Integrative genomic analyses of ZEB2: Transcriptional regulation of *ZEB2* based on SMADs, ETS1, HIF1α, POU/OCT, and NF-κB

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Abstract. Epithelial-to-mesenchymal transition (EMT) is defined as phenotypic change of epithelial cells into mesenchymal cells. EMT, allowing cellular dissociation from epithelial tissues, plays a key role in invasion and metastasis during carcinogenesis as well as in gastrulation and neurulation during embryogenesis. SNAI1/Snail, SNAI2/Slug, ZEB1/δEF1/ZFHX1A, ZEB2/SIP1/ZFHX1B, TWIST1/TWIST, and TWIST2/DERMO1 are representative EMT regulators. ZEB2 represses transcription of *CDH1, CLDN4, CCND1, TERT, SFRP1, ALPL* and miR-200b-200a-429 primary miRNA, and upregulates transcription of mesenchymal markers. ZEB2 is relatively highly expressed in brain corpus callosum and monocytes. ZEB2 is expressed in various types of human tumors, such as breast cancer, gastric cancer, and pancreatic cancer. TGFß, TNFα, IL1, AKT and hypoxia signals are involved in *ZEB2* upregulation and EMT induction; however precise mechanisms of *ZEB2* transcription remained unclear. Here, refined integrative genomic analyses of *ZEB2* gene were carried out. *ZEB2* was co-expressed with *POU3F2 (BRN2)* and *POU3F3 (BRN1)* in brain corpus callosum, spinal cord, and fetal brain, whereas *ZEB2* was co-expressed with *POU2F2 (OCT2)* in monocytes. Ets-Smad-binding CGGAGAC motif, bHLH-binding site, and POU/OCT-binding site within proximal promoter region, and NF-κB-binding site within intron 2 were completely conserved in human *ZEB2*, chimpanzee *ZEB2*, cow *ZEB2*, mouse *Zeb2*, rat *Zeb2*, and chicken *zeb2* genes. In addition, HIF1α-binding site within proximal promoter region was conserved in mammalian *ZEB2* orthologs. Consensus binding site for Hedgehog effector GLI was not identified within or adjacent to the 7-kb regions of human *ZEB2* gene. TGFß, TNFα, IL1, and hypoxia signals directly upregulate *ZEB2* to induce EMT, growth arrest, and senescence, whereas Hedgehog signals indirectly upregulate *ZEB2* via TGFß. Together these facts indicate that ZEB2, occupying the crossroads of inflammation, aging and carcinogenesis, is an important target for drug discovery.

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Introduction

Epithelial-to-mesenchymal transition (EMT) is defined as phenotypic change of epithelial cells into mesenchymal cells (1-4). Epithelial cells with E-cadherin expression are tightly held together with uniform neighboring cells to move as a sheet en block, whereas mesenchymal cells without E-cadherin expression are loosely connected with diverse neighboring cells to move individually. EMT, allowing cellular dissociation from epithelial tissues, plays a key role in invasion and metastasis during carcinogenesis as well as in gastrulation and neurulation during embryogenesis.

Downregulation of E-cadherin at the adherens junction and Claudin at the tight junction leads to the loss of epithelial phenotype, while upregulation of N-cadherin and Vimentin leads to the acquisition of mesenchymal phenotype. SNAI1/Snail (5), SNAI2/Slug (6), SNAI3 (7), ZEB1/δEF1/ZFHX1A (8), ZEB2/SIP1/ZFHX1B (9), TWIST1/TWIST (10), and TWIST2/DERMO1 (11) are representative EMT regulators. Upregulation of EMT regulators in tumor cells is associated with more malignant phenotypes in a variety of human cancer (12-16).

ZEB2 and ZEB1 are transcription factors with common domain architecture consisting of the p300-binding domain and four zinc finger domains in the N-terminal region, Smad-binding domain, Homeo domain and CtBP-binding domain in the middle region, and three zinc finger domains in the C-terminal region (17,18). ZEB2 is involved in transcriptional repression of *CDH1* (9), *ALPL* (19), *TERT* (20), *CLDN4* (21), *CCND1* (22), *SFRP1* (23) and miR-200b-200a-429 primary miRNA (24) through the recruitment of CtBP co-repressor. ZEB2 is also involved in transcriptional activation of mesenchymal markers through the recruitment of p300 co-activator in cooperation with SMAD complex or through indirect mechanisms.

ZEB2 is expressed in various types of human tumors, including breast cancer (9,25-27), gastric cancer (13,15), colorectal cancer (28), liver cancer (29,30), ovarian cancer (25), oral squamous cell carcinoma (31,32), pancreatic cancer (33), and kidney cancer (34), as summarized in Table I.

Table I. ZEB2 expression in human tumors.

Human cancer	Expression and mechanism	Author/(Refs.)
Breast cancer	Upregulation in cell lines	Comijn *et al* (9)
	Upregulation in primary tumors	Elloul *et al* (25)
	Upregulation in primary tumors	Gregory *et al* (26)
	Upregulation due to promoter hypomethylation	Rodenhiser *et al* (27)
Gastric cancer	Upregulation in primary tumors	Rosivatz *et al* (13)
	Upregulation in primary tumors	Castro Alves *et al* (15)
Colorectal cancer	Upregulation in cell lines	Guaita *et al* (28)
Liver cancer	Upregulation in cell lines	Miyoshi *et al* (29)
Ovarian cancer	Upregulation in primary tumors	Elloul *et al* (25)
Oral SCC	Upregulation in primary tumors	Maeda *et al* (31)
	Upregulation in cell lines	Taki *et al* (32)
Pancreatic cancer	Upregulation in primary tumors	Imamichi *et al* (33)
Kidney cancer	Upregulation in primary tumors	Nakada *et al* (34)

Because ZEB2 is one of key molecules involved in carcinogenesis, refined integrative genomic analyses of *ZEB2* gene were carried out in this study to elucidate the mechanisms of *ZEB2* transcription.

Materials and methods

Comparative genomic analyses. Human genome sequences corresponding to human ZEB2 RefSeq (NM_014795.2) were searched for by using BLAST programs, as previously described (35,36). *ZEB2* expressed sequence tags (ESTs) were also searched for to identify *ZEB2* splicing variants (37,38). Conserved transcription factor-binding sites within *ZEB2* promoters were then searched for based on manual inspection, as previously described (39,40).

Regulatory network analyses. Literature on ZEB2, NF-κB, TGFß, Notch and Hedgehog signaling molecules in PubMed and Medline databases was critically evaluated to extract knowledge on the regulation of NF-κB, SMAD, CSL, GLI and FOX transcription factors. The mechanisms of *ZEB2* transcription were then investigated based on our data of conserved transcription factor-binding sites within *ZEB2* promoters and in-house knowledgebase of transcription factors regulated by the stem-cell signaling network.

Results

ZEB2 splicing variants transcribed by using alternative promoters. BLAST programs using ZEB2 RefSeq (NM_014795.2) as a query sequence revealed that the *ZEB2* gene was located within human genome sequences AC009951.10 and AC010130.12. Human *ZEB2* gene, consisting of 10 exons, was found to be about 132 kb in size, and intron 2 was about 87 kb in size (Fig. 1). BLAST programs using human *ZEB2* genome sequence as a query sequence revealed that more than 50 ESTs were transcribed based on the major promoter, 5'-adjacent to exon 1, and that two ESTs were transcribed based on the minor promoter, 5'-adjacent to exon 2 (data not shown).

Comparative genomic analyses of ZEB2 orthologs. BLAST programs using human *ZEB2* genome sequence as a query sequence next revealed that chimpanzee *ZEB2* gene, cow *ZEB2* gene, mouse *Zeb2* gene, rat *Zeb2* gene, and chicken *zeb2* gene were located within NW_001232106.1, NW_001494615.2, NT_039206.7, NW_047654.1, and NW_001471733.1 genome sequences, respectively. *ZEB2* orthologs were well conserved not only within exonic regions, but also within the major promoter region and intronic regions (data not shown).

TGFß signaling and ZEB2. TGFß-binding to TGFß receptors leads to autophosphorylation of cytoplasmic serine-kinase domain of TGFß receptors, which results in phosphorylation of Smad2 or Smad3 signal transducer, and transcriptional activation of TGFß-target genes based on the Smad2-Smad4 or Smad3-Smad4 complex (41,42). TGFß signaling cascades are involved in *ZEB2* upregulation and EMT (20,43,44); however, precise mechanisms of TGFß-induced *ZEB2* upregulation remain to be elucidated.

Multiple Smad-binding elements were identified within human *ZEB2* promoter based on manual inspection (data not shown). TGFß signals and Ets1 are known to synergistically upregulate transcription of target genes involved in invasion and metastasis (45). The CGGAGAC motif, corresponding to

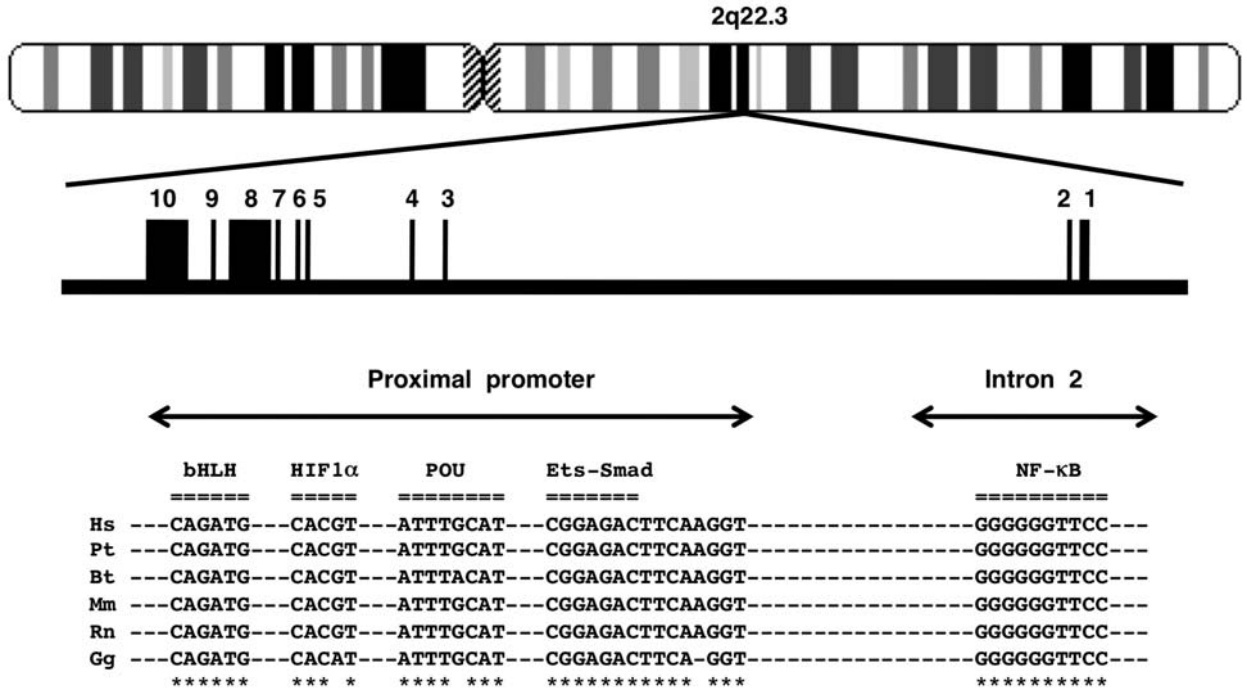

Figure 1. Integrative genomic analyses of *ZEB2*. Schematic representation of the *ZEB2* gene at human chromosome 2q22.3 is shown in the upper part. *ZEB2* gene consists of 10 exons, and intron 2 is about 87 kb in size. Conserved transcription factor-binding sites within *ZEB2* regulatory regions are shown in the lower part. Hs, human; Pt, chimpanzee; Bt, cow; Mm, mouse; Rn, rat; Gg, chicken.

Ets- and Smad-binding sites located at the -8 bp position from the transcriptional start site (TSS) of human ZEB2 RefSeq was completely conserved in chimpanzee *ZEB2*, cow *ZEB2*, mouse *Zeb2*, rat *Zeb2*, and chicken *zeb2* genes (Fig. 1).

Hedgehog signaling and ZEB2. Hedgehog-binding to Patched receptors leads to activation of Smoothened signaling, which results in transcriptional activation of Hedgehog target genes via GLI activators (46-48). Hedgehog signaling cascades are also involved in EMT (4,49). Consensus GLI-binding site was not identified within the 10-kb upstream region from the TSS of human ZEB2 RefSeq as well as within the *ZEB2* gene (data not shown). Therefore, Hedgehog-GLI signaling cascades are predicted to be involved in indirect rather than direct transcriptional activation of *ZEB2*.

Hypoxia signaling and ZEB2. HIF1 complex, consisting of HIF1α and HIF1ß/ARNT, is involved in cellular response to hypoxia through transcriptional regulation of genes involved in energy metabolism, angiogenesis, apoptosis, and EMT (50,51). HIF1α undergoes ubiquitin-mediated proteasomal degradation under normoxic condition to inhibit HIF1 activity, whereas HIF1α is stabilized under hypoxic condition to activate HIF1 activity. Hypoxia-induced HIF1α upregulation leads to invasion and metastasis of tumor cells through upregulation of EMT regulators, such as SNAI1, ZEB1, ZEB2, and TWIST1 (52,53).

Because the transcriptional mechanisms of HIF1-induced *ZEB2* upregulation remain unknown, HIF1α-binding site (hypoxia response element) within *ZEB2* promoter was next searched for. HIF1α-binding site was identified at the -876-bp position from the TSS of human ZEB2 RefSeq. The HIF1α-binding site within the proximal promoter region of human *ZEB2* gene was conserved in chimpanzee *ZEB2*, cow *ZEB2*, mouse *Zeb2*, and rat *Zeb2* genes (Fig. 1).

NF-κB signaling and ZEB2. NF-κB is a key transcription factor involved in inflammation and carcinogenesis (54-56). Because IκB tethers NF-κB in the cytoplasm to prevent its nuclear translocation and subsequent transcriptional activation of target genes, IKK-induced phosphorylation of IκB leads to its ubiquitin-mediated proteasomal degradation, and NF-κB activation. TNFα, IL1, and TGFß signals activate IKK via MAP3K7, whereas PI3K signals activate IKK via AKT.

Chua *et al* reported that TNFα, IL1 and AKT induce NF-κB activation, *ZEB2* upregulation and EMT in human MCF-10A cells, while TNFα and IL1 induce *ZEB2* upregulation in human MD-231 cells (57). Julien *et al* reported that NF-κB-binding site is not located within the human *ZEB2* promoter (58). We confirmed the absence of NF-κB-binding site within the 10-kb upstream region from the TSS of human ZEB2 RefSeq (data not shown). Moreover, we identified an NF-κB-binding site within the proximal promoter region of the mouse *Zeb2* gene; however, the NF-κB-binding site within the mouse *Zeb2* promoter was not conserved in human *ZEB2* and rat *Zeb2* promoters (data not shown).

To elucidate the mechanisms of NF-κB-mediated human *ZEB2* upregulation, we next searched for NF-κB-binding sites within the *ZEB2* gene or the 3'-adjacent region of the *ZEB2* gene. Triple NF-κB-binding sites were identified within intron 2 of the human *ZEB2* gene (data not shown). The first NF-κB-binding site at the position about 59-kb downstream from the TSS of human ZEB2 RefSeq was conserved in chimpanzee *ZEB2*, cow *ZEB2*, mouse *Zeb2*, rat *Zeb2*, and chicken *zeb2* genes (Fig. 1). The second NF-κB-binding site at the position about 66-kb down stream from the TSS of human ZEB2

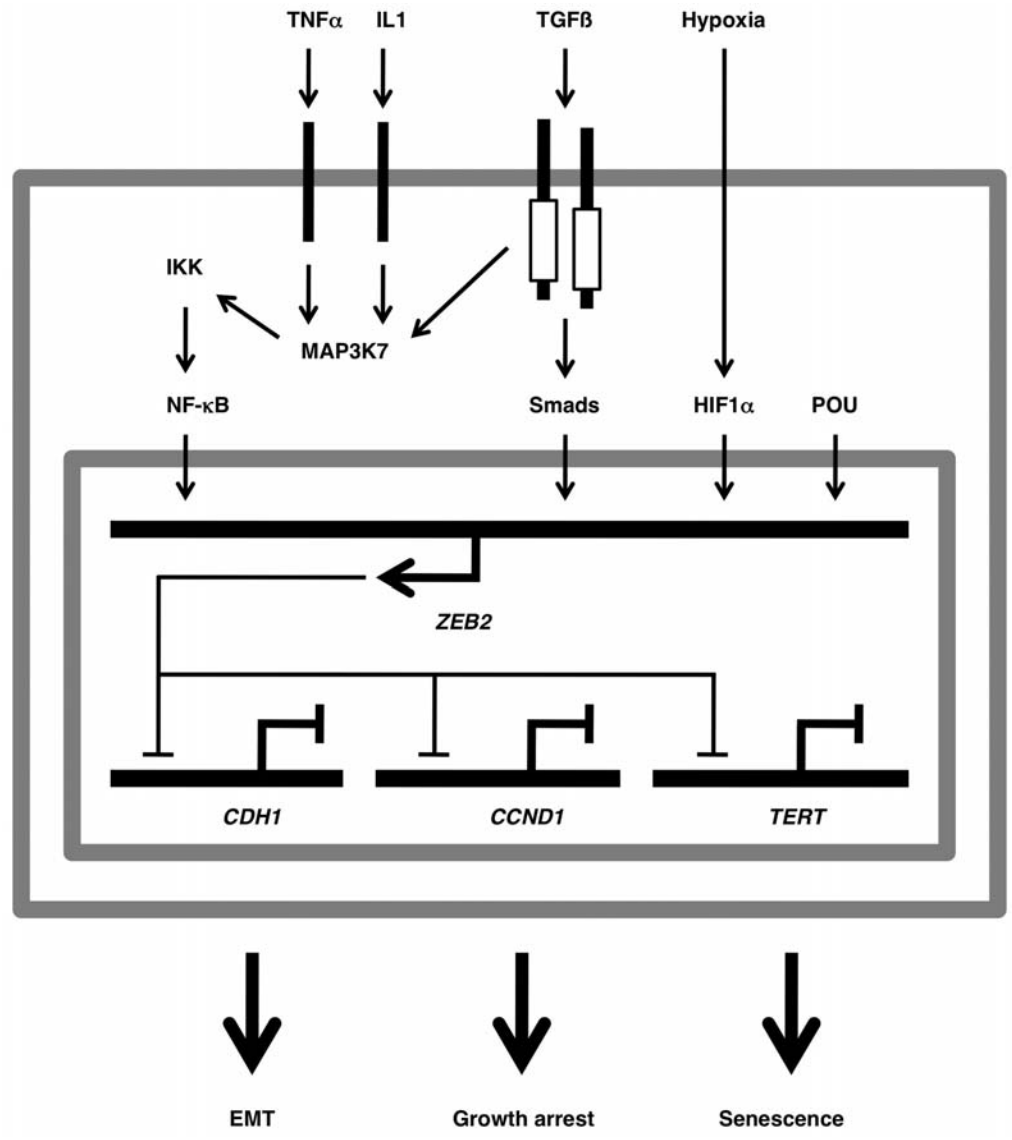

Figure 2. ZEB2 at the crossroads of inflammation, aging and carcinogenesis. Transcriptional mechanisms of *ZEB2* mRNA and effects of ZEB2 protein are summarized.

RefSeq was conserved in chimpanzee *ZEB2*, cow *ZEB2*, and mouse *Zeb2* genes, while the third NF-κB-binding site at the position about 72-kb downstream from the TSS of human ZEB2 RefSeq was conserved in chimpanzee *ZEB2*, and mouse *Zeb2* genes (data not shown). Because the first NF-κB-binding site within intron 2 of *ZEB2* orthologs was ultra-conserved in mammals and chicken (Fig. 1), it was concluded that the first NF-κB-binding site within intron 2 is involved in NF-κB-mediated *ZEB2* upregulation.

Other transcription factor-binding sites. After the identification of evolutionarily conserved Ets-Smad-, HIF1α- and NF-κB-binding sites, we then searched for other transcription factor-binding sites by using the Genetyx program. bHLH-binding site at the -940 bp position from the TSS of human ZEB2 RefSeq was conserved in chimpanzee *ZEB2*, cow *ZEB2*, mouse *Zeb2*, rat *Zeb2*, and chicken *zeb2* genes, while POU (OCT)-binding site at the -876 bp position from the TSS of human ZEB2 RefSeq was conserved in chimpanzee *ZEB2*, cow *ZEB2*, mouse *Zeb2*, and rat *Zeb2* genes (Fig. 1).

Discussion

Refined integrative genomic analyses of *ZEB2* gene were carried out in this study. Ets-Smad-binding CGGAGAC motif, bHLH- and POU/OCT-binding sites within the proximal promoter region, and NF-κB-binding site within intron 2 were completely conserved in human *ZEB2*, chimpanzee *ZEB2*, cow *ZEB2*, mouse *Zeb2*, rat *Zeb2*, and chicken *zeb2* genes (Fig. 1). In addition, HIF1α-binding site within the proximal promoter region was conserved in mammalian *ZEB2* orthologs (Fig. 1). TGFß signals activate *ZEB2* transcription via Smads, Ets1, and NF-κB; TNFα and IL1 signals activate *ZEB2* transcription via NF-κB; Hypoxia signals activate *ZEB2* transcription via HIF1α (Fig. 2). Together these facts indicate that TGFß, TNFα, IL1, and hypoxia signals directly upregulate *ZEB2*.

Hedgehog-GLI signaling cascades cross-talk with TGFß, WNT, FGF, and Notch signaling cascades to constitute the stem-cell signaling network (59-62). Consensus GLI-binding site was not identified within or adjacent to the 7-kb regions

of human *ZEB2* gene (data not shown). Because Hedgehog signals promote conversion of latent TGFß to active TGFß via Integrin αvß6 upregulation (63), Hedgehog signals are able to induce *ZEB2* upregulation indirectly through TGFß signaling cascades. TGFß signals directly upregulate *ZEB2* (Fig. 2), whereas Hedgehog signals are predicted to indirectly upregulate *ZEB2* via TGFß.

POU-binding site within the proximal promoter region of human *ZEB2* gene was conserved in mammals and chicken (Fig. 1). To investigate the relationship between POU-domain transcription factor and *ZEB2* transcription, POU family members co-expressed with *ZEB2* were also searched for in this study. *POU3F2 (BRN2)* and *POU3F3 (BRN1)* were co-expressed with *ZEB2* in brain corpus callosum, spinal cord, and fetal brain, whereas *POU2F2 (OCT2)* was co-expressed with *ZEB2* in monocytes (RefEX database). Involvement of POU family members in *ZEB2* transcription in corpus callosum and monocytes should be further investigated in the future.

Heterozygous deletions or truncating mutations of human *ZEB2* gene give rise to Mowat-Wilson syndrome, manifesting mental retardation, epilepsy, and variable congenital malformations including microcephaly, agenesis of the corpus callosum, Hirschsprung disease, congenital heart disease, hypospadias, genitourinary anomalies, short stature, and characteristic facial features (64). Mouse *Zeb2* is expressed in neural crest and neural epithelium at embryonic day 8.5, and *Zeb2* knockout mice show phenotypes similar to human Mowat-Wilson syndrome (65). Because ZEB2 is one of key regulators of EMT, germline mutations of the *ZEB2* gene results in multiple congenital malformations due to the migratory failure of neural crest cells.

Monocytes differentiate into macrophages or dendritic cells to function as antigen presenting cells regulating innate and adaptive immune responses (66,67). ZEB2 is more highly expressed in monocytes than in macrophages, whereas Smad7, functioning as an inhibitor for Smad2 and Smad3, is relatively higher expressed in macrophages than in monocytes (RefEX database). Because cellular adhesion and proliferation are promoted during differentiation of monocytes into macrophages (68), Smad7-mediated inhibition of TGFß signaling leads to ZEB2 downregulation to induce phenotypic changes from monocytes to macrophages.

EMT in physiological conditions is associated with de-differentiation and growth arrest (69). ZEB2 induces direct transcriptional repression of the *CCND1* gene, encoding Cyclin D1 (22), and *TERT* gene, encoding catalytic subunit of telomerase (20). Because constitutive downregulation of Cyclin D1 and telomerase is not preferable for tumor cells, mesenchymal-epithelial transition (MET) accompanied by ZEB2 down-regulation should occur in tumor cells at the metastasized organs to recover the expression of Cyclin D1 and telomerase.

ZEB2 also represses the *SFRP1* gene, encoding an endogenous WNT signaling inhibitor (23). Hedgehog signals induce direct upregulation of *SFRP1* gene in the earlier phase (70-72), whereas Hedgehog signals might induce indirect down-regulation of *SFRP1* gene in the later phase through the TGFß-ZEB2 signaling cascade in some situations.

This study demonstrated that TGFß, TNFα, IL1, and hypoxia signals directly upregulate *ZEB2* to induce EMT, growth arrest, and senescence. Personalized medicine is gradually gaining practicality in parallel with the development of next-generation sequence technology and the peta-scale super-computer (73). Because ZEB2 occupies the crossroads of inflammation, aging and carcinogenesis, ZEB2 should be an important target for drug discovery to promote personalized medicine.

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