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The role of canonical Wnt signaling in leg regeneration and metamorphosis in the red flour beetle *Tribolium castaneum*

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ABSTRACT

Many organisms across the Metazoa have regenerative abilities with potentially conserved genetic mechanisms that can enlighten both medicine and evolutionary studies. Here, the role of canonical Wnt signaling was examined in the red flour beetle *Tribolium castaneum* in order to explore its role during metamorphosis and larval leg regeneration. Double-stranded RNA mediated silencing of Wnt-1 signaling resulted in a loss of wings and appendages with a dramatic reduction in width, indicating that the Wnt-1 signaling pathway is necessary for proper post-embryonic appendage development in *T. castaneum*. Furthermore, disruption of canonical Wnt signaling led to the complete impairment of limb regeneration in *T. castaneum*. Our findings suggest that Wnt-1 signaling is a conserved mechanism for appendage development across all holometabolous insects and indicate that the role of Wnt-1 signaling in limb regeneration has been retained across all insects as various modes of limb development evolved. Importantly, this study shows that the availability of the genome sequence and the ease of performing leg ablations make *Tribolium* an excellent holometabolous insect model for studying regeneration.

1. Introduction

Regeneration has fascinated scientists since its discovery in hydras in the mid-18th century (Dinsmore, 1991). Regenerative abilities are widespread among a variety of organisms, ranging from planaria and echinoderms to vertebrates (Bely and Nyberg, 2010). Recent advances in molecular biology have led to a resurgence of interest in determining the genetic mechanisms underlying regeneration and the extent to which these processes are conserved among metazoans.

Regeneration is a process by which an organism replaces a lost part of the body to reform a completely new structure and can be classified into two broad categories: epimorphosis and morphallaxis. Morphallaxis is the regeneration of lost tissues that stems primarily from reorganization of existing tissues (Agata et al., 2007). A classic example of morphallaxis is of a hydra severed in half—the cells in the two remaining halves rearrange to form separate, small hydra. Alternatively, in epimorphosis, the regenerating tissues replace the lost parts through the addition of new cells to the existing structure (Endo et al., 2004). Examples of epimorphosis include arthropod and vertebrate limb regeneration.

Insects have an impressive ability to regenerate lost appendages, and this ability is seen across the entire insect phylogeny despite the various ways by which different insects develop their adult appendages. In hemimetabolous insects (insects that undergo incomplete metamorphosis), nymphs hatch out of the eggs with more or less fully developed appendages. In holometabolous insects (insects that undergo complete metamorphosis), appendages develop in several ways. The most primitive mode of appendage development is seen in beetles, such as *Tenebrio molitor*, where most of the appendages do not develop from imaginal discs, and instead develop externally during the embryonic phase and

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later transform into adult appendages during metamorphosis (Truman and Riddiford, 2002). Only a few appendages, such as the wings, develop from imaginal discs, and these imaginal discs only begin to proliferate during the late larval phase (Truman and Riddiford, 2002). In other holometabolous insects that undergo a more derived mode of appendage development, adult appendages develop from imaginal disc cells that are set aside during embryogenesis. Despite these distinct modes of development, the appendages studied to date are all capable of regenerating missing portions. In all cases, regeneration of external appendages requires a molt. Thus, only juveniles are capable of appendage regeneration, and removal of adult appendages does not lead to regeneration.

Although much progress has been made in studies of regeneration in hemimetabolous insects, much of our knowledge on holometabolous insect limb regeneration comes from studies on the imaginal discs of the fruitfly, *Drosophila melanogaster*. Studies have shown that regeneration proceeds following several steps: wound healing, blastema formation and repatterning (Bergantinos et al., 2010; Bosch et al., 2005; Endo et al., 2004; Sustar and Schubiger, 2005). Unfortunately, regeneration studies in *Drosophila* are rather difficult to perform given that imaginal discs are internal structures and cannot be readily removed. Once removed, the discs must be cultured *in vitro* or inside a host, although new methods have recently been devised to circumvent some of these issues (Smith-Bolton et al., 2009).

Regeneration can be more easily examined in insects that undergo a less derived mode of development, such as Coleoptera and Lepidoptera. In contrast to *Drosophila*, the larvae of these insects have external appendages, such as legs and antennae, that alter their morphology during metamorphosis. These larval appendages have a remarkable ability to regenerate and are easily accessible (Bodenstein, 1937, 1941). However, little is known about the molecular mechanisms underlying the regenerative process in these larval appendages. Here, we describe a new strategy to examine the molecular mechanism of regeneration using the red flour beetle, *Tribolium castaneum*. We combine leg ablation with RNA interference (RNAi) to examine the role of particular genes in larval leg regeneration. The genome of *Tribolium* is available (Tribolium Genome Sequencing Consortium, 2008), allowing us to easily identify orthologs of interest.

We focused on Wnt signaling, which regulates tissue growth in *Drosophila* and other organisms. Recent studies have highlighted the importance of Wnt signaling in regeneration across various taxa, ranging from vertebrates to planaria and insects. In hemimetabolous insects, Wnt signaling is required for leg regeneration (Nakamura et al., 2008), and a recent study indicates that the *Drosophila* ortholog of *wnt-1*, *wingless* (*wg*), is necessary for imaginal disc regeneration (Schubiger et al., 2010). Tissue damage leads to localized, regenerative cell proliferation that is characterized by a change in expression of patterning genes, including strong, elevated expression of *wg* in the regenerating tissues (Schubiger et al., 2010; Smith-Bolton et al., 2009). Gain- and loss-of-function studies reveal that *wg* expression allows for the expression of *Myc*, a transcription factor that enhances regenerative growth (Smith-Bolton et al., 2009).

In addition to regeneration, a study on Wnt signaling provides us with an opportunity to better understand its role in holometabolous insects that undergo a more primitive mode of appendage development. Wg regulates both imaginal disc allocation and tissue growth in *Drosophila* (Kojima, 2004). During early embryonic development, the thoracic imaginal primordia, the cells that will eventually give rise to the leg and wing imaginal discs, are allocated from the embryonic ectoderm by the interactions of genes that pattern the dorso-ventral (D/V) and antero-posterior (A/P) axes of the embryo. The primary signals involved in this process are *wg*, *decapentaplegic* (*dpp*), and the *Drosophila* epidermal growth factor receptor homologue (DER) (Grossmann et al., 2009; Kojima, 2004). Studies indicate that *wg* is essential for imaginal disc allocation (Cohen et al., 1993). The imaginal discs grow subsequent to their allocation, and this growth is also partially regulated by Wg (Serrano and O'Farrell, 1997; Struhl and Basler, 1993).

Much less is known about the role of Wnt signaling in holometabolous insects, such as the red flour beetle *T. castaneum* and *T. molitor*, which undergo a primitive mode of appendage development and lack embryonic imaginal disc development. Wnt signaling has been shown to play a key role in the development of legs in the embryos of *T. castaneum* (Ober and Jockusch, 2006). Downregulating the expression of *wnt-1* in *T. castaneum* embryos leads to a complete loss of thoracic appendages and changes in the expression of other genes required for limb development (Ober and Jockusch, 2006). This indicates that Wnt-1 signaling is necessary in the earliest stages of leg development in *T. castaneum*, similar to *D. melanogaster*. However, whether or not the same mechanism is also involved in late developing appendages, such as wings, is not known. It is also not clear whether larval-to-adult leg transformation requires Wnt signaling, as *Drosophila* lacks larval legs.

In the present study, we examined the role of Wnt-1 signaling during leg regeneration and postembryonic leg development in *T. castaneum*. Thus far, there are eight identified *wnt* genes in *T. castaneum*, in addition to *wnt-1*, with varying degrees of functional overlap (Bolognesi et al., 2008). In addition to Wnt-1, several other *wnt* genes are likely to act via the canonical Wnt signaling pathway. The canonical Wnt pathway is thought to proceed through binding transmembrane cell surface receptors of the *frizzled* family. This binding initiates a signal transduction pathway that stabilizes the cytosolic transcription factor Armadillo (Arm) (Brown and Moon, 1998), allowing Arm to translocate into the nucleus and activate the transcription of various genes (Bejsovec, 2005; Huelsken and Behrens, 2002). The non-canonical Wnt pathway does not involve Arm (Bejsovec, 2005). Because we wished to see if the effects of *wnt-1* knockdown were mediated by canonical Wnt signaling, we knocked down one of the two *arm* paralogs in *Tribolium* and examined its roles during leg metamorphosis and regeneration.

Through RNAi-mediated gene knockdown and larval leg ablations, we observed the role played by canonical Wnt signaling during metamorphosis and larval leg regeneration. We found that Wnt signaling is necessary for both of these processes. Our findings show that *Tribolium* is an excellent model for studies on the genetic regulation of leg regeneration.