The ARTS of Cell Death

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ABSTRACT: Although much is known regarding intestinal stem cell (ISC) self-renewal and differentiation, the specific mechanisms used for their elimination is unclear. We recently discovered that the pro-apoptotic protein ARTS, a *Septin4* isoform, interacts with X-linked inhibitor of apoptosis (XIAP) in the ISC niche to regulate stem cell survival during intestinal homeostasis and regeneration. These findings point to an intriguing avenue of translational research, examining how manipulation of stem cell apoptosis through the ARTS/XIAP module can affect stem-cell-dependent processes.

KEYWORDS: apoptosis, ARTS, XIAP, stem cells, Paneth cells, Wnt pathway, regeneration

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Introduction

Adult stem cells play an essential role in tissue homeostasis, repair, and regeneration throughout the duration of an organism's life. This inherent longevity of stem cells raises the possibility that the protective mechanisms in these cells might also be involved in various pathologies and tumorigenesis.[1,2] One fundamental mechanism for the elimination of undesired and potentially dangerous cells is apoptosis, which is a highly conserved form of programmed cell death.[3] However, despite significant advances in the apoptosis and stem cell fields, incredibly little is known regarding how stem cells undergo programmed cell death and the implications for stem-cell-dependent processes.

Emerging findings suggest that stem cells, in contrast to differentiated cells, rely more heavily on anti-apoptotic proteins for their survival.[1] One important level of negative regulation in the apoptotic pathway is mediated by the X-linked inhibitor of apoptosis (XIAP) protein, which is suppressed by inhibitor of apoptosis protein (IAP) antagonists to enable execution of the death program.[4] One particular mammalian IAP antagonist is ARTS, which is derived from the *Septin4* (*Sept4*) gene.[5] Interestingly, ARTS has been shown to function as a specific regulator of cell death in hematopoietic and hair follicle stem cells, with dramatic effects on wound healing and skin regeneration.[6,7]

One classic system that relies heavily on stem cells is the intestinal epithelium, which represents the most rapidly replenishing tissue in mammals.[8] Residing within a specialized microenvironment called the stem cell niche, the Lgr5$^+$ intestinal stem cells (ISCs) are responsible for daily replenishment of the epithelium. Importantly, the close proximity of Lgr5$^+$ ISCs to terminally differentiated secretory Paneth cells within the niche enables them to receive essential signals for their survival.[9] We recently discovered that these cells comprising the ISC niche use the pro-apoptotic factor ARTS for their elimination, which is mediated through the interaction and degradation of XIAP.[10] By performing loss-of-function studies, we found that the deletion of *Sept4*/ARTS leads to augmented proliferation and enhanced Wnt/β-catenin signaling, both in vivo and in ex vivo intestinal organoids. Strikingly, mice lacking ARTS exhibited markedly greater resistance against inflicted intestinal damage in a stem-cell-dependent fashion.

Initially, we found that ARTS is expressed in the mouse small intestinal crypt, with higher levels occurring in the Lgr5$^+$ ISC/Paneth cell zone. Importantly, in organoids expanded after multiple passages (>20 passages), we could detect a similar expression pattern of ARTS primarily localized to the de novo organoid crypts. Examining human colon tissue yielded similar results, where we could detect alternating ARTS$^+$ cells spanning throughout the crypt and particularly in the slender crypt base columnar (CBC) cells.

As a next step, we used whole-body knockout mice for the *Septin4* (*Sept4*) gene that encodes for ARTS. One particularly prominent effect we first noticed was a dramatic enlargement of the intestinal crypt base circumference and the size of individual organoids lacking ARTS. As expected, performing immunofluorescence analysis revealed a significant increase in the number of Lgr5$^+$ ISCs and Paneth cells when *Sept4*/ARTS was absent, both in vivo and ex vivo.

Our next question was whether the ISC niche expansion on loss of *Sept4*/ARTS arose as a result of resistance against apoptotic cell death. As baseline apoptosis in the intestinal crypt is constant, but occurs at a very low level, we employed a novel technique for directly perturbing and analyzing apoptosis in

freshly extracted intestinal crypts. Performing western blotting and immunofluorescence analyses revealed that both *Sept4/*ARTS$^{-/-}$ isolated crypts and intestinal organoids could better withstand treatment of staurosporine, a robust inducer of apoptosis. These analyses, particularly the use of high passage organoids, gave us an initial simplified perspective of apoptosis in the intestinal epithelium and aimed to decrease potentially confounding or influencing effects from non-epithelial or immune cells in vivo.

Having seen that loss of ARTS could bestow intestinal crypt cells with apoptotic resistance ex vivo, we next asked whether the same protection against cell death could be seen in vivo. To this end, we treated mice with a high dose of 14Gy abdominal irradiation and harvested intestines at a time point shortly thereafter where crypt apoptosis has been reported to reach peak levels.[11] Notably, we could detect dramatically less active (cleaved) caspase-3^{+} and TUNEL^{+} apoptotic crypt cells when *Sept4/*ARTS was deleted. Importantly, although loss of ARTS was able to diminish apoptosis of the ISC niche, caspase activation and anoikis at the differentiated villi tip were actually increased, presumably to counteract the increased production of Lgr5^{+} stem cell progeny. This particular result emphasized the specific role of ARTS in the intestinal crypt and indicated that differentiated epithelial cells do not require ARTS for their elimination.

In light of these particular results, and given that we observed no obvious villi lengthening but increased cell extrusion in the *Sept4/*ARTS$^{-/-}$ intestines, we next performed the BrdU label retention assay. We found that *Sept4*/ARTS$^{-/-}$ small intestines exhibited increased epithelial cell migration, with cells reaching the villi tips as little as 24 hours post pulse. Interestingly, we could detect greater numbers of total BrdU^{+} cells, suggesting that ARTS could play a role in regulating cell proliferation.

To investigate this hypothesis, we examined both intestinal wholemounts and ex vivo organoids for proliferation. In these analyses, we could detect a significantly higher number of Ki67+ and PCNA^{+} *Sept4/*ARTS$^{-/-}$ proliferative crypt cells, including crypt base hyperplasia. One particularly striking result from this study was the occasional generation of massive cystic-like organoids derived from *Sept4/*ARTS$^{-/-}$ crypts. These *Sept4*/ARTS$^{-/-}$ cystic organoids retained differentiation capacity, as demonstrated by the presence of intestinal "buds" that housed both Paneth and CBC cells, and their eventual complete differentiation into seemingly regular organoids.

One master growth signaling pathway that functions as a vital regulator of ISC proliferation and tissue homeostasis is the Wnt/β-catenin pathway.[12] As cystic organoids are often seen as a result of treating organoids with exogenous Wnt3a,[9] or can develop from ISCs harboring mutant *APC*, a key negative regulator of the Wnt pathway,[13,14] this particularly striking result was highly indicative that loss of ARTS manifests in Wnt pathway overactivation.

In addition, as Paneth cells have been shown to secrete essential Wnt ligands to neighboring Lgr5^{+} ISCs,[9] we hypothesized that their expansion in the *Sept4/*ARTS-deleted mouse could result in elevated levels of Wnt pathway activity. Indeed, performing real-time polymerase chain reaction (PCR) analysis, we could detect significantly higher transcript levels of canonical Wnt target genes both in vivo and ex vivo. In addition, immunostaining against β-catenin, an effector of the Wnt pathway, revealed higher numbers of *Sept4/*ARTS$^{-/-}$ nuclear β-catenin+ cells in vivo and in organoids.

These data initially led us to form a "chicken-or-egg"-like hypothesis, where we asked what came first: apoptotic resistance or the enhanced Wnt signaling and proliferation? To investigate this, we performed experiments in vitro, where we administered the Wnt secretion inhibitor Wnt-C59 to organoids. Initially, we found that both control and *Sept4/*ARTS$^{-/-}$ organoids depended equally on exogenous Wnt release for their expansion, which also indicated that ARTS does not function as an intrinsic inhibitor of the Wnt pathway. Importantly, when we co-administered *Sept4/*ARTS$^{-/-}$ organoids with Wnt-C59 and staurosporine and then immunostained against cleaved caspase-3, we found that inhibition of Wnt did not abrogate the apoptotic resistance conferred through deletion of *Sept4/*ARTS.

Our results indicated that the lack of ARTS-mediated apoptosis in ISCs could have critical implications for stem-cell-dependent processes, including epithelial restitution. To examine this hypothesis, we administered dextran sodium sulfate (DSS) to control and *Sept4*/ARTS$^{-/-}$ mice, a treatment that recapitulates acute inflammation and symptoms of ulcerative colitis. Notably, mice deleted for *Sept4/*ARTS retained more body weight and displayed less macroscopic and microscopic damage to the small and large intestines. We next performed fluorescent lineage tracing experiments, which enables the Lgr5^{+} ISCs and their progeny to be traced in situ at distinct time points post wound infliction. Notably, in the healing *Sept4/*ARTS$^{-/-}$ small intestinal and colon tissues, we could detect higher presence of Lgr5^{+} crypts in regions displaying greater regeneration, suggesting that their survival enabled them to drive more efficient repair and regeneration of the damaged epithelium.

Finally, in our study, we were able to show that XIAP, a robust inhibitor of downstream pro-apoptotic caspases, serves as a target for the pro-apoptotic activity of ARTS in the intestine. Using both mouse intestinal and human colon tissues, coupled with super-resolution-stimulated emission depletion (STED) microscopy, we determined that apoptotic cells displayed higher co-localization between ARTS and XIAP. To confirm that they indeed bind in intestinal crypt cells, we isolated crypts for co-immunoprecipitation experiments. However, as mutual degradation of ARTS and XIAP occurs on their binding, we used mice harboring a mutated E3 ligase *RING* domain in *XIAP* (*XIAP*$^{\Delta RING}$), which lacks ubiquitination

capacity.[15] As expected, in extracted *XIAP*$^{\Delta RING}$ crypts treated with staurosporine, we could precipitate high levels of ARTS dimers. Next, using mice deleted for *XIAP*, harboring *XIAP*$^{\Delta RING}$ or co-deleted for both *Sept4/*ARTS and *XIAP* revealed abrogation of *Sept4/*ARTS$^{-/-}$-dependent phenotypes, including diminished crypt expansion, decreased organoid growth, and greater wounding susceptibility.

Taken together, our findings indicate that ARTS interacts with XIAP in the intestinal crypt to mediate its pro-apoptotic function, which has critical implications for intestinal homeostasis and regeneration.

Of particular note, we found that deletion of *Sept4/*ARTS was able to confer protection against both radiation-induced intestinal injury and colitis pathology. Thus, our findings may have direct clinical implications for limiting barrier defects following chemoradiotherapies and in intestinal bowel diseases (IBD). Another intriguing aspect to consider is that, in the intestine, XIAP has been shown to function outside of the apoptotic realm as a key mediator of survival pathways that are critical for maintaining epithelial homeostasis and repair.[16] Whether ARTS may also function as a potential signaling nexus for non-apoptotic XIAP survival pathways remains to be investigated.

Interestingly, it has been previously reported that mice lacking *Sept4*/ARTS develop spontaneous hematological tumors, as well as display accelerated tumor progression, suggesting that its pro-apoptotic role within stem cells may serve to defend against the emergence of cancer. Pertaining to this, one may hypothesize that loss of ARTS function over an extended period of time may promote malignant transformation, as well as provide protection to transformed stem cells, thus potentiating their survival and pro-tumorigenic activity. Another important aspect to consider is that the dysregulation of Wnt signaling has been shown to robustly fuel intestinal tumorigenesis.[14] Given the dramatic increase in Wnt signaling in *Sept4/*ARTS-deleted intestinal tissues, the next important steps will be to examine how the ARTS/XIAP apoptotic module is implicated in ISC-dependent tumor initiation, cancer stem cell maintenance, and tumor replenishment.

In future, we speculate that tools promoting the inhibition of ARTS may be used in the clinic, for potentially enhancing ISC survival and promoting intestinal regeneration. However, some measure of cautious should be exerted here. Although blocking stem-cell-specific apoptosis results in elevated stem cell numbers, which is desirable for restoration of damaged tissue, expanded stem cell numbers may also heighten the probability for malignant transformation. Thus, the transient inhibition/activation of ARTS activity may serve as an attractive stem-cell-targeted approach in patients.

In summary, our findings shed important light on an apoptotic module used exclusively by the ISC niche, which plays an essential role in controlling tissue maintenance and regeneration. Future work should examine how the ARTS/XIAP module is involved in tumorigenesis and whether it may serve as a stem-cell-targeted approach for limiting barrier defects and in the treatment of intestinal pathologies.

Author Contributions

EK and YF wrote the manuscript.

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