Negative Feedback Regulation of IFN-γ Pathway by IFN Regulatory Factor 2 in Esophageal Cancers

Yan Wang, Dongping Liu, Pingping Chen, H. Phillip Koeffler, Xiangjun Tong, and Dong Xie

Abstract

IFN-γ is an antitumor cytokine that inhibits cell proliferation and induces apoptosis after engagement with the IFN-γ receptors (IFNGR) expressed on target cells, whereas IFN regulatory factor 2 (IRF-2) is able to block the effects of IFN-γ by repressing transcription of IFN-γ–induced genes. Thus far, few studies have explored the influences of IFN-γ on human esophageal cancer cells. In the present study, therefore, we investigated in detail the functions of IFN-γ in esophageal cancer cells. The results in clinical samples of human esophageal cancers showed that the level of IFN-γ was increased in tumor tissues and positively correlated with tumor progression and IRF-2 expression, whereas the level of IFNGR1 was decreased and negatively correlated with tumor progression and IRF-2 expression. Consistently, in vitro experiments showed that low concentration of IFN-γ induced the expression of IRF-2 with potential promotion of cell growth, and moreover, IRF-2 was able to suppress IFNGR1 transcription in human esophageal cancer cells by binding a specific motif in IFNGR1 promoter, which lowered the sensitivity of esophageal cancer cells to IFN-γ. Taken together, our results disclosed a new IRF-2–mediated inhibitory mechanism for IFN-γ–induced pathway in esophageal cancer cells: IFN-γ induced IRF-2 up-regulation, then up-regulated IRF-2 decreased endogenous IFNGR1 level, and finally, the loss of IFNGR1 turned to enhance the resistance of esophageal cancer cells to IFN-γ. Accordingly, the results implied that IRF-2 might act as a mediator for the functions of IFN-γ and IFNGR1 in human esophageal cancers.

Introduction

Esophageal cancers are extremely aggressive tumors, ranking the eighth most common malignancy and the sixth most frequent cause of cancer death worldwide, including two main pathologic subtypes, esophageal squamous cell carcinomas (ESCC) and esophageal adenocarcinomas (1). Although the molecular mechanisms underlying tumorigenesis of ESCCs remain largely unknown, many researches have suggest that evasion from immunosurveillance is an important mechanism for development and progression of ESCCs (2–4). In vivo, the immune system can eliminate transformed cells by recognizing the abnormal antigens expressed on the surface of these cells; this process is known as immunosurveillance or immunoediting (5, 6). Many cytokines responsible for the activation of immune system in vivo has been established to mediate the immunosurveillance, one of which is IFN-γ (5–7).

IFN-γ is a pleiotropic cytokine produced by T cells and natural killer cells and is known to play pivotal roles in eliciting responses of the immune system to tumors in vivo (8). Many researches have indicated that either neutralization of IFN-γ or inhibition of IFN-γ–mediated pathway promote the spontaneous tumor formation in vivo (5–7), which strongly supports the involvement of IFN-γ in the process of immunosurveillance. In addition, IFN-γ has direct negative effects on tumors by inhibiting cell proliferation and stimulating cell apoptosis (9). IFN-γ receptors (IFNGR) consist of two subunits: IFNGR1 (also known as the IFN-γ receptor α chain) and IFNGR2 (also known as the IFN-γ receptor β chain; ref. 10). The former is responsible for ligand binding and is required, but not sufficient, for signal transduction; the latter mainly plays a role in signaling of IFN-γ. Upon engagement with IFN-γ, IFNGRs induce the phosphorylation and activation of Janus-activated kinase 1 (JAK1), JAK2, and STAT-1 pathways to regulate transcription of many IFN-γ–inducible genes (8), two of which are IFN regulatory factor 1 (IRF-1) and IRF-2 (11, 12). IRF-1 is effectively induced in most cell types by IFN-γ (13), and when translocated into the nucleus, IRF-1 binds to promoter regions of many IFN-γ–inducible genes and activates their transcription leading to inhibition of cell proliferation and stimulation of cell apoptosis (12). IRF-1 is thus an important mediator for the antiviral, immunomodulatory, anti-proliferative, and proapoptotic effects of IFN-γ (14). IRF-2, another IRF family member, is also induced by IFN-γ but acts as an antagonist to IRF-1 to block the IFN-γ–mediated pathway (14).

Accumulating evidence has shown that IRF-1 can behave as a tumor suppressor, whereas IRF-2 has oncogenic activity (11, 12). Our recent results also established that both IRF-1 and IRF-2 are involved in the development and progression of ESCCs (15), but thus far, little attention has been paid to the influences of IFN-γ on ESCC cells. In the present work, therefore, we studied in detail the functions of IFN-γ in ESCC cells and displayed novel results that IFN-γ induced the up-regulation of IRF-2 and IRF-2 attenuated the IFN-γ–induced pathway. IRF-2, another IRF family member, is also induced by IFN-γ but acts as an antagonist to IRF-1 to block the IFN-γ–mediated pathway (14).

Materials and Methods

ESCC tissue samples and cell lines. Fifty pairs of primary ESCCs and their corresponding adjacent normal tissues, which were at least 3 to 4 cm away from the cancer, were obtained from ESCC patients treated at the First Affiliated Hospital of Zhengzhou University from 2002 to 2005 after their written informed consent. The clinical information of the patients is summarized in Supplementary Table S1, and none of the patients received any neoadjuvant therapy. Each specimen was divided into two parts: one...
was sectioned and examined historically by traditional H&E staining for the presence of >80% tumor cells (cancer sample) or only normal cells without any inflammation or tumor invasion (matched normal sample) and the other was frozen in liquid nitrogen and stored at −80°C until analysis. Our work was approved by the Institutional Review Board of the Institute for Nutritional Sciences, Chinese Academy of Sciences, ESCC cell lines EC109 and EC17 (Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences; ref. 15) were cultured in RPMI 1640 (Life Technologies), supplemented with 10% fetal bovine serum (PAA Laboratories), 10 units/ml penicillin, and 10 units/ml streptomycin, at 37°C in a humidified atmosphere containing 5% CO2.

Reagents. Rabbit anti-human IRF-1 polyclonal antibody, rabbit anti-human IRF-2 polyclonal antibody, rabbit anti-human caspase-9/p35 polyclonal antibody, and rabbit IgG were purchased from Santa Cruz Biotechnology; murine anti-human STAT-1 monoclonal antibody, murine anti-human phosphorylated STAT-1 (p-STAT-1; pY701) monoclonal antibody, goat anti-human IFNGR1 monoclonal antibody, and goat anti-human IFNGR2 monoclonal antibody were from BD Biosciences; murine anti–β-actin monoclonal antibody was from Sigma; horseradish peroxidase (HRP)–conjugated anti-murine IgG, HRP-conjugated anti-rabbit IgG, and HRP-conjugated anti-goat IgG were from Cell Signaling; recombinant (HRP)–conjugated anti-murine IgG were from Cell Signaling; ref. 15) were cultured in RPMI 1640 (Life Technologies), supplemented with 10% fetal bovine serum (PAA Laboratories), 10 units/mL penicillin, and 10 units/mL streptomycin, at 37°C in a humidified atmosphere containing 5% CO2.

Results

Gene expression and clinical significance of IFN-γ and IFNGR1 in ESCCs. Real-time PCR results showed that the level of IFN-γ mRNA was increased in 28 of 50 ESCC samples (56%) compared with the matched normal esophageal tissues (Supplementary Fig. S1A), and statistical analysis further disclosed that the IFN-γ mRNA in ESCCs positively correlated with tumor stage \( P = 0.048 \) and size \( P = 0.020 \); Supplementary Table S3). In addition, no correlations were noted between IFN-γ mRNA level and tumor differentiation \( P = 0.814 \), lymph node metastasis \( P = 0.752 \), patient age \( P = 0.793 \), or gender \( P = 0.818 \); Supplementary Table S3). We also examined the mRNA levels of IFNGR1 and IFNGR2 in the 50 pairs of samples. The results showed that the level of IFNGR1 mRNA was dramatically down-regulated in 30 of 50 ESCCs (60%) compared with the matched normal esophageal tissues (Supplementary Fig. S1B), whereas no obvious change of IFNGR2 mRNA was detected (Supplementary Fig. S1C). Furthermore, the IFNGR1 mRNA in ESCCs negatively correlated with tumor differentiation \( P = 0.048 \), stage \( P = 0.024 \), and lymph node metastasis \( P = 0.027 \); Supplementary Table S3), but no correlations were found between IFNGR1 mRNA level and tumor size \( P = 0.061 \), patient age \( P = 0.678 \), or gender \( P = 0.959 \); Supplementary Table S3). Altogether, these results suggested that the up-regulation of IFN-γ and the down-regulation of IFNGR1 might be implicated in the progression of ESCCs.

Correlations among mRNA levels of IFN-γ, IFNGR1, and IRF-2 in ESCCs. Our previous research has shown that IRF-1 and IRF-2 are frequently down-regulated and up-regulated, respectively, in ESCCs (15). Because the two molecules are important regulators in IFN-γ signal pathway (11, 12), we subsequently examined the interrelationships among IFN-γ, IFNGR1, and either IRF-1 or IRF-2. Pearson’s correlation analysis showed that the mRNA levels of
IFN-γ and IRF-2 were positively correlated \( (R = 0.350, P = 0.013) \), whereas the mRNA levels of IFN-γ and IFNGR1 \( (R = -0.504, P = 0.001) \), as well as those of IFNGR1 and IRF-2, were negatively correlated \( (R = -0.326, P = 0.021) \). However, no correlations existed between either IRF-γ \( (R = -0.214, P = 0.136) \) and IRF-1 or IFNGR1 and IRF-1 \( (R = 0.136, P = 0.347) \).

**Regulation of IRF-2 and IFNGR1 by IFN-γ in ESCC cells.** Because clinical analysis disclosed that IFN-γ, IFNGR1, and IRF-2 tightly correlated with one another, we therefore explored their relationships using EC109 cells, an ESCC cell line. The cells were treated with IFN-γ of gradient concentrations \((10–0.01 \text{ ng/mL})\), and the JAK–STAT-1 pathway in cells was clearly activated as shown by phosphorylation of STAT-1 and induction of IRF-1 (Fig. 1A; refs. 8, 11, 12). Interestingly, we found that low concentration of IFN-γ \((0.05–0.01 \text{ ng/mL})\) dramatically induced the expression of IRF-2 in EC109 cells, accompanied with the down-regulation of IFNGR1 (Fig. 1A and B), whereas treatment with higher concentration of IFN-γ \((\geq 0.1 \text{ ng/mL})\) could not induce variations of IRF-2 and IFNGR1 expression. It was notable that both inhibition of IRF-1 expression and forced expression of IRF-1 in EC109 cells could eliminate IRF-2 up-regulation induced by low concentration of IFN-γ (Fig. 1C). We additionally noticed that either treatment with high concentration of IFN-γ or forced expression of IRF-1 rapidly induced the continuous activation of caspase-9 (the production of p35 subunit; Fig. 1B and C), one of the most important executors for cell apoptosis (19, 20), whereas low concentration of IFN-γ merely induced mild and transient activation of caspase-9 just before the up-regulation of IRF-2 (Fig. 1B). Moreover, low concentration of IFN-γ did not result in the down-regulation of IFNGR1 in EC109 cells when IRF-2 expression was inhibited (Fig. 1D and Supplementary Fig. S2A). Therefore, the levels of IRF-1 and activated caspase-9 were pivotal for the regulation of IRF-2 by low concentration of IFN-γ, whereas IRF-2 was implicated in the regulation of IFNGR1 expression.

**Potential oncogenic roles of IFN-γ through up-regulation of IRF-2.** Because IRF-2 is an oncoprotein (11, 12) and is able to promote cell growth in ESCCs (15), we wanted to know whether the up-regulation of IRF-2 by IFN-γ could promote the growth of ESCC cells. TUNEL assay (for apoptosis) and MTT test (for proliferation) showed that, although high concentration of IFN-γ markedly induced apoptosis (Fig. 2A) and inhibited proliferation of EC109 cells (Fig. 2B), low concentration of IFN-γ stimulated proliferation of EC109 cells (Fig. 2A) and did not induce their apoptosis (Fig. 2B). For EC109 cells with IRF-2 knockdown; however, even low concentration of IFN-γ also dramatically caused cell apoptosis (Fig. 2C and Supplementary Fig. S2B), as well as inhibited cell growth (Fig. 2D and Supplementary Fig. S2C). Therefore, IFN-γ might possess oncogenic features by inducing the up-regulation of IRF-2 under appropriate conditions.

**Suppression of IFNGR1 transcription by IRF-2.** The above experiments showed that the IRF-2 could regulate the expression of IFNGR1. Because IRF-2 is known as a transcription inhibitor (21), we concluded that IRF-2 might directly repress the IFNGR1 transcription. Consistent with this speculation, we found that

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**Figure 1.** Regulation of IRF-2 and IFNGR1 by IFN-γ. A, protein levels of p-STAT-1, IRF-1, and IRF-2, and IFNGR1 in 109/WT cells after treatment with various concentrations of IFN-γ for 0, 4, 8, 24, and 48 h. The results show density quantification of Western blot bands normalized by the value of β-actin in each sample. Columns, mean of four or five independent experiments; bars, SD. Statistical differences were determined using ANOVA and Student’s t test. *, \( P < 0.05 \) and ***, \( P < 0.01 \) are set for significant and highly significant difference, respectively. B, representative Western blot results indicate the levels of p-STAT-1, IRF-1, IRF-2, IFNGR1, caspase-9 precursor (caspase-9), and activated caspase-9 (p35) in 109/WT cells after exposure to either 10 or 0.05 ng/mL IFN-γ. C, Western blotting shows levels of IRF-1, IRF-2, and p35 in EC109 cells transfected with control siRNA of IRF-1 (109/C1), IRF-1 siRNA (109/I1), 109/V0, and IRF-1–expressing pcDNA3.1 (109/V1), respectively, after exposure to 0.05 ng/mL IFN-γ for 0, 4, 8, 24, and 48 h. D, Western blotting shows levels of IFN-2 and IFNGR1 in EC109 cells transfected with control siRNA of IRF-2 (sequence 1, 109/C2-1) or IRF-2 siRNA (sequence 1, 109/2i-1) after exposure to 0.05 ng/mL IFN-γ for 0, 4, 8, 24, and 48 h. 0 h, the time point just before the addition of IFN-γ. β-Actin is loading control of protein samples.
overexpression of IRF-2 resulted in the down-regulation of IFNGR1 mRNA and protein in ESCC cells (Fig. 3A), whereas inhibition of IRF-2 expression increased the endogenous levels of IFNGR1 mRNA and protein (Fig. 3A and Supplementary Fig. S2D). In another ESCC cell line, EC109, similar regulation of IFNGR1 by IRF-2 was also detected (Supplementary Fig. S3). However, we could not find the regulation of IFNGR1 by IRF-2 in some other cancer cell lines, for example, 293T, HeLa, MCF-7 (breast cancer), DU145 (prostate cancer), and U251 (glioma; Supplementary Fig. S3).

Because IRF-2 affected the levels of IFNGR1 mRNA, we next examined the effect of IRF-2 on IFNGR1 promoter activity. Several reports have characterized this promoter, including identification of a variety of transcriptional factor binding sites (16, 22). According to those studies, we constructed a luciferase reporter gene system that was attached to various lengths of the IFNGR1 promoter (Fig. 3B). These constructs were cotransfected into EC109 cells with either IRF-1–expressing pcDNA3.1 (V1), IRF-2–expressing pcDNA3.1 (V2), or empty pcDNA3.1 (V0) as control, and luciferase activity was measured 24 h later. The reporter gene containing the promoter fragment −840 to −1 bp showed 2.5-fold down-regulation of luciferase activity by V2 cotransfection compared with either V0 or V1 cotransfection (Fig. 3B). Removal of the 5′ region from −840 to either −240 or −160 bp resulted in a 2.5-fold and 3-fold decrease of promoter activity, respectively, with cotransfection of V2. Inhibitory activity of IRF-2 reached its peak (−90% inhibition) with deletion of −840 to −100 bp. Inhibitory activity of IRF-2 disappeared when −840 to −70 of the 5′ region of the promoter was removed. These results suggested that an element located between positions −100 and −70 played a role in the ability of IRF-2 to blunt the promoter activity of IFNGR1 (Fig. 3B). IRF-1 had no effect on luciferase reporter activity irrespective of the length of the IFNGR1 promoter (Fig. 3B). In additional experiments, we deleted the C-terminal region of IRF-2 that is a crucial region for IRF-2 transcriptional activity (12), and the result showed that IRF-2–mediated repression of IFNGR1 promoter was lost with deletion of this region of IRF-2 (Fig. 3C), which further supported the transcriptional inhibition of IFNGR1 by IRF-2.

Identification of IRF-2 recognition site in IFNGR1 promoter.

A careful examination of the promoter region from −100 to −70 revealed a putative IRF-1 and IRF-2 binding site −86AAGTGA. This motif is a known conserved IRF-binding site in the promoters of many IFN-inducible genes (23–25). A series of linker scanning mutants were generated by the introduction of a 6-bp XbaI linker sequence (TCTAGA) into the IFNGR1 promoter from −104 to −69 (Fig. 4A). Mutation of the core motif from −86 to −81 in pGL-M1 entirely rescued the inhibition of promoter activity by IRF-2 and increased both basal and induced promoter activity (Fig. 4A). Mutation of the sequence immediately upstream of the core motif (pGL-M2 and pGL-M3) enhanced the inhibition mediated by IRF-2, whereas mutation of the six nucleotides downstream of the AAGTGA (pGL-M5) partly attenuated the inhibition by IRF-2. Mutation of sequences, either further upstream (pGL-M4) or downstream (pGL-M6 and pGL-M7), had no effect on regulation of promoter activity by IRF-2.

More directly, CHIP assay in ESCC cells examined the binding of IRF-2 to the IFNGR1 promoter. In wild-type EC109 cells (109/WT), the PCR product representing the region (−160 to −40) of the IFNGR1 promoter was detectable after immunoprecipitation by the IRF-2 antibody and the PCR product was further increased in EC109 cells with IRF-2 overexpression (Fig. 4B). The results suggested that endogenous IRF-2 effectively bound the DNA sequences that included −86AAGTGA in IFNGR1 promoter. In contrast, no PCR products were visible when using the primers for the region (−620 to −500) of the IFNGR1 promoter (Fig. 4B). Likewise, no PCR band was detected after immunoprecipitation using the IRF-1...
antibody and IgG (data not shown). Additionally, the IRF-2 antibody was no longer able to immunoprecipitate a product in the EC109 cells with IRF-2 knockdown (Fig. 4B). After the induction of IRF-2 by IFN-γ (0.05 ng/mL), the PCR product representing the potential IRF-2-binding region in the IFNGR1 promoter was also elevated (Fig. 4C), which further supported our hypothesis that the induced IRF-2 by IFN-γ could bind to the IFNGR1 promoter and regulate IFNGR1 expression.

**Involvement of IFNGR1 in IRF-2-mediated IFN-γ resistance of ESCC cells.** Because IFNGR1 is a key initiator for IFN-γ pathway, its down-regulation should lead to the attenuation of IFN-γ-elicited pathway, as well as enhancement of cellular resistance to IFN-γ. Thereby, we firstly measured the phosphorylation of STAT-1 after IFN-γ treatment, which is the marker of IFN-γ pathway (26, 27). Without IFN-γ treatment, STAT-1 levels were similar among 109/WT cells, EC109 cells with transfection of empty pcDNA3.1 (109/V0), EC109 cells with transfection of IRF-2 expression vector clone 1 (109/V2-1), and EC109 cells with transfection of IRF-2 expression vector clone 2 (109/V2-2), and no p-STAT-1 was detected (Fig. 5A). At 1 and 2 h after IFN-γ addition (10 ng/mL), the level of p-STAT-1 in 109/WT was obviously higher than those in 109/V2 (Fig. 5A). At 4 h after addition of IFN-γ, p-STAT-1 reached the highest levels, but still markedly lower in 109/V2 than in 109/WT (Fig. 5A). At 8 and 12 h, p-STAT-1 was still obvious in 109/WT, but had become either very weak or undetectable in 109/V2-1 and 109/V2-2, respectively (Fig. 5A). These results clearly suggested that IRF-2 overexpression did weaken the IFN-γ pathway as expected. In addition, MTT assay showed that the growth of EC109 cells was effectively inhibited by IFN-γ (10 ng/mL), whereas IRF-2 overexpression largely rescued the growth inhibition of EC109 cells by IFN-γ (Fig. 5B). In addition, simultaneous overexpression of IFNGR1, but not IFNGR2, obviously eliminated the protective roles of IRF-2 (Fig. 5B). Therefore, IRF-2 could enhance the resistance of ESCC cells to IFN-γ largely mediated by the suppression of IFNGR1 transcription.

**Discussion**

In the present study, our clinical data showed that IFN-γ expression frequently increased in the ESCC samples, and its up-regulation positively correlated with tumor stage and size, suggesting that IFN-γ could foster the growth of ESCC cells. Although the results seemed largely contrary to the general conception that IFN-γ functions as an antitumor cytokine (8, 28, 29), some researches have reported that IFN-γ also possesses the activity to promote cell growth under some conditions (30–36). Additionally, the clinical analysis disclosed that the expression of IFN-γ positively correlated with that of IRF-2, and 

**Figure 3.** Regulation of IFNGR1 by IRF-2. A, left, expression of IRF-2 and IFNGR1 in 109/WT (lane a), 109/V0 (lane b), EC109 with IRF-2 overexpression clone 1 (lane c), and clone 2 (lane d); right, expression of IRF-2 and IFNGR1 in 109/WT (lane e), 109/C2b-1 (lane f), and 109/2i-1 (lane g). β-Actin is loading control of protein or DNA samples. B, top, schematic representation of each IFNGR1 promoter construct; bottom, each IFNGR1 promoter construct was transiently cotransfected into EC109 cells with empty pcDNA3.1 (V0), IRF-1–expressing pcDNA3.1 (V1), or IRF-2–expressing pcDNA3.1 (V2), and the luciferase activity of the cell lysate was determined 24 h after transfection. C, luciferase assay of pGL-840 after cotransfection with empty vector (V0), full-length IRF-2 (1–349; V2), or repression domain-deleted IRF-2 (1–109; V2ΔC). The value of pGL-840 cotransfected with V0 was used to normalize results of the other IFNGR1 promoter constructs. Columns, mean from four independent assays; bars, SD.
low concentration of IFN-γ hardly induced activation of caspase-9 in spite of the clear activation of IFN-γ pathway. In addition, low concentration of IFN-γ no longer increased IRF-2 expression in ESCC cells with forced overexpression of IRF-1 that has been proved to result in severely spontaneous apoptosis and proliferative inhibition of ESCC cells (15). Therefore, there might be competitive relationship between up-regulation of IRF-2 and induction of apoptosis by IFN-γ or IRF-1: strong apoptosis could block induction of IRF-2 and lead to cell death; weak apoptosis could not interfere with IRF-2 up-regulation, and up-regulated IRF-2 would protect cell from apoptosis.

Accumulated evidence from clinical analysis has disclosed that inflammation is a key risk factor for ESCCs (2), and IFN-γ is implicated in esophageal inflammation (38–41). In light of our observation that low concentration of IFN-γ could induce IRF-2 expression, we speculated that the IFN-γ produced during esophageal inflammation might, at least in part, be responsible for the transformation from esophagitis to ESCCs by stimulating the expression of IRF-2. Although we did not investigate the derivation of the up-regulated IFN-γ in ESCCs in the present studies, T lymphocytes have been reported to infiltrate into the tumor masses of ESCCs (42, 43), which are important origins of IFN-γ in vivo (44).

Our clinical data also showed that ESCC samples frequently had decreased expression of IFNGR1, and therefore, the tumors might be expected to have resistance to IFN-γ, which is consistent with the observation that IFN-γ has no therapeutic efficacy to ESCCs.

Figure 4. Identification of IRF-2 recognition site in IFNGR1 promoter. A, the 6-bp nucleotide sequences in pGL-840 that were replaced with an XbaI site are underlined, and the resulting mutant constructs are named correspondingly. Luciferase assay of pGL-840 and a series of mutant pGL-840 constructs after cotransfection with either V0 or V2. The value of pGL-840 cotransfected with V0 was used to normalize results of the other IFNGR1 promoter constructs. Columns, mean from four independent assays; bars, SD. B, CHIP assay in 109/WT, 109/V2, and 109/2i cells was performed to examine the binding site of IRF-2 in the IFNGR1 promoter. P1 and P2 represent the region −160 to −40 and −620 to −500 of IFNGR1 promoter, respectively. C, CHIP assay using 109/WT cells after exposure to either 10 or 0.05 ng/mL IFN-γ for 24 h.

Figure 5. ESCC cell growth under IFN-γ presence. A, the levels of STAT-1 and p-STAT-1 in 109/WT (lane a), 109/V0 (lane b), EC109 with IRF-2 overexpression clone 1 (lane c), and clone 2 (lane d) with the presence of 5 ng/mL IFN-γ for 1, 2, 4, 8, and 12 h. p-Actin is loading control of protein samples. B, MTT assay was used to measure the cell growth of 109/WT, 109/V0, 109/V2, 109/V2 with cotransfection of IFNGR1 expression vector, 109/V2 with cotransfection of IFNGR2 expression vector under the presence of 10 ng/mL IFN-γ, as well as 109/WT without IFN-γ treatment as control. Columns, mean of four or five independent experiments; bars, SD.
Our results additionally displayed that the down-regulation of IFNGR1 was tightly associated with clinicopathologic features of ESCCs, which suggested that the loss of IFNGR1 was involved in the development and progression of ESCCs. Thus far, many researches support that the evasion from immunosurveillance is an important mechanism for development and progression of ESCCs (2, 4). Correspondingly, tumor cells adopt a variety of mechanisms to escape from the immunosurveillance (5, 6). IFN-γ produced by immune cells and IFNGR1 widely expressed by target cells have central roles in these processes. Genetically engineered animals that lack either IFN-γ or IFNGR1 have a higher incidence or progression of tumors (5–7), which suggests that the down-regulation of IFNGR1 potentially enhances the ability of cancer cells to escape from immunosurveillance. Therefore, the loss of IFNGR1 in ESCCs, as we found through clinical analysis, might ensure ESCC cell growth in vivo by protecting tumor cells against IFN-γ–induced immunosurveillance.

Interestingly, we found that IRF-2 suppressed the expression of IFNGR1 by binding to a specific motif in the IFNGR1 promoter, and the analysis of clinical ESCC samples also disclosed that expression of IFNGR1 mRNA inversely correlated with the mRNA level of IFN-γ. suggesting that IRF-2 could also inhibit IFNGR1 in vivo. A variety of studies have shown that IRF-2 functions as an inhibitory transcription factor, and many genes have been reported to be negatively regulated by IRF-2 (23, 24, 46, 47). The promoters of these genes have an AAGTGA hexamer nucleotide consensus-binding motif for IRF-2 (23–25, 46, 47), similar to what we found in the promoter of IFNGR1. Our results certainly did not exclude the possibility that IRF-2 bound to this motif by interacting with other regulatory proteins, as have been shown under some conditions (48–50), because besides the canonical binding sequences, upstream and downstream bases were also used by IRF-2 for its full inhibitory activity. The location of AAGTGA in the IFNGR1 is adjacent to the binding site of the cAMP-responsive element binding protein/CRE-BP1/c-Jun transcription factors, and IRF-2 might hinder these two transcription factors. Additionally, the AAGTGA includes the initiation of transcription, and binding of IRF-2 might also directly interfere with the binding of RNA polymerase to the IFNGR1 gene. In some other kinds of cancer cells, we could not find the regulation of IFNGR1 by IRF-2, which further supported our speculation that there might be several factors to regulate IFNGR1 and IRF-2 was not the main regulator for IFNGR1 in those cells.

Previous studies have shown that IRF-1 and IRF-2 regulate the same genes but with entirely opposing effects, and IRF-2 mediates its inhibitory function merely by blocking the binding of IRF-1 to the promoters of target genes (23–25, 46, 47). Therefore, just the DNA-binding domain of IRF-2 is able to display a similar function to that of intact IRF-2 (12, 14, 21). Our results, however, showed that the expression of IFNGR1 was affected by IRF-2, but not by IRF-1, and the entire IRF-2 protein was required for the regulation of IFNGR1. Hence, the studies disclosed a novel action manner of IRF-2, which was independent of IRF-1.

In summary, our results initially established the positive regulation of IRF-2 by IFN-γ and the negative regulation of IFNGR1 by IRF-2, implying a novel negative feedback mechanism by which IRF-2 blocked the IFN-γ–induced signal transduction (Fig. 6). Moreover, IFN-γ and IFNGR1 was found correlated with progression of ESCCs, and IFN-γ might play potential oncogenic roles by increasing IRF-2 level. Considering these findings, we hypothesized that the induction of IRF-2 by IFN-γ might drive the transformation from esophageal inflammation toward ESCCs, and the down-regulation of IFNGR1 by IRF-2 might subsequently facilitate the escape of ESCC cells from immunosurveillance. Our results, therefore, implied that IRF-2 and the IFN-γ signal pathway might be potential targets for ESCC therapy.

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