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ISG15 and ISGylation: Emergence in the cytoskeleton dynamic and tumor microenvironment

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Abstract: Cytoskeletal remodeling affects the shape, adhesion, and motility of cells. Cytoskeletal dynamics are modulated by matrix proteins, integrins, and several cytokines in the tumor microenvironment. In this scenario, signaling is activated by integrins and interferons, which can induce ISG15 gene expression. This gene encodes a ubiquitin-like protein that functions as a protein modifier via the ISGylation system. Furthermore, non-conjugated ISG15 acts as a cytokine-like protein. In this viewpoint, the interplay between free ISG15, protein ISGylation, and cytoskeletal dynamics in the tumor microenvironment is discussed.

Introduction

A tumor is not isolated but surrounded by an entire microenvironment with which it interacts, maintaining reciprocal communication. Different elements comprise the tumor microenvironment, such as different cell types, including vascular cells, cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), and other immune cells. The tumor microenvironment contains various molecules implicated in cellular communication, such as extracellular matrix (ECM) proteins, integrins, cytokines, and several molecules contained in extracellular vesicles (EVs) (Zhang *et al.*, 2021). In the tumor microenvironment, biochemical factors and biomechanical stimuli are generated by ECM. Both elements trigger signaling that activates cytoskeleton remodeling, influencing tumor cell decisions by regulating cell morphology, spreading, migration, metastasis, and invasion (Li and Wang, 2020).

Integrins are heterodimers composed of α and β chains that form transmembrane receptors to recognize various surface molecules of other cells, EMC proteins, and specific soluble proteins. Integrins mediate cell adhesion to the ECM and act as mechanosensors because integrins can sense mechanical forces in the cells and their tumor microenvironment. Hence, integrins transduce signaling from the extracellular to the intracellular space and vice versa, presenting in all the cells of the surrounding tumor microenvironment (Li and Wang, 2020; Tang *et al.*, 2021). Several types of integrins are differentially expressed in diverse cell types. For example, cells of the immune system can present integrins β2 and β7, while leukocytes and lymphocytes display LFA-1 (αLβ2, CD11a/CD18). In summary, integrins represent a system that senses and manages responses to modifications of the tumor microenvironment (DiPersio and Longmate, 2017).

Furthermore, immune cells contribute to the tumor environment by secreting and recognizing cytokines (Labani-Motlagh *et al.*, 2020). The primary role of immune system cells in the tumor microenvironment is to target and eliminate tumor cells. However, tumor cells can acquire immune system evasion ability (Lei *et al.*, 2020). In all these scenarios, ISG15 is emerging as a critical element in cytoskeleton dynamics and the tumor microenvironment, as discussed below.

Interferon-Stimulated Gene 15 (ISG15)

Interferons (IFNs) are a family of proteins having antiviral, antiproliferative, and proapoptotic activities through the activation of a JAK-STAT system that leads to the expression of interferon-stimulated genes (ISGs) (Schoggins, 2019; Jorgovanovic *et al.*, 2020). Many ISGs encode proteins that can promote or inhibit tumor growth. ISG15 is one of the most enigmatic interferon-stimulated genes because of its expression, modulation, and function. For instance, ISG15 is expressed in vertebrates and encodes a ubiquitin-like protein. ISG15 protein can be detected in two forms: conjugated and free.

a) Conjugated ISG15: ISG15 is covalently associated with lysine residues of its target proteins. The covalent interaction

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between ISG15 and its target proteins occurs *via* a catalytic process known as ISGylation, which is mediated by an E1-activation, E2-conjugation, and E3-ligase enzymes for ISG15. Hence, ISGylation is a posttranslational modification, and can be reverted by the USP18 enzyme, which removes ISG15 from its target proteins (Tecalco-Cruz, 2020) (Fig. 1).

b) Free ISG15: ISG15 is not covalently bound to other proteins. Moreover, free ISG15 can be detected as a free protein localized intracellularly and/or extracellularly.

ISGylation has garnered increasing attention in recent years, based on the fact that ISGylation can increase or reduce protein stability or protein-protein interactions. ISGylation is detected in the nucleus and cytoplasm, but a limited number of targets have been identified. For example, cytoskeleton-associated proteins such as IQGAP1 (Cerikan *et al.*, 2016); and nuclear proteins with activity of transcription factors, such as STAT1 and IRF3, are modified by ISGylation (Shi *et al.*, 2010; Ganesan *et al.*, 2016).

Instead, free ISG15 acts as a cytokine because it is produced and secreted from immune system cells, is recognized by the LFA-1 integrin-type receptor and has pleiotropic activities (Swaim *et al.*, 2017, 2020). Thus, the multifunctionality of ISG15 associated with its activity as a cytokine, and as a protein modifier exclusive of vertebrate organisms, distinguishes it from other ISGs.

ISG15 Emerging in the Tumor Microenvironment Stage

ISG15 is upregulated in most of the cancer types and is associated with a pro-tumor action. Nevertheless, the proteins that are ISGylated in these cancer types are not completely defined. Instead, it has been found that free ISG15 is secreted by cancer cells such as melanoma, esophageal squamous cell carcinoma (ESCC), pancreatic ductal adenocarcinoma (PDAC), and nasopharyngeal carcinoma (NPC) cells (Padovan *et al.*, 2002; Sainz *et al.*, 2014; Chen *et al.*, 2016; Yuan *et al.*, 2018). In PDAC and NPC, ISG15 secretion enhances the cancer stem cell phenotype. Tumor-associated macrophages (TAMs) in PDAC and NPC also secrete ISG15, which is associated with poor patient survival (Sainz *et al.*, 2014; Chen *et al.*, 2016). The presence of ISG15 in the tumor microenvironment promotes the induction of macrophages that exhibit an M2-like phenotype, cell migration, and tumor progression (Chen *et al.*, 2020).

Moreover, it has been demonstrated that free ISG15 is secreted by cells such as monocytes and lymphocytes and is then recognized by the LFA-1 integrin receptor present in natural killer (NK) cells, inducing the secretion of cytokines such as IL-10 and IFN-γ (Swaim *et al.*, 2017). The communication between immune cells and tumor cells through extracellular ISG15 may play central role in the tumor microenvironment.

In addition, EVs are released from different cell types and transport diverse molecules. Consequently, EVs are important for cell communication in the tumor microenvironment and modulate several actions such as angiogenesis, cell differentiation, metabolic reprogramming, and treatment resistance (Zhang *et al.*, 2021). As a regulation point, multivesicular bodies (MVB) can be degraded or secreted via exosomal EVs. Interestingly, ISGylation of MVB proteins such as TSG101 is related to the inhibition of exosomal EV secretion, promoting the lysosomal degradation of MVB (Villarroya-Beltri *et al.*, 2016). Thus, ISGylation may also modulate the communication of the tumor microenvironment cells via removal of EVs.

However, it is important to mention that antitumor activity of ISG15 has been reported in ovarian, cervical, and blood cancer cells (Mao *et al.*, 2016; Zhou *et al.*, 2017; Yeung *et al.*, 2018). Although the mechanisms that define the pro and antitumor effects of ISG15 are not clear, this fact suggests a complex crosstalk between ISG15-associated mechanisms and several oncogenic pathways that are dependent on cell type. Accordingly, the molecular target partner for ISGylation may differ and/or be sequential to other modifications or signals in different cancer types.

ISG15 and ISGylation in the Cytoskeleton Dynamic

Few ISGylation targets have been identified, and some of them include NMIIA, IQGAP1, and Filamin B, proteins linked to the actin cytoskeleton (Desai *et al.*, 2006; Jeon *et al.*, 2009; Lee *et al.*, 2010).

For example, IFN-γ has been reported to induce changes in the morphology of breast cancer cells, which seems to be related to the induction of ISGylation, promoting changes in the cytoskeleton and the distribution of F-actin and stress fibers (Tecalco-Cruz *et al.*, 2019).

The actomyosin complex is the association between NMIIA and F-actin, and is essential for the organization of the cytoskeleton. Interestingly, IFN-γ induces NMIIA ISGylation, modulating the interactions of NMIIA with some cytoskeletal proteins. Also, NMIIA ISGylation could be a key mechanism during cell spreading, a process essential for cell movement

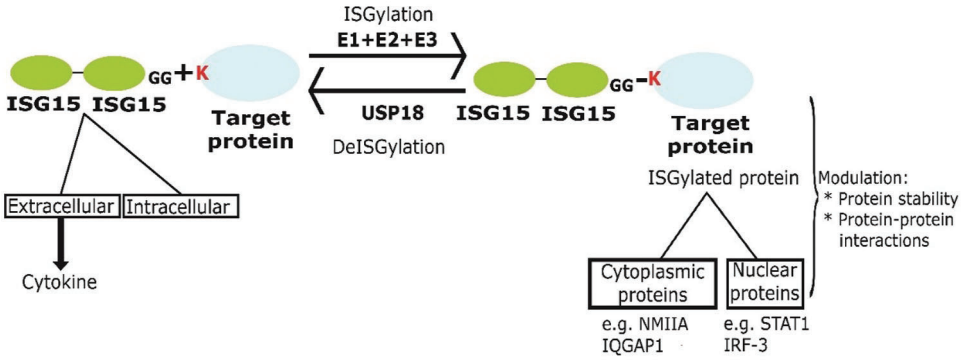

FIGURE 1. Free ISG15 and ISGylation system. Subcellular distribution and functions of free or conjugated ISG15.

and migration (Cruz-Ramos *et al.*, 2019). Hence, in the attachment of the cells at the substrate, as well as the detachment, cell spreading is an initial step, and myosin proteins modulate this event (Betapudi *et al.*, 2006; Cai *et al.*, 2006; Betapudi, 2010). Particularly, it has been suggested that the ISGylation of NMIIA can occur in the spreading of breast cancer cells induced by fibronectin, an extracellular matrix protein (Cruz-Ramos *et al.*, 2019). In addition, fibronectin-binding integrins of αv and β1-class activate MRTF-A/SRF transcription factors to induce ISG15 expression, which has been associated with IQGAP1 ISGylation in MDA-MB-231 breast cancer cells (Cerikan *et al.*, 2016; Hermann *et al.*, 2016).

Therefore, extracellular matrix proteins such as fibronectin have an effect on ISG15 expression, increasing the ISGylation of cytoskeleton proteins such as NMIIA and IQGAP1, modulating cytoskeletal organization, cell morphology, and spreading required for cell migration. Thus, the regulation of cytoskeleton by ISGylation is connected to factors or signals from the tumor microenvironment.

ISG15 and ISGylation in the Breast Cancer Context

In vitro and *in vivo* studies have shown the implications of ISG15 in a breast cancer context by using RNA interference specific for ISG15. *In vitro*, a reduction in proliferation and migration when the levels of ISG15 are decreased has been observed. Also, a reversion of epithelial-mesenchymal transition of breast cancer cells occurs when the levels of ISGylation are reduced. A similar phenotype was observed using RNA interference to reduce the expression of UBCH8 (conjugation enzyme for ISGylation) (Desai *et al.*, 2012; Burks *et al.*, 2014). These results suggest the pro-tumor potential of ISGylation in breast cancer cell lines.

In contrast, *in vivo* mouse models, xenotransplantation of breast cancer cells with a decrease in ISG15 expression generated an increase in tumor incidence and size, suggesting an antitumor role for ISG15 (Burks *et al.*, 2015, 2019). These differences in pro-and antitumor activities of ISG15 *in vitro* (2D cell culture) compared with *in vivo* (xenotransplantation in athymic mice) models may be related to the lack or existence of a tumor microenvironment, respectively. Thus, factors from the tumor environment may trigger signaling with the ability to regulate ISG15 actions.

These studies have analyzed the actions of ISG15 by reducing its expression via RNA interference but not discern between the implications of the free and conjugated ISG15 forms. Nonetheless, an *in vivo* study showed that the injection of exogenous free ISG15 close to breast cancer cell xenotransplantation significantly reduced tumor development, suggesting its antitumor function by favoring NK cell infiltration into the tumor (Burks *et al.*, 2015). Moreover, free ISG15 is a potent adjuvant for cytotoxic T lymphocyte (CTL) responses through an NK cell-dependent pathway (Iglesias-Guimarais *et al.*, 2020). These data indicate that the administration of ISG15 in the tumor microenvironment may have an antitumor function.

Potential Implications of ISG15 as a Factor of Tumor Microenvironment Remodeling

Tumor cells can sense the rigidity and extracellular composition of the surrounding microenvironment, inducing changes in the cytoskeletal organization of tumor cells to adapt or respond to external circumstances. According to previous reports, various elements of the microenvironment, including integrins and IFNs, can induce the expression of ISG15, generating various possible routes to modulate the cytoskeleton dynamic and tumor microenvironment (Sainz *et al.*, 2014; Hermann *et al.*, 2016) (Fig. 2).

Thus, it is possible to propose:

1) ISGylation modulates the cytoskeleton dynamics and exosome secretions, repercuting in the cell behavior, cell signaling and cell-cell communication in the microenvironment.

2) The proteome modifications by ISGylation of cells that integrate the tumor microenvironment are incompletely defined, but ISGylation may determine specific cell responses.

3) Transcriptional and epigenetic regulators may also be modified by ISGylation, modulating the transcriptome, and generating a gene expression signature that directs cell fate.

4) Free ISG15 stimulates cytokine production in some cells. For instance, ISG15 induces the production of IFN-γ in NK cells, suggesting that free ISG15 affects the cell secretome of the tumor microenvironment.

5) Free ISG15 can also recruit cells from the immune system to fight against tumors. Therefore, free ISG15 may improve immunotherapy in several cancer types.

6) Free ISG15 can also mediate a signaling that suppresses the immune response in some cancer types under specific conditions.

Although in general, it would be assumed that free ISG15 and ISGylation have antitumor and tumor-promoting functions, respectively, the type of tumor and the composition of the tumor microenvironment may determine the free ISG15/ISGylation action.

Knowing the integration of the modulation and functionality of ISG15 and the elements of the tumor microenvironment will enable the identification of new therapeutic targets, biomarkers, and markers of tumor progression, sensitivity, or treatment resistance. For instance, the deregulation of ISG15 expression in various cancer types and its presence at the extracellular level suggests its potential use as a biomarker.

Furthermore, the effects reported for ISG15 indicate its potential as a therapeutic agent. For example, decreasing ISG15 in PDAC triggered PDL-1 downregulation and a more effective anti-PD1 immunotherapy (Burks *et al.*, 2019), suggesting its potential as a therapeutic strategy.

Further research is required to understand the mechanisms of regulation and action of ISG15 in the tumor microenvironment. The modulation of the tumor microenvironment by ISG15 and its collaboration with other factors may influence the decision of cell programs on continuing proliferation, migration, invasion, senescence or death.

In conclusion, ISG15 is an interesting molecule because of its multifunctionality in vertebrate organisms, high modulation capacity by various factors, and central participation in cancer pathologies. The impact of ISG15 on the proteome, secretome, and transcriptome could be dependent on the cellular context. The high functional complexity requires elucidation in specific contexts. Nevertheless, the studies suggest that ISG15 and ISGylation have a central role in the cytoskeleton dynamic and tumor microenvironment.

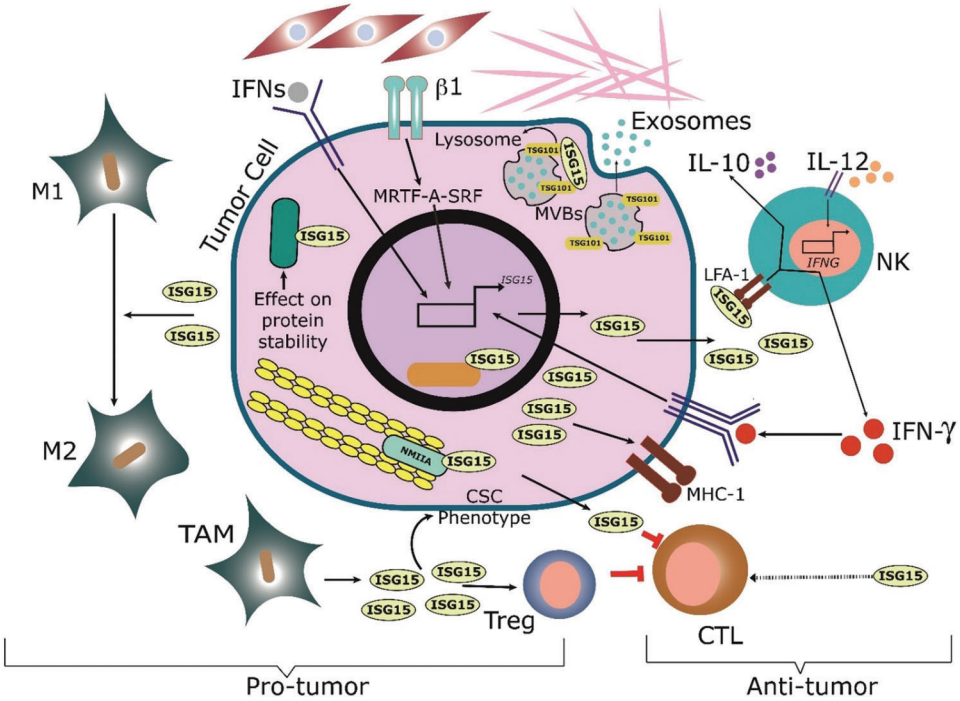

FIGURE 2. Free ISG15 and ISGylation in the cytoskeleton dynamic and tumor microenvironment. ISG15 and ISGylation have a pro-tumor and anti-tumor role. ISG15 induces the conversion from the M1 phenotype (pro-inflammatory and anti-tumor macrophages) to the M2 phenotype (anti-inflammatory and pro-tumoral macrophages). TAMs (tumor-associated macrophages) and tumor cells release ISG15 to the extracellular microenvironment. ISG15 can promote a CSC (cancer stem cell) phenotype. ISG15 inhibits CTLs (cytotoxic T lymphocytes) and activates Treg (regulatory T cells). Then, Treg cells inhibit CTLs. IFNs/IFN-γ pathways and β1 (integrin beta 1)-dependent signaling induce ISG15 expression (long arrows). MRTF-A-SRF, a β1-activated transcription complex, induces ISG15 expression (empty square = ISG15 promoter region). ISG15 is associated with the presence of MHC-1 (major histocompatibility complex class I). ISGylation promotes the MVBs (multivesicular bodies) degradation via lysosome, inhibiting exosome secretion. NMIIA (non-muscle myosin IIA), an actin-associated protein, is modulated by ISGylation. Cytoplasmic proteins (green box) and nuclear proteins (orange box) are regulated by ISGylation. Natural killer (NK) cells have a receptor that recognizes ISG15 (LFA-1), promoting the secretion of interleukin 10 (IL-10) and IFN-γ. IL-12 induces the expression of IFN-γ (IFNG gene. Empty Square = *IFNG* promoter). Exogenous ISG15 seems to activate CTLs (dashed arrow). Cancer-associated fibroblasts and extracellular matrix are shown above.

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