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EXTRA VIEW

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How tissue damage MET metabolism: Regulation of the systemic damage response

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ABSTRACT

Living organisms experience tissue damage from both, the surrounding environment and from inside their bodies. Tissue repair/regeneration is triggered by local tissue injury to restore an injured, or lost, part of the body. Tissue damage results in a series of responses, not only locally but also systemically in distant tissues. In our recent publication, we established a "dual system" that induces spatiotemporal tissue damage simultaneously with gene manipulation in surrounding tissues. With this system, we demonstrated that appropriate regulation of methionine metabolism in the fat body is required for tissue repair in Drosophila wing discs, thus highlighting the importance of systemic damage response (SDR) in tissue repair. This "Extra View" aims to discuss our recent reports that propose methionine metabolism to be an essential part of SDR, together with related topics in several model organisms.

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Introduction

Organisms are inevitably affected by their internal and external environments. In multicellular organisms in particular, numerous tissue interactions establish a complex internal environment and are crucial for protection against various stresses. One of the most severe insults to the body is tissue injury. Tissue injury often occurs from the outside because of physical injury. A wound is defined as a disruption of the outer physical barrier, causing not only a local wound response (LWR) but also a systemic wound response (SWR). Green and Ryan observed that when potato or tomato leaves were injured either physically or by herbivore attack, proteinase inhibitors accumulated not only at the site of injury but also in uninjured leaves.[1] Extensive studies in plants have identified systemic defense mechanisms that underlie this wound response. When tomato leaves are injured, the jasmonic acid biosynthetic pathway is locally activated at the wound site. Jasmonic acid then acts as a mobile wound signal and travels to other parts of the body to induce genes that encode plant defensins and anti-microbial peptides, in preparation for the next injury.[2]

A systemic response upon tissue injury is not only restricted to the wound but also involves a wide range of tissue damage responses as suggested in various reports, including those discussed below. Thus, we will use the terms systemic damage response (SDR) and local damage response (LDR) in the following sections. SDR is a reasonable homeostatic mechanism for multicellular organisms because the influence of local damage is not always confined to the damaged region (Table 1). For instance, local tissue injury potentially causes infection, body fluid leakage, or chronic pain, which would require the systemic response in an organism. Thus, investigation of SDR is significant for understanding the physiology of multicellular organisms. To elucidate the molecular mechanisms of SDR, it is essential to analyze damaged tissues and responsible tissues separately. Recent

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development of genetic tools has enabled us to manipulate genes tissue-specifically and tissue-independently. In our recent paper, we established a "dual system" using *Drosophila* genetics to study tissue repair/regeneration from the viewpoint of SDR.[3] Here, we introduce our recent studies of SDR and methionine metabolism, and discuss related studies and future prospects.

Establishment of a new Drosophila model to study the functional SDR for tissue repair and regeneration

The imaginal discs of *Drosophila*, epithelial sheets fated to become adult organs, are known to have a remarkable ability to repair after tissue damage.[4-6] In our study, we established a temporal cell ablation system in imaginal discs by using a temperature-sensitive form of the diphtheria toxin A domain (DtA^{ts}).[3,7] DtA^{ts} could induce cell death at low temperatures (18°C), but was inactive at high temperatures (29°C) [7,8], thus enabling us to control the timing of induction of tissue injury by simply placing DtA^{ts}-expressing larvae at 18°C (Fig. 1A). In previous studies, a similar system for spatiotemporal genetic ablation was established, based on the Gal4/UAS/$Gal80^{ts}$ system.[9,10] The Gal4/UAS system is a budding yeast-based binary expression system for tissue-specific gene manipulation.[11] Gal4 binds to UAS (upstream activating sequence) and induces transcription of downstream target gene. $Gal80^{ts}$, a temperature-sensitive Gal4 suppressor, controls the Gal4/UAS-based gene expression in a temperature dependent manner.[12] Tissue injury can be regulated both spatially and temporally by ectopic expression of cell death-inducing genes using these sophisticated genetic tools. However, simple utilization of $Gal80^{ts}$ system does not allow the uncoupling of tissue damage induction and the manipulation of genes of interest (i.e. tissue repair genes) using the currently available genetic resources, such as the UAS-RNAi library. Alternative approaches involve using whole body loss-of-function mutations [9,13], but this cannot tell us specifically where in the organism the gene works. Therefore, the new system is required to test the non cell-autonomous function of the genes/pathways in surrounding tissues.

Drosophila has genetic tools available for tissue-specific gene manipulation with several binary genetic systems in addition to the Gal4/UAS system. Systems such as the LexA[14] or Q systems[15,16] can be utilised independently of Gal4/UAS. The combination of 2 systems makes it possible to control gene A in tissue X while manipulating gene B in tissue Y in the same animal (Fig. 1B). In previous studies, the combination of binary genetic systems have been utilised to screen for functional ligand receptors. For example, the Gal4/UAS and LexA systems were combined successfully to identify the receptor of *Drosophila* insulin-like peptide 8 (Dilp8).[17] In our research, we overexpressed DtA^{ts} in imaginal discs to control temporal damage using the Q system, while simultaneously manipulating Gal4/UAS-mediated gene expression in non-ablated tissues (Fig. 1C). Such a "dual system" enables us to search for non-autonomous factors contributing to local tissue repair, even by genome wide RNAi screening, for example.

Involvement of methionine metabolism in SDR

Methionine is an essential amino acid, which must be regularly consumed through our diet. In addition to

Table 1. Selected examples of SDR in *Drosophila*.

Damage	Response	Mediator	Output	Reference
Genetic disc injury	Change of methionine metabolism in the fat body	probably not DAMPs	Supporitng effects on disc repair	3
Necrosis in wings	Inflamatory response and enhanced SAM metabolism in the fat body	DAMPs?	Protecting from wasting phenotype	24
Integumental wounding	Enterocyte cell death	ROS	Gut renewal and host survival	37, 38
	ISC turnover	Upd		38, 39
	JNK activation in neuron	Hayan-dependent ROS	Cytoprotection in neuron and host survival	37, 40
Tumour formation in discs	JAK/STAT signaling in haemocyte and the fat body	Upd	Haemocyte proliferation and attachmeht to injured tissue	42
	Haemocyte proliferation and immune response in the fat body	Spz from Haemocyte to the fat body	Affecting tumor cell death	43
Traumatic brain injury	Intestinal barrier dysfunction	Neuroendocrine signals or nervous system	Leakage of bacteria and glucose in gut	45, 46

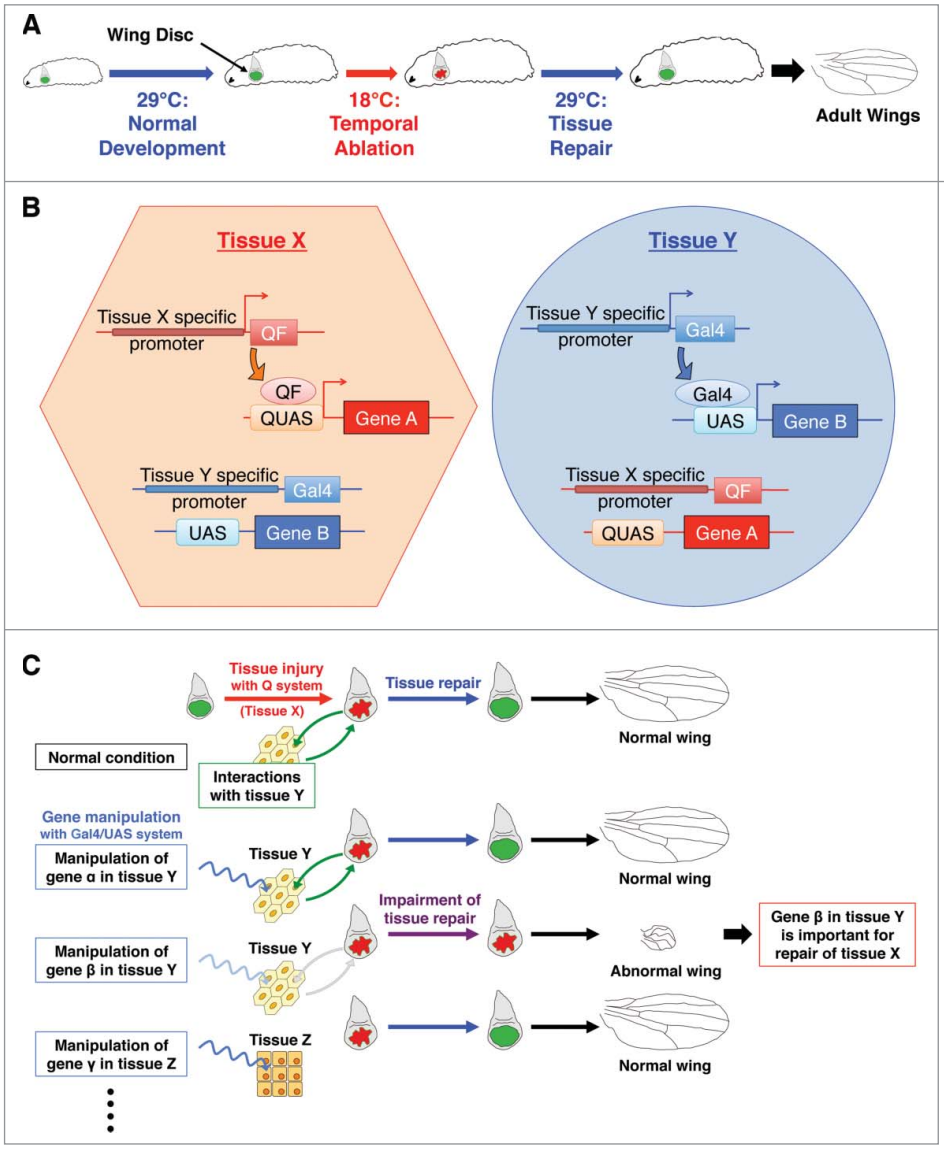

Figure 1. Dual system for studying systemic damage responses during tissue regeneration (A) Schematic view of temporal ablation with DtA^{ts}. DtA^{ts} induces cell death at low temperatures (18°C) and is inactivated at high temperatures (29°C). Thus, by shifting temperatures, a temporal ablation can be induced. With wing disc specific DtA^{ts} expression, local tissue injury and subsequent tissue repair can be observed in wing discs. The extent of wing regeneration can be estimated by observing the adult wing phenotype. DtA^{ts}: temperature-sensitive form of the diphtheria toxin A domain. (B) Independent tissue-specific gene manipulation by the combination of binary systems. Combination of Gal4/UAS, LexA, or Q system makes it possible to manipulate gene expression in independent tissues. For instance, tissue X specific gene promoter can induce the expression of QF only in tissue X and QF subsequently binds to QUAS, which results in the expression of gene A with no induction of other extrinsic binary system like Gal4/UAS system. Similarly, tissue Y specific gene promoter regulates the expression of gene B through the tissue specific expression of Gal4 and following binding to UAS. (C) Identifying genes required for systemic damage response. Combining a Gal4-based genetic manipulation and a Q-based DtA^{ts} ablation, the systemic factors required for disc repair can be investigated in uninjured and remote tissues. Manipulation of different genes in different tissues can be performed by changing the combination of Gal4 drivers or UAS lines. Together with a UAS-RNAi library, this dual system makes it possible to perform genetic screening for studying the tissue interactions involved in tissue repair.

being a protein constituent, methionine is a precursor of S-adenosylmethionine (SAM), a versatile metabolite required for methylation of many substrates, such as nucleic acids, metabolites, lipids, and proteins, including histones. Methionine, together with ATP, is converted to SAM by a highly conserved enzyme called methionine adenosyltransferase (MAT). In *Drosophila*, only one SAM synthase (*sams*) functions to produce SAM (Fig. 2A). The product of SAM-dependent methylation is S-adenosylhomocysteine (SAH), which in turn inhibits methyltransferase activity. Thus, the SAM/SAH ratio can be regarded as a

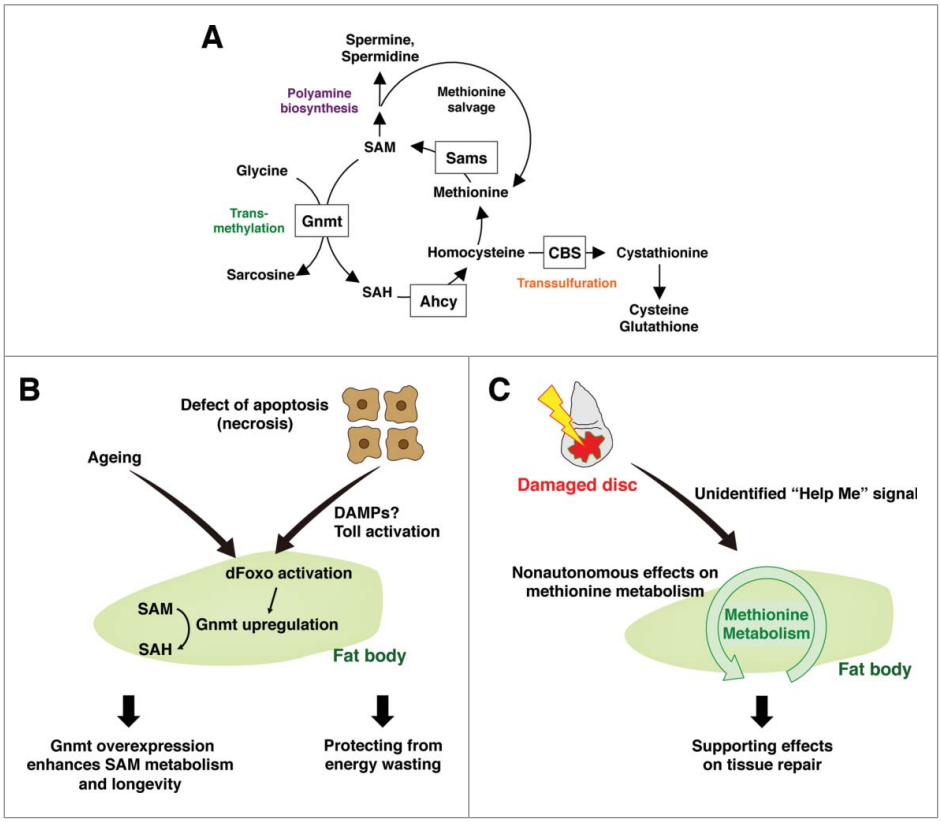

Figure 2. Systemic damage responses of the fat body methionine metabolism (A) Methionine metabolism in *Drosophila*. Methionine is converted to SAM by Sams. SAM provides a methyl group for various methyltransferases including Gnmt, and becomes SAH. SAH is further metabolized into homocysteine, which is either regenerated into methionine, or converted into cystathionine through the transsulfuration pathway. SAM also provides an aminopropyl group to synthesize polyamines, such as spermidine and spermine. SAM: S-adenosylmethionine, Sams: SAM synthase, Gnmt: glycine n-methyltransferase, SAH: S-adenosylhomocysteine, CBS: cystathionine β synthase, Ahcy: adenosylhomocysteinases. (B) Enhancement of SAM metabolism in the fat body affects energy wasting and aging. Defects in apoptosis cause necrosis that triggers a systemic immune response including Toll activation in the fat body. dFoxO is activated concomitantly, and induces Gnmt that converts SAM into SAH, possibly functioning as an adaptive response against energy wasting. As aging proceeds, Gnmt is upregulated in the fat body however SAM level is also increased. Enhancing the dFoxO-Gnmt axis rescues age-dependent SAM accumulation and extends the lifespan. (C) Fat body methionine metabolism remotely regulates local tissue repair. Local disc injury non-autonomously affects methionine metabolism in the fat body via an unidentified "Help Me" signal. In turn, appropriate regulation of methionine metabolism in the fat body is required for sufficient tissue repair in damaged discs.

methylation index. SAH is converted to homocysteine (Hcy) by adenosylhomocysteinases (Ahcy). Hcy is either metabolised to cystathionine through the transsulfuration pathway (TSP) via an enzymatic reaction mediated by cystathionine β synthase (CBS), or recycled into methionine. Cystathionine is further converted to cysteine and contributes to redox homeostasis mainly via *de novo* glutathione (GSH) production.[18,19] A computational estimate suggested that there are approximately 200 putative methyltransferase genes in the human genome.[20] Of these, glycine N-methyltransferase (Gnmt) seems to be the predominant consumer of SAM for converting glycine into sarcosine in liver, a tissue where ~85% of methylation reaction takes place.[21] Thus, Gnmt is believed to tightly regulate SAM levels, and the amount of SAM is dramatically increased in Gnmt loss of function mutants.[22-24]

From transcriptome and metabolome analyses in our previous study, we unexpectedly observed that apoptosis-deficient flies displayed altered methionine metabolism, triggered by necrotic tissue damage in adult wings.[24] Necrotic wings induce Gnmt in the fat body, which contributes to a reduction in SAM and induction of SAH, leading to a decreased methylation index. Gnmt induction in apoptosis-deficient mutants is due to FoxO activation in the fat body, in response to the systemic activation of innate immunity by necrosis in wing epidermal cells. Contrary to our initial assumption that Gnmt induction and a reduced methylation index may contribute to the pathological phenotypes, the decreased SAM levels seem to be an

adaptive response to suppress the energy wasting in necrotic mutants[24]. FoxO-induced Gnmt is not only observed in necrotic models, but also in various conditions including epidermal physical injury (pricking, Obata and Miura, unpublished), starvation,[24] and aging.[25] Considering that Gnmt is also transcriptionally induced by Nrf2/CncC,[25] a transcriptional factor responsible for either oxidative or xenobiotic stress response, Gnmt-mediated changes in methionine metabolism might be a general mechanism as a part of SDR.

To test whether methionine metabolism is also affected by local tissue damage in our new larval model developed to study systemic control of tissue repair, we quantified the amount of methionine metabolites in the larvae expressing DtA^{ts} in the imaginal discs. As we expected, we found a decrease in methionine and SAM, and an increase in SAH in the fat body after disc ablation, particularly in the early stage of disc repair (6 h after ablation).[3] These data suggest that methionine metabolism in the fat body responds to local tissue damage in wing discs. Notably, Gnmt expression did not change at either the transcript or the protein level following ablation, unlike the necrosis model in adult flies. This implies that SAM consumption by methyltransferases other than Gnmt was up-regulated in the fat body. Instead, DtA^{ts} ablation in wing discs upregulated *sams* in the fat body, probably as an adaptive response against SAM reduction, which is also similar to the adaptive increase of MAT2A expression observed after partial hepatectomy in the rat.[26] Interestingly, a decline in SAM and an increase in SAH were also observed during liver regeneration. As for rat liver regeneration, hepatic SAM levels fell to about 40% of baseline at 12 and 24 h following partial hepatectomy (PH), which is coincident with an increase in SAH and a decrease in hepatic DNA methylation.[26] This is strikingly similar to the non-autonomous changes seen in the *Drosophila* fat body during the early stage of disc repair, although mammalian liver cases have been studied in a tissue-autonomous context. The question then arises, how is SAM converted to SAH? It has been reported that methylation of a histone and a cytosolic 20-kDa protein peaked at about 24 h after PH.[27,28] The identity of the 20-kDa protein is still unknown, and its contribution to liver regeneration has not been determined, but it has been suggested that the increased methylation is associated with cell proliferation and transformation.[27,28] It is unclear whether the fat body simply works as a source of SAM for methylation in regenerating discs or if some methylated factors in the fat body directly or indirectly support disc repair. Therefore, the precise identification and analysis of methylation target(s) or other downstream targets of methionine metabolism are required for further understanding of the process.

Functional analysis of methionine metabolism in tissue repair

Functional contributions to tissue repair could be assessed by combining Q-dependent DtA^{ts}-induced wing disc ablation and a fat body Gal4 driver.[3] As we reported, both knockdown and overexpression of *gnmt* or *sams* in the fat body impaired the repair of wing discs, while these genetic manipulations had no apparent effects on normal wing development. This indicates that appropriate regulation of methionine metabolism in the fat body is only necessary for disc repair. Additionally, overexpression of a dominant negative form of the insulin receptor (InR^{DN}) in the fat body did not affect the adult wing morphology. This negates the idea that simple fat body dysfunction causes the impairment of disc repair and sheds light on the importance of methionine metabolism as a systemic modulator of tissue repair.[3]

Although it is still unclear how both up- and down-regulation of *gnmt* or *sams* impairs disc repair, both *MAT 1A*$^{-/-}$ and *GNMT*$^{-/-}$ mutant mice showed impaired liver regeneration.[29,30] Therefore, the effect of methionine metabolism on tissue repair must be evolutionally conserved. In the case of *MAT 1A*$^{-/-}$ mice, S phase hepatocytes were significantly decreased in the injured liver, while hepatic apoptosis was markedly increased in the liver of *GNMT*$^{-/-}$ mice. Although it has not been determined whether methionine metabolism metabolites work in damaged or uninjured tissues, further studies will reveal the exact molecular mechanisms that underlie tissue regeneration.

Potential trigger of methionine metabolism changes in SDR

In animals, tissue damage leads to the release of damage- (or danger-) associated molecular patterns (DAMPs) and activation of the innate immune response.[31,32] DAMPs release is often followed by non-apoptotic cellular damage, including plasma membrane rupture and swelling of cells, which are

hallmarks of necrosis and associated with pathological conditions such as traumatic injury[33] and spontaneous tumor formation.[34] The larval haemolymph of the apoptosis-deficient mutant contains DAMPs that activate the Toll innate immune pathway.[35] In *Drosophila*, DAMPs activate a proteolytic cascade of haemolymph to generate the mature form of the Toll ligand, Spätzle (Spz).[36] Spz activates an innate immune reaction not only in blood cells but also in non-circulating cells, such as the fat body. Activated Spz overexpression leads to induced FoxO-Gnmt in adult flies, suggesting that systemic immune activation is sufficient to alter methionine metabolism.[24] However, DtA^{ts}-induced tissue damage in the wing discs did not induce immune activation, at least in the fat body (Kashio, Obata, and Miura, unpublished). This is consistent with the fact that Gnmt expression is not induced in these animals. Thus, additional systemic mediator(s) other than DAMPs, may exist and function as "Help Me" signals from damaged discs to other tissues, which would positively regulate SDR in tissue regeneration.

SDR studies and their relevance to methionine metabolism

The *Drosophila* model has contributed significantly to studies of SDR.[37] When integumental wounding by simple pricking was performed in adult *Drosophila*, an inter-tissue reaction was observed in the intestine and neurons. In the intestine, enterocytes, gut epithelial cells, reacted to integumental wounding. The IL-6-like cytokine Upd-3 was induced, and caspases were activated in the enterocytes. When caspase activation was prevented in enterocytes, animals became susceptible to integumental wounding.[38] Another paper demonstrated that wounding of the cuticle induced the production of Upd-2, -3 by haemocytes through JNK activation, which activates JAK/STAT signaling in visceral muscles and intestinal stem cells and regulates gut epithelial renewal.[39] These studies showed that SDR in the gut is required for organismal survival against integumental wounding. Integumental wounding activated the local production of reactive oxygen species (ROS) by dual oxidase (DUOX) or systemic ROS production through activation of pro-phenoloxidase by the haemocyte-derived phenoloxidase-activating enzyme Hayan, a CLIP-domain-containing serine protease. Integumental wounding activated neuronal JNK, and phenoloxydase-mediated ROS was required for this JNK activation.[40] Hayan mutants are susceptible to wound-induced lethality and can be rescued when the neuronal antioxidant pathway is reduced or JNK activity is enhanced in neurons.[40] ROS could be one of the upstream mediators of distant tissue reactions because ROS is also required for caspase activation in enterocytes after wounding.[38]

Drosophila metastatic tumors (Ras^{V12}/$scribble^{-/-}$ clones in imaginal discs) or neoplastic tumors in $scribble^{-/-}$ mutants disrupt the basement membrane.[41] As in wounding, JNK and Upd-3 are up-regulated in tumors. Circulating haemocytes adhere to tumors, and Upd-3 is induced in the haemocytes. This represents positive feedback between Upd-3 expression and systemic activation of JAK/STAT signaling. Importantly, the fat body, a tissue distant from the injured site, is also engaged in the amplification loop of JAK/STAT signaling. Haemocytes proliferate via JAK/STAT signaling and attach to tumors to restrict their growth. A pinching method was used to induce physical aseptic tissue damage of the imaginal disc, and haemocyte recruitment to the damage site and amplification of JAK/STAT signaling were initiated.[42] Vidal's group also showed that imaginal disc tumors non-autonomously trigger a tumor suppressor response via Toll activation in the fat body.[43] These studies of SDR in *Drosophila* reveal bi-/multi-directional tissue interactions during the tissue damage response and following homeostatic regulation in the host. While it has not been studied whether methionine metabolism is also involved in tumor-inducing SDR, some cancers are reported to have a common feature of an absolute requirement for methionine, known as methionine dependency.[44] It would be interesting to determine whether methionine metabolism is altered in tumor-dependent SDR and involved in tumor progression.

Traumatic brain injury (TBI) in *Drosophila* revealed a secondary, non-mechanical injury in the intestine.[45] TBI increased intestinal permeability, and glucose feeding stimulated intestinal permeability, which enhanced lethality after TBI. Leakage of bacteria from the gut lumen then activated an innate immune response. Although the precise role of this immune response in survival after TBI has not been determined,[46] this observation provides a clear example of TBI-induced SDR in the gut. Intriguingly, the human patient with TBI had altered amounts of methionine and SAM in their blood,[47] which is similar to the case observed in *Drosophila* disc repair. The

direct relevance to methionine metabolism on TBI-induced SDR has not been studied yet, but this suggests the evolutionally conserved significance of methionine metabolism in SDR.

Future directions

Some diseases or metabolic abnormalities cause perturbation of methionine metabolism. In humans, diabetes causes an increase in the amount of Met and SAM.[48] Interestingly, diabetic patients also exhibit impaired wound healing. This wound healing defect is reportedly caused by an impairment in nitric oxide (NO)-mediated endothelial progenitor cells homing to the injured site.[49] It was also reported that 5-methyltetrahydrofolate, the predominant form of dietary folate, regenerated tetrahydrobiopterin (BH_4), a cofactor for producing NO with endothelial nitric oxide synthase (eNOS).[50] Although it is unclear whether eNOS function is directly affected by methionine metabolism, it remains likely that a diabetes-induced wound-healing defect is related to disruption of methionine metabolism because methionine metabolism is linked to folate metabolism in mammal. On the contrary, a low nutritional condition induced by a diet lacking in protein also impaired wound healing, but supplementation with Met was able to rescue the formation of connective tissue.[51] It is possible that Met regulates the rate of available protein utilization for connective tissue generation, or it may be utilised as a source of SAM, polyamine, or sulfate at the wound site. This report demonstrated the specific role of methionine metabolism in tissue repair and highlighted the potential of methionine metabolism regulation as a clinical target.

A number of reports have revealed the underlying mechanisms and importance of SDR, but many crucial questions remain unanswered. How is SDR induced? Why is SDR required? What are the mediators among the tissues? One major approach for understanding the mediators is to focus on the secreted factors. In a recent study, *in vitro* screening was used to show that myristoylated alanine-rich C kinase substrate (MARCKS)-like protein, a secreted factor, is required for the initial cell cycle response during axolotl appendage regeneration.[52] Mutant zebrafish, lacking most haematopoietic tissues, showed a regeneration defect accompanying apoptosis in regenerating fin after amputation.[53] In the tail explant culture, extracts from the bodies of wild-type zebrafish larvae rescued the apoptosis of regenerative cells in the haematopoietic tissue-deficient mutant, implying that diffusible factor(s) from haematopoietic tissue support the survival of regenerative cells.[53] These results prompted the functional screening of secretory factors (i.e., the secretome[54]) and the use of *Drosophila* genetics to understand the molecular mechanisms of SDR. Studies that focus on tissue interactions and the internal milieu (i.e., systemic biology) will further reveal the complex systems that multicellular organisms use for survival. Ultimately, such studies will contribute to pharmaceutical progress as well as to understanding the essential homeostatic mechanisms of multicellular organisms.

Abbreviations

LWR	local wound response
SWR	systemic wound response
SDR	systemic damage response
LDR	local damage response
DtA^{ts}	temperature-sensitive form of the diphtheria toxin A domain
Dilp8	*Drosophila* insulin-like peptide 8
SAM	S-adenosylmethionine
MAT	methionine adenosyltransferase
sams	SAM synthase
SAH	S-adenosylhomocysteine
Hcy	homocysteine
Ahcy	adenosylhomocysteinases
TSP	transsulfuration pathway
CBS	cystathionine β synthase
GSH	glutathione
Gnmt	glycine N-methyltransferase
PH	partial hepatectomy
InR^{DN}	dominant negative form of the insulin receptor
DAMPs	damage- or danger- associated molecular patterns
Spz	Spätzle
ROS	reactive oxygen species
DUOX	dual oxidase
TBI	traumatic brain injury
VZ	ventricular zone
NPCs	neural precursor cells
NO	nitric oxide
BH_4	tetrahydrobiopterin
eNOS	endothelial nitric oxide synthase

MARCKS	myristoylated alanine-rich C kinase substrate
Upd	unpaired
JNK	Jun: c-Jun N-terminal kinase
JAK/STAT	Janus kinase/signal transducer and activator of transcription

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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