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Identification of the *Lysyl Oxidase* Gene as a Target of the Antioncogenic Transcription Factor, IRF-1, and Its Possible Role in Tumor Suppression[1]

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ABSTRACT

The transcriptional activator IFN regulatory factor 1 (IRF-1) and its antagonistic repressor IRF-2 are regulators of the IFN system. IRF-1 also manifests tumor suppressive activity, and its inactivation could contribute to the development of human hematopoietic malignancies. Here, we report the identification of the *lysyl oxidase* gene as a target gene of IRF-1. An IRF response element was identified in the *lysyl oxidase* gene promoter. We also demonstrate that the transformed phenotype of *ras*-expressing embryonic fibroblasts with a null mutation in the *IRF-1* allele could be suppressed by the expression of the *lysyl oxidase* cDNA, implicating its potential role in tumor suppression. Thus, the regulation of the *lysyl oxidase* gene by IRF-1 could contribute to the multistep process of malignant transformation.

INTRODUCTION

IFNs represent a family of cytokines that manifest antiviral activities on a diverse range of target cells. Type I IFNs (IFN-α and IFN-β) are produced by a variety of cells by viruses, whereas the production of type II IFN (IFN-γ) is restricted to activated T lymphocytes and natural killer cells. In addition, IFNs have been under intensive research in the context of regulation of cell growth; they usually function as "negative growth factors" (reviewed in Refs. 1–4).

Two novel DNA binding factors, a transcriptional activator, IRF-1,[4] and its antagonistic transcriptional repressor, IRF-2, have been discovered and characterized as regulators of the IFN system (4–7). Expression of the *IRF-1* and *IRF-2* genes is induced by viruses and IFNs (5, 6). The two factors share high homology in their NH_2-terminal regions, and these regions contain DNA-binding domains (8). In fact, the DNA sequence elements, termed IRF-Es (consensus sequence, G(A)AAA$^{G}/_{C}{}^{T}/_{C}$GAAA$^{G}/_{C}{}^{T}/_{C}$) bind both factors, and they are found within the promoters of *IFN-α*, *IFN-β* and many IFN-inducible genes (6, 8). Series of expression studies have shown that IRF-1 and IRF-2 act as a transcriptional activator and a repressor, respectively, for type I IFNs and IFN-inducible genes (9, 10). Recently, antioncogenic and oncogenic functions of IRF-1 and IRF-2, respectively, have been demonstrated in NIH3T3 cells (11). The antioncogenic function of IRF-1 was further revealed by the following observations: expression of activated c-Ha-*ras* oncogene in *IRF-1*-deficient (IRF-$1^{-/-}$) primary EFs results in cell transformation, and the transformed phenotype can be suppressed by the expression of the *IRF-1* cDNA (12). Furthermore, IRF-1 is able to suppress cell transformation by c-*myc* or *fos*B (13).

The human *IRF-1* gene has been mapped to chromosome 5q31.1, a region frequently deleted in a significant number of leukemia or preleukemic myelodysplastic syndromes (14). In fact, *IRF-1* is the gene consistently deleted at one or both alleles in patients with leukemia and myelodysplastic syndromes characterized by 5q aberrations (14). Moreover, accelerated exon skipping of *IRF-1* mRNA may cause the inactivation of IRF-1 and could contribute to the development of human hematopoietic malignancies (15).

It is likely that IRF-1 exerts its antioncogenic function by activating a target gene(s). Although some IFN-inducible genes, such as *2′,5′ oligoadenylate synthetase* and *double-stranded RNA-dependent protein kinase* genes, are implicated as antioncogenic, these genes are expressed in an IRF-1-independent manner (16, 17). Here, we attempted to identify genes that are differentially expressed in wild-type and IRF-$1^{-/-}$ EFs, using the mRNA differential display system (18). We report the isolation of one gene that was differentially expressed only in wild-type EFs but not in IRF-$1^{-/-}$ EFs. From our sequencing results, this gene was found to be identical to the mouse *lysyl oxidase* gene.

Lysyl oxidase was discovered as an extracellular copper-dependent enzyme that catalyzes the oxidative deamination of peptidyl lysine to α-aminoadipic-δ-semialdehyde, the precursor to the covalent cross-linkages responsible for the integrity of extracellular matrix (19–21). It initiates the biosynthesis of the cross-linkages in tropocollagen and tropoelastin units (20). Lysyl oxidase is able to act on collagen and elastin, both of which are important components in maintaining the stability of the microfibrillar network present in the cytoskeleton (20).

The isolation and identification of *lysyl oxidase* in the course of the differential display system suggest the possibility that *lysyl oxidase* is one of the genes that may involve in tumorigenesis by IRFs. In fact, this gene was identified in the mouse *ras* recision gene (*rrg*) and implicated in the reversion of *ras*-transformed NIH3T3 cells (22, 23). We discuss our findings in the context of the antioncogenic function of IRF-1.

MATERIALS AND METHODS

mRNA Differential Display. Poly(A)$^+$ RNA from wild-type and IRF-$1^{-/-}$ EFs (24) was isolated by the guanidinium-thiocyanate method, followed by using the mRNA Purification Kit (Pharmacia). mRNA differential display was performed essentially as described in the manufacturer's protocol (RNA map; GenHunter Cooperation; Refs. 18 and 25), except for the following modifications. Sixty ng of poly(A)$^+$ RNAs isolated from wild-type or IRF-$1^{-/-}$ EFs were used on one set of reactions. Control reactions were conducted in the absence of reverse transcriptase or RNA. Water instead of cDNA was added to control tubes during the PCR amplification. We performed reaction mixtures (20 μl) in duplicate, each containing arbitrary decanucleotides as 5′ primers (2 μl) and $T_{12}MN$ as 3′ primers (2 μl), where *M* represents a mixture of A, C, and G, and *N* represents A, C, G, or T nucleotide. Duplicate samples from both wild-type and IRF-$1^{-/-}$ EFs were analyzed simultaneously by electrophoresis in 6% polyacrylamide gel. Gels were autoradiographed for about 18 h. To confirm the reproducibility of the amplification for the selected bands, we repeated the above-mentioned reactions at least twice.

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[4] The abbreviations used are: IRF, IFN regulatory factor; EF, embryonic fibroblast; EMSA, electrophoretic mobility shift assay.

Northern Blot Analysis. The procedure for Northern blot analysis is described (10). The specific activity of each probe was approximately 6×10^8 cpm/μg. The amounts and integrities of RNA in each slot were confirmed by visualizing 28S ribosomal RNA with methylene blue staining and by mRNA levels of β-actin.

EMSA. Extracts of *Escherichia coli* strains harboring either mouse *IRF-1* cDNA expression plasmid or a control plasmid without cDNA were used in EMSA with ^{32}P-labeled synthetic oligonucleotide containing one IRF-E motif from the *lysyl oxidase* promoter (see Fig. 2*A*) as a probe (10).

cDNA Library Construction and Isolation of Clone. Poly(A)$^+$ RNA (8 μg) purified from wild-type EFs was used to make a random primed cDNA library and was cloned into λgt 11 vector (Stratagene). ^{32}P-labeled TA-cloned DNA (Invitrogen) was used as a probe to screen for a full-length clone, which was then sequenced and checked with the Genbank for any homology with known genes. One full-length cDNA clone obtained was found to encode mouse lysyl oxidase. This cDNA was then subcloned into *Eco*RI sites of pBluescriptII (Stratagene) and named pBS-LyO.

Plasmid Construction. The *lysyl oxidase* expression vector, under the control of the *β-actin* promoter (pLyO), was constructed by ligating the following two pieces of DNA: the *Hin*dIII-XbaI backbone fragment from pAct-C (10), the *Hin*dIII site of which was blunted by T4 DNA polymerase, and the *Eco*RV-*Xba*I fragment from pBS-LyO. The *lysyl oxidase* gene promoter-luciferase fusion construct was made by ligating a *Bam*HI-*Hin*dIII fragment that contains the human IFN-β promoter (−55 to +19), a SacI-*Hin*dIII fragment of Picagene vector (Wako, Japan), and *Sac*I-*Bam*HI synthetic oligomer, shown in Fig. 2*A*.

Luciferase Assay. Transfection using P19 embryonic carcinoma cells and luciferase assay was performed as described (10, 26).

Colony-forming Assay in Methylcellulose Gel. IRF-1$^{-/-}$ EFs (5×10^5 cells/10-cm dish) expressing an activated form of c-Ha-*ras*, *ras* EF11 (12) were cotransfected with 15 μg of pLyO or pAct-C (see "Plasmid Construction") and 0.3 μg of pMiwhph (27), using the calcium phosphate method (10). Seventy-two hours after transfection, the cells were suspended with 1.3% methylcellulose gel dissolved in the medium containing 200 μg/ml hygromycin and overlayed on an agarose bed composed of 0.53% agarose and culture medium. Colonies were scored 3 weeks after plating.

RESULTS

mRNA Differential Display. To identify genes regulated by IRF-1, we compared mRNA differential display patterns from wild-type and IRF-1-deficient EFs. We detected a total of 26 differentially expressed genes using all of the primer combinations (see "Materials and Methods"), and only two cDNAs were identified with corresponding mRNAs that down-regulated in IRF-1$^{-/-}$ EFs. In particular, one cDNA gave a signal that was dramatically affected in IRF-1$^{-/-}$ EFs (Fig. 1*A*), and its expression was confirmed by other litters (both wild-type and IRF-1$^{-/-}$ EFs). A 250-bp fragment was isolated and sequenced. Sequence analysis showed that this cDNA encodes mouse *lysyl oxidase* (data not shown). We obtained a 1.5-kb cDNA containing the coding region of lysyl oxidase by screening with a cDNA library (see "Materials and Methods"). This fragment was then used as a probe in the Northern blot analyses as well as in the construction of the expression vector. Results of Northern blot analyses using either the 250-bp or the 1.5-kb fragment show similar differential expression (Fig. 1*B*; data not shown).

An IRF Response Element Is Present in the *Lysyl Oxidase* Gene Promoter. Because the expression of *lysyl oxidase* is down-regulated in the IRF-1$^{-/-}$ EFs, we suspected the presence of an IRF-binding motif (IRF-E) in the promoter of *lysyl oxidase*. Upon inspection of the sequence of the putative transcription control region (28), we found an IRF-E at position −886 to −898 of the mouse *lysyl oxidase* gene promoter (Fig. 2*A*). We then attempted to determine whether this sequence can bind to IRF-1 by EMSA. As shown in Fig. 2*B*, formation of the labeled DNA-IRF-1-protein complex was observed only in the *E. coli* extract expressing IRF-1, and no bands were detected in the

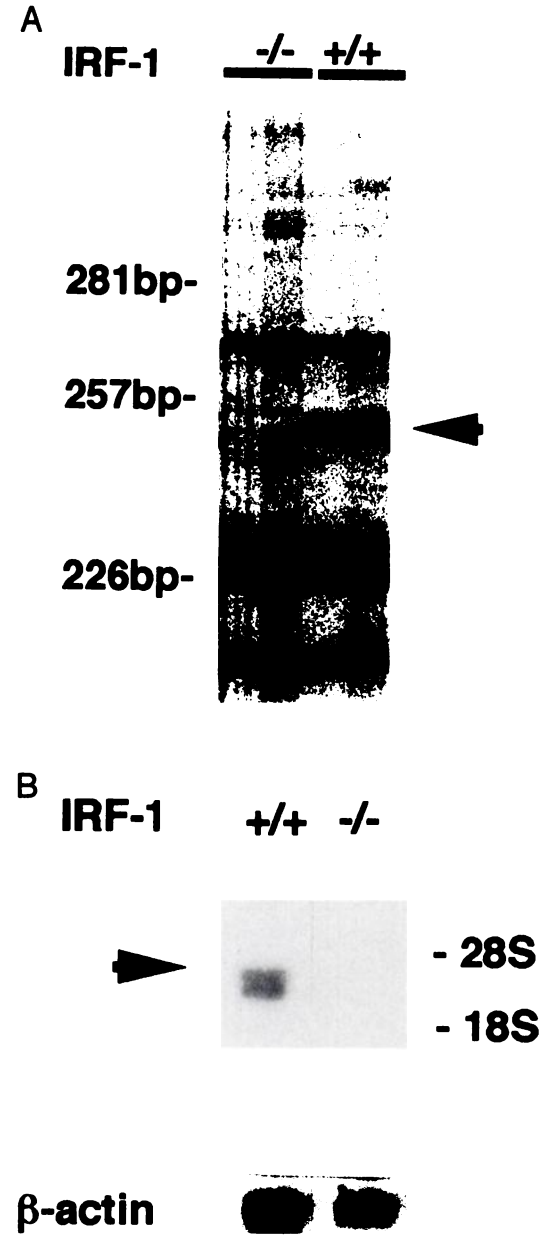

Fig. 1. Differential expression in wild-type and IRF-1$^{-/-}$ EFs. *A*, mRNA differential display of wild-type and IRF-1$^{-/-}$ EFs. Duplicate samples were loaded. *Arrow*, cDNA isolated from the gel. *B*, Northern blot analysis using the *lysyl oxidase* cDNA as a probe. Three μg of total RNA were loaded per lane. *Arrow*, mRNAs hybridizing to the *lysyl oxidase* probe. The filter was then reprobed with *β-actin* to confirm the RNA content in each slot.

control lanes. This formation was reduced accordingly, with decreasing amounts of extracts. This binding activity was efficiently inhibited by an excess of synthetic DNA fragment containing one IRF-E (data not shown). We also constructed the reporter gene that comprises the luciferase gene driven by a promoter and the above-described synthetic oligomer. P19 embryonal carcinoma cells, which are devoid of detectable IRF activity (10), were cotransfected with the reporter gene and the IRF expression plasmids. The reporter gene was activated by *IRF-1* cDNA expression, and this activation was repressed when the *IRF-2* cDNA was coexpressed (Fig. 2*C*). These results indicate the possibility of a tight association of lysyl oxidase with IRFs.

Down-Regulation of Lysyl Oxidase mRNA Expression in NIH3T3 Cells Overexpressing IRF-2. Previously, it was shown that overexpression of the repressor IRF-2 in NIH3T3 cells resulted in cell

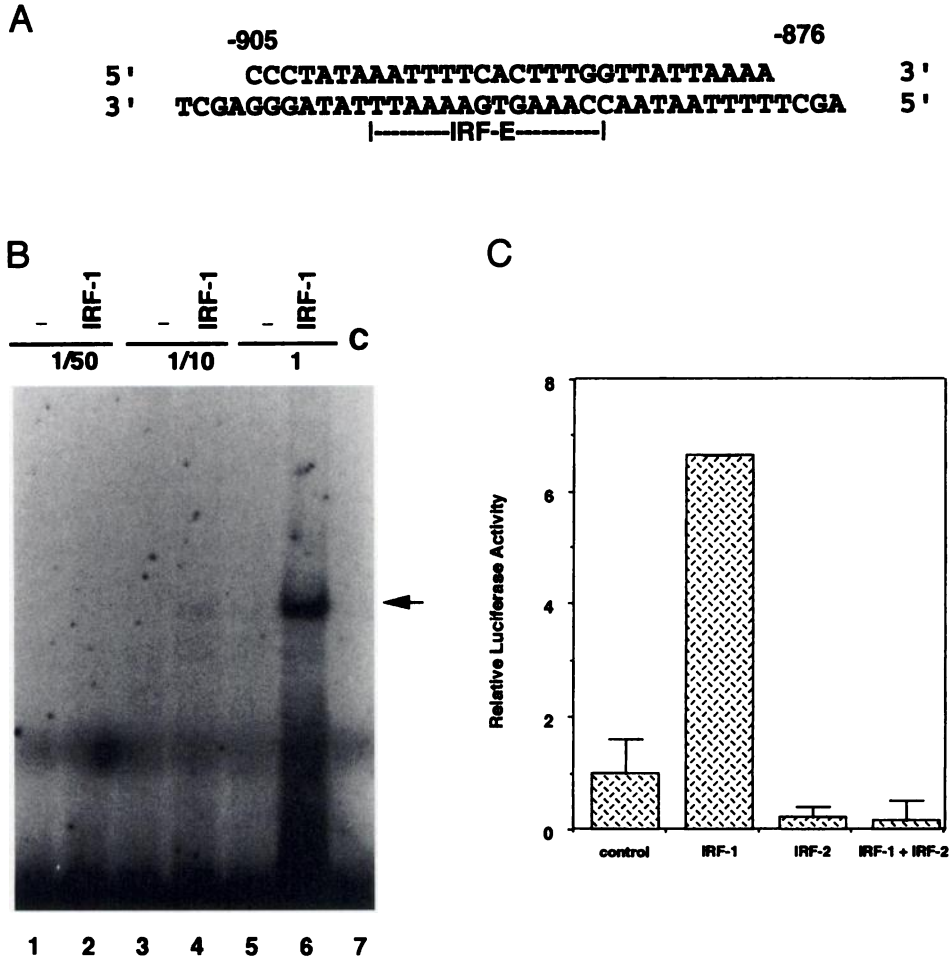

Fig. 2. *A*, sequence of the synthetic oligomer containing IRF binding element (IRF-E) from the mouse *lysyl oxidase* promoter. *B*, binding of IRF-1 to the *lysyl oxidase* promoter. EMSA was performed using the synthetic oligomer in *A* as a probe. Increasing amounts (1/50, 1/10, and 1) of recombinant *E. coli* extracts expressing either a control (*Lanes 1, 3,* and *5*) or the IRF-1 cDNA expression plasmid (*Lanes 2, 4,* and *6*) were loaded. *Lane 7*, negative control, in which only the probe is loaded. *Arrow*, location of labeled DNA-IRF-1-protein complex. *C*, levels of luciferase activities in transfected P19 cells. P19 cells were transfected with 5 μg of the luciferase reporter gene and 5 μg of the effector genes. The transfected effector genes are as follows: control, 5 μg of pAct-C (10); IRF-1, 2.5 μg of pAct-C and 2.5 μg of pAct-1 (10); IRF-2, 2.5 μg of pAct-C and 2.5 μg of pAct-2 (10); IRF-1 + IRF-2, 2.5 μg of pAct-1 and 2.5 μg of pAct-2. Transfections were duplicated, and the assay was repeated, with results essentially reproducible. *Bars*, SE.

transformation, whereas concomitant overexpression of IRF-1 can revert this phenotype (11). We next attempted to determine whether the *lysyl oxidase* gene expression is affected in these cells. As shown in Fig. 3, the expression of *lysyl oxidase* mRNAs is down-regulated in the NIH3T3 cell lines overexpressing IRF-2 (R25 and R27) as compared to the control cell lines (C2 and C3) by about 3-fold. Interestingly, the expression of the *lysyl oxidase* mRNA in the reverted cell lines R25–2 and R27–3, each overexpressing IRF-1, was brought to a level similar to that of the control cell lines (Fig. 3).

Inhibition of Colony Formation of the Ha-*ras*-transformed IRF-1$^{-/-}$ EFs by the Expression of *Lysyl Oxidase* cDNA. To determine whether lysyl oxidase manifests tumor suppressor activity, IRF-1$^{-/-}$ EFs expressing an activated form of c-Ha-*ras* oncogene (12) were transfected with a control plasmid (pAct-C) or a *lysyl oxidase* expression plasmid (pLyO). As shown in Table 1, the transfection of the *lysyl oxidase* cDNA resulted in a dramatic inhibition of colony formation as compared to the control. The colony formation potential of the cells transfected by the *lysyl oxidase* construct was reduced approximately 3-fold. This is an indication of the tumor-suppressive characteristic of lysyl oxidase.

DISCUSSION

IRF-1 was originally characterized as a positive regulator of transcription in both type I IFN and IFN-inducible genes (9, 10, 16, 24, 29). It has been reported that IRF-1 functions as a tumor suppressor and that its functional loss is able to predispose the primary mouse EFs to tumorigenic transformation by activated c-Ha-*ras* oncogene *in vitro* (12). The fact that tumorigenic transformation by *ras* normally requires the cooperation of a second oncogene strongly indicates that IRF-1 is one factor that is responsible for the prevention of cell transformation by *ras* alone (12). It has been suggested that the target genes of IRF-1 for IFN response and tumor suppression may be distinct (12); thus, the identification of a target gene that possesses tumor-suppression activity is definitely important in the study of genes crucial for the mediation of tumor suppression by IRF-1.

In this study, we identified *lysyl oxidase* as one of several genes down-regulated in IRF-1$^{-/-}$ EFs, using the mRNA differential display method (18). The expression of *lysyl oxidase* was dramatically reduced in the absence of IRF-1 (Fig. 1). An IRF-E motif is present in the *lysyl oxidase* promoter (Fig. 2*A*), and IRF-1 was able to bind to this sequence (Fig. 2*B*). Moreover, the *lysyl oxidase* promoter-luciferase construct showed a dramatic activation when transfected with IRF-1 (Fig. 2*C*). These results indicate that *lysyl oxidase* is under the control of IRF-1.

The expression of *lysyl oxidase* is decreased by about 3-fold in NIH3T3 cells overexpressing IRF-2 (Fig. 3). We think that the physiological balance of IRF-1 and IRF-2 is vital to the well-being of the cell, and any alteration is able to seriously disturb the system. In fact, it was shown previously that overexpression of the repressor IRF-2 in NIH3T3 cells resulted in cell transformation, whereas concomitant overexpression of IRF-1 can revert this phenotype (11). It is presumed that the overexpression of IRF-2 suppresses the cell growth-restraining function of IRF-1 (11); thus, the down-regulation of *lysyl oxidase* in these cells supports the notion that its expression is indeed related

to IRF-1. As IRF-1 and IRF-2 possess antagonistic roles, a transcriptional repressor IRF-2 would negatively regulate *lysyl oxidase*. This is supported by the observation that when IRF-1 cDNA was introduced into cell lines overexpressing IRF-2, mRNA expression of *lysyl oxidase* reverted to about the same level of expression observed in the parental NIH3T3 cells (Fig. 3). Results from the luciferase assay also indicate that IRF-2 is able to suppress the expression of *lysyl oxidase* (Fig. 2*C*). These results strongly indicate that *lysyl oxidase* truly is an IRF-1-target gene.

The transfection of *lysyl oxidase* cDNA resulted in a dramatic inhibition of colony formation by at least 3-fold when compared to the cells transfected with the control vector (Table 1). This result further supports the role of lysyl oxidase in tumor suppression. Even in the absence of IRF-1, it alone is able to "rescue" the cells, thereby decreasing its potential in tumor formation activities. Previous results indicated the profound inhibition of colony formation ability when IRF-1 was added into IRF-$1^{-/-}$ EFs expressing c-Ha-*ras* (15), clear evidence that IRF-1 has tumor suppressor activity. Our results here indicate that *lysyl oxidase* could be a target gene of IRF-1 in tumor suppression.

Fig. 3. *A*, Northern blot analysis of *lysyl oxidase* expression in NIH3T3 cells overexpressing IRF-1 and IRF-2. *C2* and *C3* are controls, containing plasmid devoid of cDNA insert. *R25* and *R27* are cell lines overexpressing IRF-2. *R25–2* and *R27–3* were derived from parental cell lines R25 and R27, respectively, with introduction and increased expression of IRF-1 (11). Five μg of total RNA were loaded per lane. The filter was stained by methylene blue to show total RNA before being probed with *lysyl oxidase* cDNA. *B*, changes in the level of lysyl oxidase mRNA expression from *A*. mRNA levels were quantitated by an imaging analyzer (Bas2000, Fuji). *Bars*, SE.

Table 1 *Suppression of colony-forming ability by pAct-C or pLyO*

Transfected construct[a]	No. of colonies	
	pAct-C	pLyO
Experiment 1	790	277
	594	235
	723	326
Experiment 2	739	258
	794	421
	612	191

[a] Transfected constructs are as follows: pAct-C, control β-actin promoter vector; pLyO, an expression vector containing the coding region of *lysyl oxidase*. Colonies were scored from three independent plates per experiment.

Evidence has been provided showing that lysyl oxidase might play an important role in tumor suppression. The involvement of lysyl oxidase has been demonstrated as a phenotypic suppressor of transformation by the *ras* gene product $p21^{ras}$ (22, 23, 30). Moreover, the down-regulation of lysyl oxidase upon cellular transformation is reexpressed after prolonged treatment of these transformed cells with mouse IFN-α/β (22). Thus, lysyl oxidase is an important marker in neoplastic transformation as well as in the reversion of cellular transformation. The level of lysyl oxidase was found to be markedly decreased in Menkes' disease, X-linked cutis laxa, Marfan's syndrome, and various diseases, all of which share certain abnormalities in the lung and skin of these patients. Depletion of copper from the diet can induce lathyrism, a condition characterized by a variety of connective tissue abnormalities, including aortic rupture, skeletal deformities, and skin fragility (20, 21, 31, 32). Its role in several pathological diseases indicates its importance not only in maintaining the condition of connective tissue matrix but also in the prevention of uncontrolled growth characteristic of oncogenic cells as well as normal cell function.

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