Regulatory Network of ARF in Cancer Development

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INTRODUCTION

Uncontrolled cell proliferation is one of the hallmarks of cancer. Aberrant growth signals or oncogenic stimuli including RAS or c-MYC elicit hyper-proliferation of cells. These processes are normally blocked by a primary fail-safe program including senescence, which is an irreversible cell cycle arrest that restrains aberrant tumor progression (Collado and Serrano, 2010; He and Sharpless, 2017; Lindstrom and Wiinan, 2003; Serrano et al., 1997; Zindy et al., 1998). By-pass of the fail-safe program allows cell transformation, with progression of tumorigenesis in normal cells (Brown et al., 1997; Chen et al., 2005; Eischen et al., 1999). The expression of the INK4b/ARF/INK4a gene locus is a well-characterized mechanism used by cells to respond to oncogenic stimuli by instigating cellular senescence (oncogene-induced senescence, OIS) (Evan et al., 1992; Ferbevre et al., 2002; Lin et al., 1998; Serrano et al., 1997; Vafa et al., 2002; Zhu et al., 1998).

The INK4b/ARF/INK4a gene locus located on chromosome 9p21 in humans is one of the most frequently deleted locus in human cancers. This site encodes potent tumor suppressors including p15ink4b, p14ARF, and p16ink4a. p15ink4b has a physically distinct open reading frame and p14ARF and p16ink4a share exons 2 and 3, following the first exon 1β and α, respectively (Gil and Peters, 2006). Although they share exon 2 and 3, p14ARF and p16ink4a encode totally different amino acids because of an alternative reading...
Fig. 1. The INK4a/ARF/INK4b locus and tumor suppressive functions of ARF. The INK4a/ARF/INK4b locus encodes potent tumor suppressors including p15ink4b, p16ink4a and p14ARF. ARF stimulates cellular senescence and apoptosis through p53-dependent or -independent pathway, thus suppressing the tumor formation.
cently, it was reported that isoforms of human DMP1 differently regulate ARF transcription. DMP1α stimulates ARF transcription and DMP1β antagonizes its action on ARF expression by sequestering DMP1α from the ARF promoter region (Tschan et al., 2015). In addition to these observations, it was reported that transforming growth factor-beta (TGFβ) signaling activates ARF transcription via a direct interaction of Smad2/3 with the ARF promoter and activation of the p38 mitogen-activated protein kinase (p38 MAPK) pathway (Zheng et al., 2010). TGFβ induces the binding of Smad2/3, histone H3 acetylation, and recruitment of RNA polymerase II to the ARF locus, inducing ARF transcription in MEFs (Zheng et al., 2010). Although the mode of action of the p38 MAPK signaling pathway in transcriptional activation of ARF remains unclear, ARF transcription induced by TGFβ and RAS signaling is attenuated by the treatment of MEFs with the p38 MAPK inhibitor SB203580 (Zheng et al., 2010). Another previous report described that Wip1 (Wild-type p53-induced phosphatase 1) phosphatase suppresses ARF expression by inhibiting p38 MAPK signaling (Bulavin et al., 2004). HKR3 (Human Krüppel-related 3) is also a positive regulator of ARF expression. HKR3 binds to the ARF promoter with coactivator p300 and activates its transcription (Yoon et al., 2014). Furthermore, acute myeloid leukemia-1 (AML1) activates its transcription through an association with the ARF promoter, thus eliciting cellular senescence in MEFs and human cells. However, the t(8;21) translocation induced fusion protein AML-ETO, which is present in ~12-15% of AML patients, acts as a repressor of ARF transcription (Linggi et al., 2002).

Negative regulators of ARF transcription are as varied as its positive regulators. The polycomb group gene BMI-1 (B cell-specific moloney murine leukemia virus integration site 1) was identified as a transcriptional repressor of ARF (Jacobs et al., 1999a). BMI-1 deficient MEFs manifest premature senescence and impaired proliferation. Both are rescued by the simultaneous deletion of ARF (Jacobs et al., 1999a). Another study demonstrated that BMI-1-containing polycomb-repressive complex 1 (PRC1)-mediated ARF transcription repression requires the EZH2 (the histone methyltransferase enhancer of zeste homolog 2)-containing PRC2 complex to maintain the tri-methylation levels of histone H3 on Lys 27 (H3K27me3) in the ARF locus (Bracken et al., 2007). Another polycomb group gene, CBX7, also prolongs cell proliferation with reduced ARF expression in human cells and MEFs (Gil et al., 2004). In addition, Twist-1, the basic helix-loop-helix (bHLH) transcription factor, recruits EZH2 to the promoter region of ARF, which represses its transcription via the increased H3K27me3 on the ARF locus, and suppresses cellular senescence in human bone marrow (BM)-derived mesenchymal stem stromal cells (Cakouros et al., 2012). The T-box member Tbx2 also negatively controls the transcription of ARF, which stimulates immortalization of MEFs (Jacobs et al., 2000). Recently, D Dayde and colleagues described that mutant EGFR-L858R inhibits ARF transcription. Vsp34 enhances EGFRI-L858R nuclear trafficking and its accumulation to the ARF promoter attenuates ARF transcription in response to EGFR stimulation in lung tumor cells (Dayde et al., 2016) (Table 1).

| Table 1. Transcriptional regulators of ARF. |
|-----------------------------|-----------------------------|-----------------------------|
| Transcription factor | Regulation of ARF transcription | Ref. |
|-----------------------------|-----------------------------|-----------------------------|
| c-Myc | + | 5 |
| FoxO | + | 53 |
| E2F1 | + | 55 |
| E2F3a | + | 56 |
| DMP1a | + | 57, 59 |
| AML | + | 63 |
| p38 | + | 60 |
| Smad2/3 | + | 60 |
| HKR3 | + | 62 |
| BMI-1 | - | 64 |
| E2F3b | - | 56 |
| DMP1b | - | 59 |
| AML/ETO | - | 63 |
| CBX7 | - | 66 |
| Twist-1 | - | 67 |
| Tbx2 | - | 68 |
| mutant EGFRs | - | 69 |

Positive and negative transcriptional regulators of ARF are indicated in the table. +, up regulation; -, downregulation

Although many studies of transcriptional regulation of ARF have been done since the 1990s, studies on its post-translational regulation have been conducted only relatively recently. Further research focused on identifying post-translational events that regulate ARF protein stability has defined several regulators of ARF and their importance in tumor suppression.

**POST-TRANSLATIONAL REGULATION OF ARF**

Although it has been widely assumed that ARF expression is mainly regulated at the transcriptional level, research that focused on the post-translational regulation of ARF revealed that ARF proteins can be ubiquitinated and degraded via proteasomal degradation (Kuo et al., 2004; Sherr, 2006). In 2004, Kuo and colleagues have identified that human ARF, a lysine less protein, and mouse p19ARF, which has a single lysine, can be polyubiquitinated at their N-terminal amino group, followed by proteasomal degradation without any clue to the enzymes mediating this process (Kuo et al., 2004). The first ubiquitin E3-ligase was identified in 2010. The authors observed that TRIP12 induces ARF ubiquitination and proteasomal degradation, which leads to the activation of cellular proliferation. The enzyme was designated ULF (Ubiquitin ligase for ARF). ULF-mediated ARF degradation is negatively regulated by nucleophosmin (NPM) and c-Myc through direct interaction, underscoring the importance of transcription-independent regulation of ARF under oncogenic stress (Chen et al., 2010). Another study described that USP7 accelerates ARF degradation through deubiquitinination and stabilization of TRIP12, and promotes the progression of hepatocellular carcinoma (Cai et al., 2015). The-
These findings emphasize the impact of ARF ubiquitination and degradation in cancer development. Subsequently, the second ubiquitin E3-ligase of ARF Makorin 1 (MKRN1) was identified (Ko et al., 2012). Ablation or knock-out of MKRN1 can induce cell growth retardation and cellular senescence through the ubiquitination-dependent degradation of ARF in gastric cancer cell lines, human normal cells, and mouse embryonic fibroblasts (MEFs). MKRN1 ablation can reduce tumor growth of p53 positive and p53 negative gastric cancer cells through the induction of ARF-mediated cellular senescence in a xenograft model. Furthermore, MKRN1 was reported to be highly expressed in well-differentiated gastric carcinoma and was negatively correlated with ARF expression. These results indicate the significance of the post-translational regulation of ARF protein in tumorigenesis. Siva1 was also identified as a ubiquitin E3-ligase of ARF, which inhibits p53 function through ARF polyubiquitination and degradation (Wang et al., 2013). Interestingly, distinct from these mechanisms of ARF degradation mediated by E3 ligases, chaperon HSP90 and ubiquitin E3-ligase, CHIP (Carboxyl terminus of Hsc70-interacting protein) cooperatively induce ubiquitination independent lysosomal degradation of ARF. HSP90 and CHIP form a ternary complex with ARF and accelerate the ubiquitination-independent lysosomal degradation (Han et al., 2017). Ablation or knock-out of CHIP, and ablation or inhibition of HSP90 by its inhibitor, geldanamycin (GA), can induce cell growth retardation and cellular senescence in human normal cells and MEFs. Non-small cell lung cancer (NSCLC) patients with a high expression of HSP90 and CHIP, and low expression of ARF have a significantly worse overall survival rate, and these expression patterns have been implicated as independent prognostic factors. Interestingly, the presence of ARF was reported to significantly increase the sensitivity of NSCLC cancer cells to GA treatment, regardless of endothelial growth factor receptor (EGFR) mutation, ALK (Anaplastic lymphoma kinase) fusion and p53 status, which are frequently identified genetic alterations in NSCLC, suggesting that ARF could be an important criterion for an effective therapeutic strategy using HSP90 inhibitors. HSP90 is recognized as a potential oncoprotein that stabilizes various growth factor receptors including EGFR and signaling molecules, such as phosphoinoside-3-kinase (PI3K) and AKT, and numerous oncoproteins including mutant p53 (Kamal et al., 2004). In addition to these functions, these findings suggest a new concept, in which HSP90 can induce the degradation of the ARF tumor suppressor, independent of ubiquitination via chaperone-mediated autophagy (CMA). This event, along with oncoprotein stabilization, stimulates tumorigenesis.

ALTERATION OF ARF IN HUMAN CANCERS

The identification of numerous genetic and epigenetic alterations of the ARF locus has provided convincing support for the notion that ARF functions as a tumor suppressor. The low expression of ARF mRNA is frequently observed in human cancers and is caused by promoter hyper-methylation and deletion of the genetic region. The ARF promoter contains a CpG island that can be silenced by hyper-methylation. Inactivation of ARF by this epigenetic alteration has been amply described in numerous human cancers including breast, bladder, colon, liver, gastric, lung, oral, prostate and brain cancers (Chaar et al., 2014; Dominguez et al., 2002; 2003; Esteller et al., 2000; Hsu et al., 2004; Iida et al., 2000; Konishi et al., 2002; Lee et al., 2006; Sailasree et al., 2008; Shintani et al., 2001; Silva et al., 2003; Tannapfel et al., 2002a; Tannapfel et al., 2002b; Zochbauer-Muller et al., 2001). This alteration that induces ARF inactivation has emerged as a predictor of the poor prognosis of many cancer patients. INK4a and ARF hyper-methylation are associated with frequent lymph node metastasis and higher tumor
grade in colon cancer, and significantly correlate with worse prognosis in breast, head and neck, colon and bladder carcinomas (Dominguez et al., 2003; Kawamoto et al., 2006; Kim et al., 2000; Lee et al., 2006). The chromosome 9p21 segment, where the \(\text{INK}4a/\text{ARF}\) gene is located, is one of the major deletion sites (Cairns et al., 1994; Dominguez et al., 2003). Homozygous deletion at the \(\text{INK}4a/\text{ARF}\) locus and loss of heterozygosity affecting ARF low expression have been detected in many cancers (Berggren et al., 2003; Hsu et al., 2004; Ito et al., 2004; Kasahara et al., 2006; Konishi et al., 2002; Sailasree et al., 2008; Shintani et al., 2001; Silva et al., 2003; Tannapfel et al., 2002b). These alterations of promoter hyper-methylation and genetic deletion found in cancers often correlate with the low expression of ARF in cancers. Conversely, the overexpression of ARF mRNA without any genetic or epigenetic alteration in its locus has been described in several cancers (Ito et al., 2004; Silva et al., 2003). As ARF responds to various oncogenic stimuli including c-MYC, RAS, and E2F1 to accelerate the fail-safe program, such as apoptosis and senescence, against a hyper-proliferation, these mechanisms probably could occur in these cancers. An ARF germline mutation is frequently detected in familial melanoma. These mutations include short deletion or short insertion in exon 1 beta and missense mutation in exon 2, and functionally impair ARF (Hewitt et al., 2002; Randerson-Moor et al., 2001; Rizos et al., 2001a, 2001b). ARF mutations are detected in other cancer types, albeit rarely (Berggren et al., 2003; Ito et al., 2004). The alteration status of post-translational mechanisms affecting ARF protein expression in cancer has not been firmly addressed compared to its genetic or epigenetic alterations. As the importance of post-translational regulation of ARF emerges, the status of protein expression of ARF and its regulators in cancers should be pursued. Indeed, several studies showed the possibility of ARF disruption at the post-translational level in cancers in the late 1990s. The loss of ARF protein expression was identified in several hematopoietic tumor cell lines, which have abundant beta transcripts (Della Valle et al., 1997). Another study reported the complete absence of ARF protein expression in small cell lung cancer cells, but not in normal cells surrounding tumor cells, with frequent uncoupling between transcripts and the ARF (Gazzeri et al., 1998). These reports imply the possibility of the disruption of translational or post-translational regulation of ARF in cancers. The relationships between ARF protein expression and clinical outcome and prognosis in cancers have been addressed. The loss of ARF protein expression correlates with poor outcome of squamous cell carcinoma of the anterior tongue (Kwong et al., 2005). Absent expression of ARF protein is associated with ovarian carcinomas compared to borderline and benign tumors, which suggests that ARF abnormalities occur later in carcinogenesis (Cabral et al., 2016). ARF expression has been negatively correlated with the expression of its E3 ubiquitin ligase, MKRN1, in gastric cancer. MKRN1 overexpression with low expression of ARF has been significantly associated with well-differentiated gastric carcinoma (Ko et al., 2012). The inverse association between protein expression levels of ARF and CHIP, which induce lysosomal degradation of ARF with the aid of HSP90, has been detected in NSCLC. Furthermore, the high expressions of HSP90 and CHIP, and low expression of ARF have been associated with a significantly worse overall survival rate and was an independent prognostic marker in NSCLC (Han et al., 2017). In contrast with normal cells, c-MYC expression has been reversely correlated with USP10 and ARF in NSCLC. Patients with high expression of c-MYC and low expression of USP10 and ARF have significantly worse overall survival. This status is an independent prognostic factor with respect to overall survival in NSCLC (Ko et al., 2018). These results suggest that the post-translational regulation of ARF is disrupted in human cancers and might be an important mechanism that affects tumor progression and clinical outcomes of cancers. On the contrary, several studies described that overexpression of ARF proteins heralds a worse clinical outcome in cancers. Breast cancer patients with increased expression of ARF displayed a high risk of disease recurrence, with no influence on the survival rate (Davy et al., 2009). ARF is a nucleolar protein that sequesters MDM2 into the nucleolus and activates p53. Interestingly, in these tumors ARF was expressed in the cytoplasm rather than the nucleolus. Cytoplasmic expression of ARF has been detected in several human cancers including NSCLC, pancreatic, gastric and prostate cancer (Cabral et al., 2016; Jarrard et al., 1998; Klump et al., 2003; Ko et al., 2012; Vonlanthen et al., 1998). These results might be possibly related to the functional impairment of cytosolic ARF (Rizos et al., 2000; Weber et al., 2000b). Further studies identifying the complex mechanisms regulating ARF expression, stability, localization and function, and the impact of their disruption on cancer development with multidisciplinary approach are needed for a better understanding of the pathological function of ARF, which could utilize our scientific knowledge for clinical application.

CONCLUSIONS

Tumor suppressive functions of ARF have been demonstrated. ARF is deregulated through gene silencing by promoter hyper-methylation, genetic locus deletion and mutation in numerous human cancers. Research on ARF regulation focused on its transcription have identified numerous positive and negative transcriptional factors of ARF. In addition to transcriptional regulation, post-translational control mechanisms of ARF, which are directly linked to their suppression of tumorigenesis, are being elucidated. Recent research has identified several E3 ubiquitin ligases and deubiquitinases, which control ARF protein stability. The findings suggest the importance of the post-translational regulation of ARF in tumor suppression. They can regulate cellular senescence and tumor growth by controlling the stability of ARF protein in cells and mice xenograft models. Supporting these results, the negative correlation between E3-ubiquitin ligases and ARF protein levels or decreased expression of deubiquitinase and ARF has been detected in several human cancers, and these expression patterns are associated with a significantly low survival rate. Although the post-translational regulators of ARF and their roles in tumorigenesis have been defined, more regulators need to be discovered and how they can be
fine-tuned to regulate ARF protein stability upon oncogenic stimuli remains unclear. The presence of various factors affecting ARF stability could be context-dependent and their association with a variety of cancer should be further pursued.

As ARF is one of the well-established tumor suppressors and low levels of ARF protein and mRNA have emerged as an important potential prognostic marker in several human cancers, restoring ARF activity would be a promising therapeutic strategy for cancers. Indeed, given that treatment of GA has a significant cytotoxic effect on ARF-positive NSCLC cell lines compared to ARF-negative cells due to the increased protein stability of ARF, various HSP90 inhibitors including GA analogs developed as cancer drug for clinical trials could be a good therapeutic strategy for NSCLC with high expression of HSP90 and low expression of ARF. Future studies focusing on identifying the complex networks that regulate ARF proteins and their disruption in human cancers will hopefully provide promising therapeutic targets for cancers harboring low expression of ARF proteins.

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