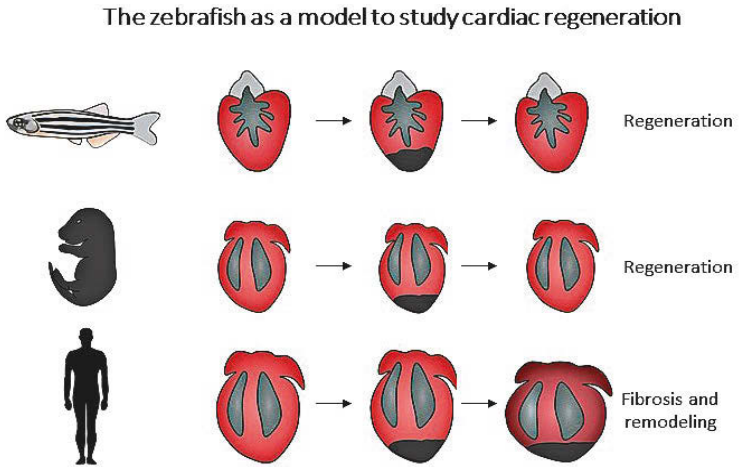

Figure 12. Zebrafish as a model to study heart regeneration. *Zebrafish as well as neonatal mice regenerate their hearts after sever injury. Adult human hearts undergo fibrosis and remodelling upon injury.*

The incorporation in the last decade of a mammalian model to study heart regeneration has been very important to the field (Figure 12). The groups of Eric Olson and Enzo Porrello described that during their first week after birth, mice can regenerate the heart with a similar efficiency as zebrafish (Porrello et al., 2011). During the first week of age, neonatal cardiomyocyte also re-enter the cell cycle and divide upon cardiac lesion. Indeed, several pathways and mechanisms of regeneration are conserved between mouse and zebrafish, supporting the possible translational impact of studies on heart regeneration in the zebrafish. While classically the human heart has been considered to be postmitotic, there is a physiological turnover of cardiomyocytes, which is particularly prominent in

the first two decades of life. Indeed, some reports estimate that the cardiomyocyte pool is replaced twice during human lifespan (Bergmann et al., 2009; Bergmann et al., 2015). While after cardiac injury, there is a statistically significant increase in cardiomyocytes undergoing cell cycle reentry, the numbers are far too small to support heart regeneration. The finding that some mammals have the capacity to regeneration early on in life, but that this program is repressed in the adult, might indicate that there is a therapeutic window to re-activate a naturally repressed mechanism. A close interaction of researchers working with different species and the combination of approaches ranging from basic to translational science hopefully pave the way not only to fully uncover the mechanisms of heart regeneration but also to design strategies to promote it.

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