Interrogating Precision Metabolite Signaling Actions Promoting Cardiovascular Health & Translation

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1. OVERVIEW & IMPACTS.

We here unite YA's latest precision metabolite–target mapping technologies (2024:*Cell*[1]), FS's advanced clinically-relevant immuno-cardiovascular models, and LH's extensive experience in precision therapies. The goal is to decode cell-specific metabolite–proteome signaling mechanisms that support cell–cell metabolic crosstalk essential for regenerative healing. Our collective drug discovery expertise further promises novel mechanism-guided therapeutic avenues.

2. SIGNIFICANCE.

Single-cell sequencing and spatial-omics tools can identify changes in local proteome, (epi)transcriptome, and metabolome makeup. However, these methods cannot decode how individual localised metabolites modulate local proteome functions and signaling networks. Many innate metabolites (hereafter, reactive metabolites "REMs") can directly modify cellular proteins, to drive non-enzymatic post-translational modifications that influence disease outcomes[2-9]. However, pinpointing specific biologically-relevant REM-modifications with spatiotemporal precision remains highly challenging. This is because simply flooding cells/animals with REMs (*the approach underpinning all state-of-the-art proteomics target-ID methods*) drowns relevant events in a sea of nonspecific modifications and off-target signaling, giving poor control over location and timing. YA Group has pioneered novel tools to map the molecular targets and precision signaling activities of REMs in an organ-specific, time-resolved manner (e.g., **2024**:*Cell*[1]; Z-REX, **2023**:*Nat Protoc*[10]). Using these methods, we have also decoded specific REM-modifications of proteins that alter cell-specific processes of conserved importance in physiology and drugs' mode-of-action[1,11,12]. We have also shown how such knowledge directly guides precision drug design[12,13] and novel biomarkers, including in immune cells[11]. In unison with FS Group, we will now apply these tools in leading disease models in cardiac regeneration. We will deliver for the first time comprehensive mechanistic maps of the locale, timing, specific REMs' targets, and downstream functional changes crucial to cardiac healing. (Note: Identity of specific endogenous REMs and synthetic analogues of interest are not disclosed for confidentiality, but our choice is guided by escalating importance of certain REMs in cardiovascular diseases). In collaboration with LH, we will exploit our newly-discovered disease-modifying REM-dependent pathways, ligands, and targets in human-derived samples to enable streamlined precision interventions.

3. HYPOTHESIS.

Efficient crosstalk between injured cardiomyocytes and local interacting cells is essential for regenerative healing. For instance, FS showed functional involvement of macrophages in facilitating perfect heart repair in regeneration-competent systems such as neonatal mice hearts: such functions

are lacking in non-regenerative adult mammalian and human hearts (2020:Nat.Commun[14]). Distinct classes of REMs are increasingly implicated in wound-resolving phenotypes of the damaged heart: including protection against hypoxic heart injury; and boosting heart function post injury in rodents and human-derived cardiovascular disease models. Altered macrophage functions are implicated in many instances, but mechanisms remain unclear. We **hypothesise** that profiling cell-specific REM-responsive proteins and metabolite–proteome signaling changes during cardiac healing will uncover druggable regulators hidden from conventional and spatial omics experiments. ***Models.*** We will deploy human (h)iPSC-derived *in-vitro* co-cultures[15] and state-of-the-art 3D cardiac organoids[16] (**Fig.1**), in which we can functionally mimic ischemic reperfusion injury (I–R) and myocardial infarction (MI)[17]. Commercial hiPSCs will be used to produce these models, as they furnish more reproducible outcomes, and thus robust conclusions in systematically testing our hypothesis.

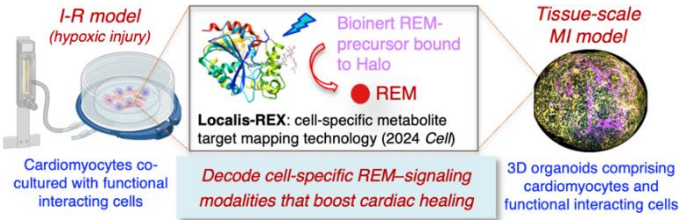

Fig.1. Overview of the enabling technology integrated into the two functional mechanistic models as described in the proposal. Localis-REX (2024 Cell[1]) will enable cell-specific identification of druggable targets/pathways with precise timing, locale, contexts, and REM-chemotypes. A parallel validation technology (T-REX, not illustrated here, but see, for instance, 2023 Nat Protoc[10]) will mechanistically link on-target REM-modifications to specific cardiac-healing associated phenotypic outputs. 3D cardiac organoid image was reproduced from FS-Group's recent work, 2023 Front Cardiovasc Med[16]).

4. AIMS.

YA-lab's unpublished work has shown how their latest tools are uniquely able to map functional REMs' direct targets cell-specifically, including cardiomyocytes and immune cells. Our ***Specific Aims*** include: mapping cardiac healing-associated phenotypic changes, driven by cell-specific REM upregulation **(Aim:I)**; multiplexed quantitative mapping of cell-specific REMs' targets with an unprecedented spatiotemporal resolution **(Aim:II)**, and mechanistically linking specific phenotypes to specific REM—protein target modifications for modality-agnostic therapeutic exploitations **(Aim:III)**.

5. APPROACH.

FS-lab established protocols will help us hit the ground running in achieving functional human iPSC-derived co-cultures and organoids. In both systems, lentiviral transgene overexpression routinely used by us[11,18,19], will achieve cell-type-specific expression of requisite engineered protein tags alongside fluorescent-protein markers required for functional integration of YA-lab technology (**Fig. 1**). **Aim:I** (phenotypic mapping): In live co-culture, modeling on established hypoxic stress-protocols[15,20], we will execute (against relevant untreated controls) cell-specific upregulation of individual REMs at distinct post-stress stages of relevance to I-R injury. Extent of cardiomyocyte healing will be assessed using established workflows[21] targeted at known downstream markers. In functional 3D organoids, following injury (established protocols[17]), we will execute controlled REM-upregulation at specific cell-types at distinct stages relevant to post-MI-injury immune responses phases[22,23]. We will map injury-associated phenotypes routinely assessed in FS Group. **Aim:II** (REMs' targets mapping) will follow cell sorting, lysis, and subsequently, YA-lab-established quantitative proteomics target-ID workflows, yielding cell-specific REM/target pairs[1,10]. **Aim:III** (mechanistic studies and target/pathway validations) will deploy conceptually-similar strategies as in YA's & FS's previous publications[1,10-12,16,18,19,24-28]. YA-Group's established workflow to validate REM-target engagement in cultured human cells (cell-type to be guided by discovery in Aim:I-II)[1,12,19,25] will quantitatively score top protein-hits, against their respective REM-ligand occupancy. We will further deploy YA-lab's established modification-site-mapping mass-spectrometry workflow to identify REM-binding site. We will then use this holistic information to create functional KO/KI lines for functionally-validated top hits. Compared with control lines, KO/KI lines are expected to be recalcitrant to REM-induced molecular-level changes, measured by qPCR,

western blot, scRNA-Seq assays. We will also assess in KO/KI vs. control cells cardiomyocyte proliferation, scratch-wound migration, and established functional measurements in cardiac organoids[23,27,28]. Our mechanistic data are directly translatable for ***wider therapeutic benefits*** as hypoxic stress and injured cardiac organoids are features of I–R and MI, respectively. Cardiac organoids functionally recapitulate tissue-scale hallmarks of MI injury, including cardiotoxicity, cardiac, and fibrotic effects, and are thus excellent mechanistic models for understanding REM mechanism of action. Novel mechanistic biomarkers, REM-signal propagators, protein–protein interactions, and REM-inspired small-molecule modulators will be further developed.

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Supervisor's recent relevant publications

Yimon Aye (primary supervisor):

• Liu et al. & Aye, **2024 Cell** "**Organ-specific electrophile responsivity mapping in live *C. elegans***" The Aye lab presented how a method designed to construct a functionally-annotated map of chemical actionability on locale proteomes in whole live organisms helps discover localized metabolite-protein signaling, not easily mapped by any other approaches.

• Huang et al. & Aye, **2023 Nat Protoc** "**Z-REX: shepherding reactive electrophiles to specific proteins expressed tissue specifically or ubiquitously, and recording the resultant functional electrophile-induced redox responses in larval fish"** The Aye lab established a general technology to perturb a single protein-target with reactive electrophiles in live fish and simultaneously monitor local and global cell-type-specific, on-target electrophile-mediated signaling responses in vivo.

• Poganik et al. & Aye **2021 Nat Commun** "**Wdr1 and cofilin are necessary mediators of immune-cell-specific apoptosis triggered by Tecfidera"** Leveraging their in-house-developed precision REX-technologies, the Aye lab mapped the Keap1-targeted drug-modification-specific changes in Keap1's interactomes & downstream transcriptomes. This strategy discovered a novel innate-immune-cell-

specific electrophilic drug (Tecfidera) responsive Keap1-Wdr1-mitochondrial-targeted apoptosis pathway. Fundamentally, this study documents that substoichiometric single-protein–electrophile engagement is sufficient to trigger phenotypic-level responses in whole animals.

• Fu et al. & Aye **2018 Nat Chem Bio** "**Nuclear RNR-α antagonizes cell proliferation by directly inhibiting ZRANB3"** RNR-enzyme-complex is upregulated in cancer but expression of one RNR-subunit (RNR-α) is tumor-suppressive: the mechanistic basis remained unknown for decades. The Aye lab discovered that dATP/related FDA-approved antileukemic nucleoside drugs induce RNR-α-specific hexamerization and rapid nuclear translocation, whereby nuclear RNR-α displaces ZRANB3 from PCNA. This novel pharmaceutically-relevant tumor-suppressive nuclear signaling axis resolves the long-debated tumor-suppressor role of RNR-α.

• Long et al. & Aye **2017 Nat Chem Bio** "**Akt3 is a privileged first responder in isozyme-specific electrophile response"** This work spotlights how single-protein-specific electrophile modification method (T-REX) enables novel target/ligand-discovery and mechanistic validations. Leveraging T-REX, they uncovered enhanced electrophile-sensitivity of Akt3-kinase over Akt2/Akt1, and Akt3-C119-specific electrophile-signaling mechanisms in cells/zebrafish. This discovery further guided the development of first-in-class Akt-isoform-specific inhibitors (2020 ACS Cent Sci).

Filipa Simões (co-supervisor)

• Lin HC, Makhlouf A, Vazquez Echegaray C, Zawada D, **Simões F.C.#** (2023). Programming human cell fate: overcoming challenges and unlocking potential through technological breakthroughs. Development 150(24):dev202300.

• Reyat JS, di Maio A, (...), Psaila B, **Simões F. C.**, Rayes J, Khan AO. (2023). Modelling the pathology and treatment of cardiac fibrosis in vascularised atrial and ventricular cardiac microtissues. Front Cardiovasc Med. 10:1156759. This work presented a novel methodology for the generation of chamber-specific cardiac microtissues that is highly scalable and allows for the multi-parametric assessment of cardiac remodelling and pharmacological screening.

• **Simões, F.C.*,** Cahill, T.J.*, et al. (2020). Macrophages directly contribute collagen to scar formation during zebrafish heart regeneration and mouse heart repair. Nature Communications 11, 600, PMCID: PMC6992796. (This study was highlighted in Nature Reviews Cardiology, F1000Prime and the press, with over 250 citations to date). This seminal work showed that macrophages directly contribute collagen to the forming cardiac scar. This new evolutionarily-conserved role contrasts with the current model of scarring and is likely applicable across organ systems and fibrotic disease.

• **Simões, F.C.#** and Riley, P.R.# (2022). Immune cells in cardiac repair and regeneration. Development 149 (8): dev199906.

• Weinberger, M.*, **Simões, F.C.***, Gungoosingh T, Sauka-Spengler T, Riley PR. (2024). Distinct epicardial gene regulatory programs drive development and regeneration of the zebrafish heart. Developmental Cell S1534-5807(23)00692-5. This study unravelled cis-regulatory elements and gene circuits that are unique to the adult regenerative epicardium when compared to its developmental counterpart, findings that challenge the view that regeneration simply recapitulates developmental genetic programmes.