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Citation

Rietjens, R. G. J., Wang, G. Q., Berg, B. M. van den, & Rabelink, T. J. (2024). Spatial metabolomics in tissue injury and regeneration. *Current Opinion In Genetics & Development*, 87. doi:10.1016/j.gde.2024.102223

Version: Publisher's Version

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Downloaded from: <https://hdl.handle.net/1887/4107381>

Note: To cite this publication please use the final published version (if applicable).

Review

Spatial metabolomics in tissue injury and regeneration

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Tissue homeostasis is intricately linked to cellular metabolism and metabolite exchange within the tissue microenvironment. The orchestration of adaptive cellular responses during injury and repair depends critically upon metabolic adaptation. This adaptation, in turn, shapes cell fate decisions required for the restoration of tissue homeostasis. Understanding the nuances of metabolic processes within the tissue context and comprehending the intricate communication between cells is therefore imperative for unraveling the complexity of tissue homeostasis and the processes of injury and repair. In this review, we focus on mass spectrometry imaging as an advanced platform with the potential to provide such comprehensive insights into the metabolic instruction governing tissue function. Recent advances in this technology allow to decipher the intricate metabolic networks that determine cellular behavior in the context of tissue resilience, injury, and repair. These insights not only advance our fundamental understanding of tissue biology but also hold implications for therapeutic interventions by targeting metabolic pathways critical for maintaining tissue homeostasis.

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Current Opinion in Genetics & Development 2024, 87:102223

This review comes from a themed issue on **Cell Reprogramming, Regeneration and Repair**

Edited by **Anne Grapin-Botton** and **Jonathan Loh**

For complete overview of the section, please refer to the article collection, "[Cell Reprogramming, Regeneration and Repair \(2024\)](#)"

Available online 19 June 2024

<https://doi.org/10.1016/j.gde.2024.102223>

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Introduction

Adaptations in cellular metabolism are key within the tissue microenvironment (TIM) to maintain homeostasis or to

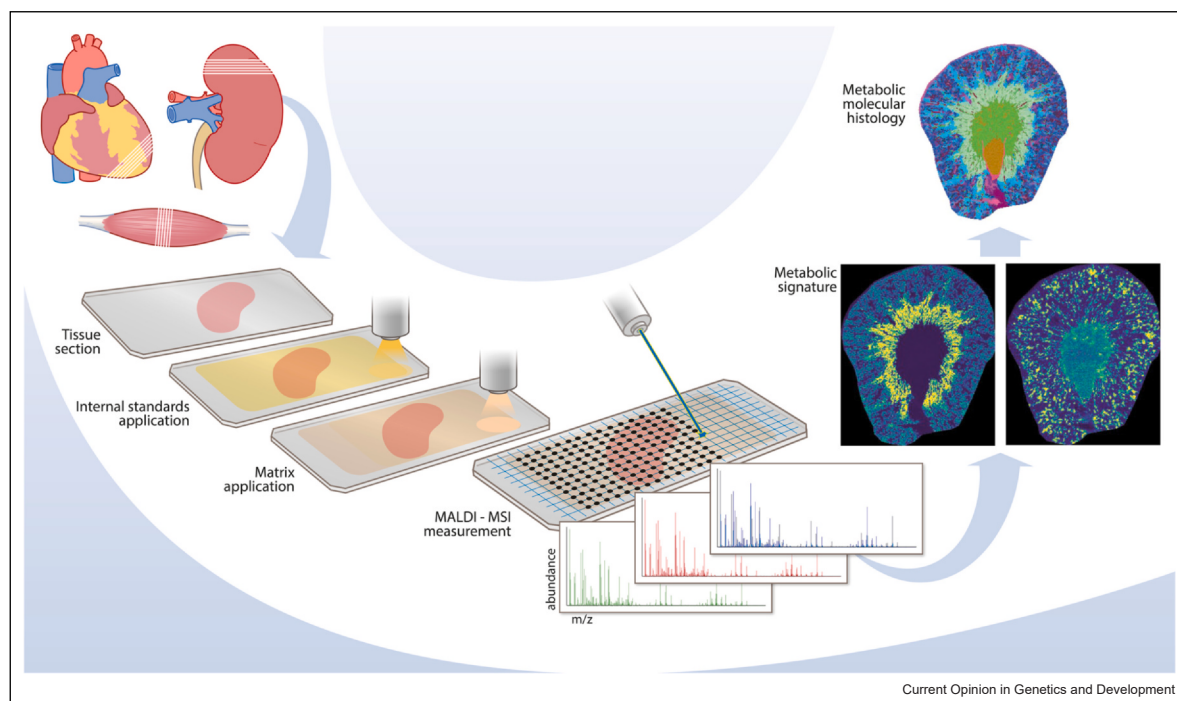
initiate a response to injury [1]. Changes in metabolic fluxes determine chromatin accessibility through direct metabolite modifications of histones and determine subsequent downstream transcriptional responses and ultimately cell fate decisions [2,3]. Being informed about the metabolic interaction between cells in a TIM is thus relevant to understanding processes, such as homeostasis, injury, and regeneration. Although conventional (single cell) metabolomics approaches provide extensive cellular metabolic profiles, information of spatial heterogeneity and composition of the TIM is lost. We here discuss the current state of the art with respect to spatial metabolomics, an approach that allows *in situ* interrogation of cellular metabolism.

The rise of spatial metabolomics

The field of spatial metabolomics has made rapid advancement in recent years, with mass spectrometry imaging (MSI) being the predominant enabling technology. To date, MSI remains the workhorse in spatial metabolomics studies [4]. Initially, these studies were limited by spatial resolution and molecular class identification, but rapid progression in MSI instrumentation now allows imaging of thousands of molecules such as metabolites, lipids, proteins, and glycans at a (sub)cellular resolution [5,6]. MSI methods can vary in, for example, ionization technology or method of detection, which is all well described by Reyzer & Caprioli [7]. In this review, we mainly focus on the matrix-assisted laser desorption ionization (MALDI) method, which has seen extensive technological advancement and is the most commonly used technique in biological research settings [8]. In MALDI-MSI, a chemical matrix is applied to a tissue section, thereby extracting analytes from the tissue and forming *in situ* molecule-matrix crystals (Figure 1). These crystals desorb the laser energy, causing the molecules to ionize and making them amenable for measurement by mass spectrometry (MS). As this method does not require any labeling, MALDI-MSI is a powerful tool that enables unlabeled investigation of the metabolic content of biological tissues in a spatial manner.

As for every analytical technique, MALDI-MSI has its limitations and technical challenges [9]. These can be attributed to the sample preparation process (e.g. post-mortem degradation, molecular delocalization, or matrix application), MALDI-MSI measurement (e.g. spectral or spatial resolution, ionization bias, or in-source fragmentation), and metabolomics data analysis (e.g. analyte annotation, absolute quantification, and multiomics

Figure 1



Metabolic molecular histology. Sections of fresh frozen tissues are applied to a slide for the MALDI-MSI pipeline. For qMSI, internal standards are first applied (refer to [Box 1](#) for details). In all MALDI-MSI experiments, matrix is uniformly applied to the section, facilitating extraction of analytes from the tissue and formation of crystals to make them amenable for MS analysis. In the mass spectrometer, spectra are recorded in a spatially correlated manner by imposing a virtual coordinate grid on the tissue. This enables visualization of the analyte distribution within the tissue context. Subsequent dimensionality reduction and segmentation allow for the reconstruction of metabolic molecular histology. The molecular histology images were adapted from previous reports [18].

integration). [Box 1](#) describes these current challenges and limitations in more detail.

The tissue microenvironment and metabolic molecular histology

The importance of the molecular context in which cells reside is widely recognized in cancer studies, where the tumor microenvironment significantly affects disease progression and therapeutic outcome [10,11]. Similarly, the TIM plays a crucial role in tissue regeneration postinjury, with altered metabolite availability and oxygen tension necessitating metabolic adaptations to meet cellular energy demands [12]. Recent studies highlight the pivotal role of metabolite pool alterations in processes beyond metabolic energy transduction, including nutrient sensing and storage, cell survival and differentiation, and immune activation and cytokine secretion [13–16]. Characterization of the TIM using spatial metabolomics is crucial for our fundamental understanding of the role of metabolic processes in tissue homeostasis.

The lipid membrane composition has proven to be a powerful tool for dissecting the complex cellular composition of the TIM. Spatial segmentation based on membrane lipids has been used in multiple instances to reveal a so-called metabolic molecular histology ([Figure 1](#)) [17–19]. This histology allows researchers to identify different cell populations and states within these populations, both in health and disease, which are not always revealed with conventional histology approaches [20]. In the kidney, this approach leads to the finding of lipid signatures for different renal cells in specific nephron segments and visualized metabolic alterations in cells both preceding tissue injury as well as the altered post-injury TIM [17–19]. For the liver, specific hepatic lipid patterns were identified, corresponding to the liver zonation pattern found with immunofluorescent staining [21]. Another study focusing on chronic muscle injury revealed that degenerated muscle regions could be distinguished from healthy muscle regions based on their lipidomic profile [22]. These examples highlight the strength of spatial lipidomics in its ability to characterize the cellular composition of the TIM.

Box 1 Limitations and challenges of spatial metabolomics.

Spatial metabolomics comes with limitations and challenges, both in experimental considerations as well as data processing and analysis. Given the highly dynamic nature of metabolism, quality of the starting material greatly impacts the resulting quality and resolution of the image [41]. Postmortem degradation and molecular delocalization need to be prevented as much as possible by swift tissue handling, freezing, and storage, as well as employing properly optimized MALDI matrix application methods [42]. Proper sample handling and preparation methods, combined with matrix crystal size, laser spot diameter, and MALDI stage movement together, determine the resolution of the resulting MALDI images.

Next, spectral resolution and quality need to be considered, which are for a significant part determined by the MS instrumentation. Spectral resolution is critical for metabolite annotation, as insufficient resolution may combine multiple molecules into a single peak, and mass accuracy is equally essential for confidently assigning identities to mass features. Quality of the spectra always needs to be assessed to be able to filter out ions that are caused by, for example, in-source fragmentation, adduct formation, or natural isotope abundance. Currently, merely empirical and fragmentary knowledge is available about these spectral artifacts, necessitating manual curation of the data [43].

Finally, a significant hurdle in the spatial metabolomics field is quantification. Quantitative MSI (qMSI) requires strategies to deal with variations in ionization efficiency between molecules, which can be achieved through the use of internal standards [44–46]. Use of internal standards, on the one hand, allows correction for unwanted signal variability and, on the other hand, is required for absolute quantification via calibration curves. qMSI has been applied successfully to investigate drug delivery but also to visualize metabolic fluxes in a complex tissue [47,48]. Furthermore, the possibility of omics-scale qMSI for lipid analysis has been reported using a combination of 13 internal standards for different lipid classes [49]. The challenge remains to apply a similar approach for metabolites due to the variety in molecular structure.

Recently, a collaborative effort by the MSI field has been done to evaluate various spatial metabolomics protocols and technologies [50]. These efforts, combined with further development of methods, will help to establish standard operating procedures, to obtain reproducible and reliable data, and to overcome the limitations and challenges of the technique.

An additional layer of information that can be added to the molecular metabolic histology is that of spatial proteomics. Imaging mass cytometry and multiplexed ion beam imaging by time of flight started to pave the way to bring this into practice; however, these techniques remain limited due to necessity for highly specialized instrumentation and low throughput [23,24]. Recently, a new MALDI-MSI-based spatial multimomics technique has been developed, which combines spatial metabolomics with spatial proteomics using mass-tag-labeled antibodies on post-MSI tissue. This technique, called MALDI-immunohistochemistry (IHC), is based on photocleavable mass tags that are amenable for measurement with MSI instrumentation, which enables highly multiplexed IHC combined with conventional MSI [25–27]. Addition of proteomics to the molecular metabolic histology allows for a deeper and more comprehensive understanding of the TIM.

Once the composition of the TIM is dissected, the metabolic state of different cell types can be interrogated to decipher the metabolic networks important for tissue homeostasis. As spatial metabolomics allows hundreds of molecules to be measured at the same time, the technique can be applied to look for either metabolic signatures or metabolites that are specific for cell populations or injured sections of the TIM. The strength of the technique is that there is no need for labeling, which makes it applicable to a large variety of tissues and pathologies, which is illustrated by the following examples. A study investigating the early effects of myocardial ischemia revealed that MSI could detect the ischemic cardiac region, whereas this was challenging before due to lack of diagnostic markers [28]. One example of a metabolite that was clearly increased in the ischemic region is a toxic degradation product of nicotinamide adenine dinucleotide, which could potentially serve as a new diagnostic marker. In a second study, MSI was

applied in a rat model of ischemic brain injury [29]. In this model, glutamate abundance seemed to be decreased in the infarct area, whereas it was increased in the peri-infarct area [29]. Interestingly, cell therapies tested in this study showed a location-specific effect; information that would be lost with a regular metabolomics strategy. These examples showcase that the identified signature metabolites might provide new therapeutic ways to specifically target the affected TIM upon injury to promote tissue regeneration and homeostasis.

Metabolic dynamics unveiled: mass spectrometry imaging meets isotope tracing

Conventional spatial metabolomics studies with MSI have been restricted to capturing metabolite pool sizes, which allows construction of the metabolic molecular histology but is limited to providing static ‘snapshot’ of cellular metabolic states. Metabolism is a highly dynamic process, which necessitates a method to elucidate the direction and turnover rate of metabolic pathways. A promising method to achieve *in situ* measurements of metabolic dynamics, is to combine stable isotope tracing with subsequent MSI analysis [18,30–32]. In this approach, a stable isotope-enriched metabolic substrate is introduced in a biological system, allowing the uptake, turnover, and incorporation of this labeled substrate into downstream metabolites to be measured with MSI (Figure 2). For example, U-¹³C₆-glucose as a substrate facilitates tracing of glycolysis, the tricarboxylic acid (TCA) cycle and other metabolic pathways related to glucose metabolism. The most common ways of introducing the stable isotope are through *in vivo* infusion (Figure 2a) or *ex vivo* tissue culture (Figure 2b). The integration of stable isotope tracing with MSI not only expands our understanding of metabolic dynamics, but it also paves the way to reveal metabolic pathways, which

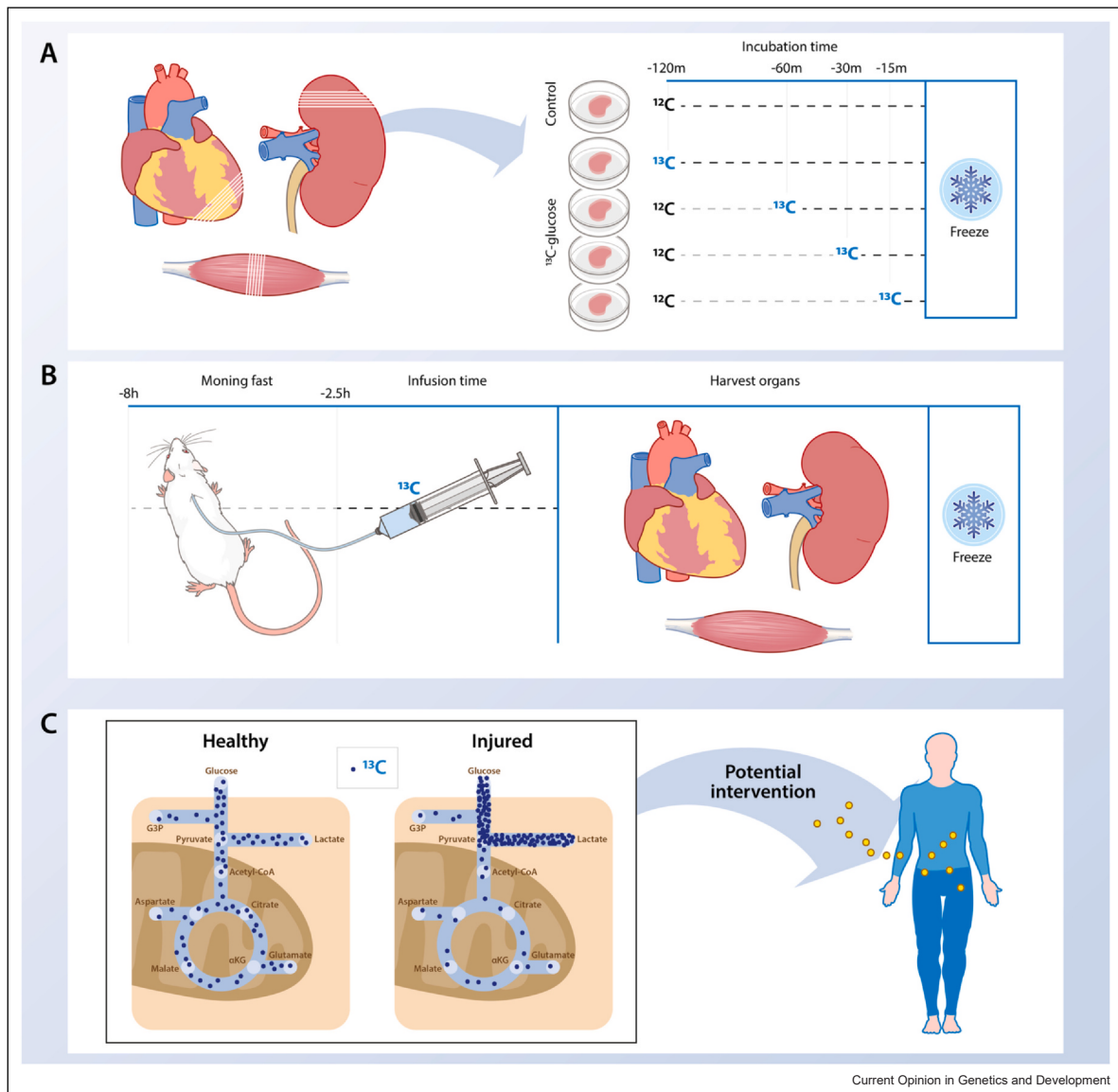
could be targeted for therapeutic interventions aiding tissue homeostasis (Figure 2c).

Role of metabolic flux in tissue injury and regeneration

The applicability and biological relevance of dynamic metabolic measurements have been recently exemplified in various publications [18,30,33]. Work by the groups of Davidson and Rabinowitz unveiled regional metabolic preferences in the kidney, highlighting a clear glycolytic and

gluconeogenic preference of the medulla and cortex, respectively [30]. By increasing the spatial resolution, Wang et al. were able to interrogate metabolic preferences at the single-cell level [18]. This study revealed a higher glycolytic flux and altered ^{13}C -enrichment of TCA cycle intermediates in the maladapted proximal tubule cells after ischemic reperfusion injury compared with their healthy counterparts. Strikingly, unexpected metabolic anomalies were identified in proximal tubule cells with a seemingly normal phenotype in the recovery phase, emphasizing the impact of the TIM.

Figure 2



Dynamic metabolic measurements. (a) Ex vivo tissue slices are incubated at various time points with the ^{13}C -enriched stable isotope. After the experiment is done, the sections are frozen down and are subjected to the MALDI-MSI workflow as depicted in Figure 1. (b) The ^{13}C -enriched nutrient is infused in the animal, after which the organs are harvested and frozen down for subsequent MALDI-MSI analysis as depicted in Figure 1. (c) Dynamic metabolic calculations can be performed to interrogate the metabolic heterogeneity between healthy and injured tissue. This comparison can reveal potential new therapeutic interventions, promoting tissue regeneration and homeostasis postinjury.

In parallel, spatial dynamic metabolomic measurements were used to improve *in vitro* generation of kidney organoids [33]. By understanding the metabolic trajectory of the developing human kidney, a defect in metabolic maturation of the kidney organoids was identified. Addition of the metabolite butyrate to the culture media resulted in enhanced *in vitro* differentiation and maturation of proximal tubules. Similar approaches applied to the TIM may reveal novel metabolic targets crucial for tissue regeneration after injury.

An exciting yet challenging frontier in this line of technology involves integrating *in situ* isotope tracing with metabolic flux analysis. In a recent publication, fractional fluxes — the fraction of a metabolite pool, which is newly synthesized during the isotope labeling period — were calculated and visualized throughout the brain to investigate the degree of metabolic heterogeneity in the tumor microenvironment compared with surrounding healthy tissue [31]. By adjusting established tools typically used for liquid chromatography (LC)/MS-based metabolomics analysis toward application for MSI, the work of Schwaiger-Haber et al. introduced a workflow that allows fractional fluxes of metabolic pathways to be quantitatively compared between different conditions. Applying this method in a tissue regeneration context allows quantitative evaluation of therapeutic interventions on tissue architecture restoration. Taking it one step further, application of flux modeling algorithms on spatial isotope tracing data sets holds promise for spatially resolved flux measurements during tissue injury and regeneration. A major hurdle in accurate modeling of metabolic fluxes is the need for absolute quantification, which MSI does not provide. Up until now, nonspatial LC/MS-based methods are necessary in parallel to ensure reliable quantification results. Insights derived from such experiments can provide a foundation for developing targeted therapeutic interventions that restore homeostasis and facilitate tissue repair [34,35].

Spatial metabolomics in the multiomics era

Similar to other omics technologies, spatial metabolomics generates extensive data sets, containing molecular information from a considerable number of pixels. With spatial resolution improving, the number of pixels per data set is increasing drastically. Furthermore, the unprocessed data sets contain nonessential information, such as adducts or fragments formed during the MALDI process. Spatial metabolomics data sets can reach terabits in size, underscoring the need for tailored computational methods for effective MSI data processing and analysis. Given the technical artifacts present in the data, such as adduct formation and ion suppression, there is no single software program available yet that is able to provide a ready-to-use analysis platform. One approach

for handling an MSI data set is to treat it as a transcriptomics data matrix using pipelines such as *Seurat* and *Scanpy*, where each pixel represents a cell and each mass is a feature, which eliminates the need for dedicated image analysis pipelines [36,37]. After processing, the spatial coordinates of pixels can be reintroduced to reconstruct the spatial images. Ongoing efforts are expected to establish more streamlined pipelines to improve MSI data processing in the future.

In the broader omics landscape, integrating data from various modalities has a prominent focus, aiming to construct a comprehensive, multimodal view of cellular states [38,39]. Combining metabolomics with other omics modalities is crucial, as these layers, though distinct, are closely connected in affecting cellular function. Epigenomic analysis can reveal how differences in metabolism influence the chromatin landscape, whereas proteomics (e.g. through MALDI-IHC) can reveal which enzymes might be responsible for different metabolic dynamics. The ability to integrate spatial metabolomics with other omics modalities relies on advancing computational methods. One example is the single-cell spatially resolved metabolic (*scSpaMet*) framework, which incorporates MSI with imaging mass cytometry protein maps to enable correlation of features as well as downstream analysis of the joint data [40]. Given the complex, multidimensional, ill-understood nature of the multiomics data, another promising computational approach is to apply deep learning methods, which are known for their capacity to identify correlations in this type of data. This application of artificial neural networks offers a promising avenue for extracting new biological insights from complex multimodal data sets.

Conclusions

Metabolism plays a critical role in determining cellular behavior and function in the process of tissue homeostasis, injury, and regeneration. Spatial metabolomics approaches are well suited to study metabolism *in situ*, enabling exploration of intricate metabolic networks within tissue complexity. This review highlighted three key aspects of application of spatial metabolomics. First, the utility of metabolic molecular histology was discussed in revealing cell states. Second, the significance was underscored of dynamic metabolic measurements in elucidating altered metabolic processes during tissue injury and regeneration, with implications for refining differentiation strategies and identification of treatment opportunities. Finally, it was explained how the integration of spatial metabolomics with other omics technologies calls for advancements in bioinformatic tools for data interpretation. Insights from this new player in the field of (spatial) omics hold great promise for advancing our

fundamental understanding of tissue biology as well as for identifying potential new therapeutic interventions improving tissue regeneration after injury.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest with the contents of this article.

Acknowledgements

We thank Manon Zuurmond (LUMC, Leiden, The Netherlands) for illustration of the figures and Bram Heijis (Bruker Daltonics GmbH & Co. KG, Bremen, Germany) for providing feedback. The Novo Nordisk Foundation Center for Stem Cell Medicine (reNEW) is supported by Novo Nordisk Foundation grants (NNF21CC0073729).

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