Identification and expression analysis of zebrafish testis-specific gene 10 (tsga10)

SHOHREH ASGHARI-GIVEHCHI1,2 and MOHAMMAD HOSSEIN-MODARRESSI*,3

1Department of Medical Genetics, School of Medicine, International Campus, Tehran University of Medical Sciences, Tehran, Iran, 2Keenan Research Center for Biomedical Science, Li Ka Shing Knowledge Institute, St. Michael’s Hospital, Toronto, Ontario, Canada and 3Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

ABSTRACT Several clinical studies suggest that testis-specific gene antigen 10 (TSGA10) is a cancer-testis antigen with a discernible expression pattern in the testis. Recent studies have highlighted that TSGA10 overexpression in HeLa cells impairs the transcriptional activity of hypoxia-inducible factor alpha (HIF-1α) and inhibits angiogenesis. In this study, we used the zebrafish as a powerful model organism to identify and characterize the orthologue of TSGA10. We analyzed the gene expression pattern by RT-PCR and whole mount in situ hybridization and overexpressed the tsga10 protein by mRNA microinjection. Our results revealed that during early development, tsga10 expression is enriched, but gradually subsides between 0 and 72 hours post fertilization (hpf). There was no detectable transcript at the larval stages. In adult fish, we found high expression levels of tsga10 in the testis and unfertilized egg and low levels of gene expression in the brain, eyes and muscle. Overexpression of tsga10, using tsga10 mRNA microinjection into one-cell stage embryos, resulted in angiogenic and morphological defects at 24 and 48 hpf. This study clarified the expression pattern of tsga10 in different developmental stages and adult tissues, suggesting that tsga10 may have a related biological role in different cell types and tissues. Our results indicate that tsga10 mRNA at embryonic stages is maternally deposited, indicating a transient functional role during embryogenesis. Our findings suggest that tsga10 is a human orthologous gene relevant for future studies to elucidate its mechanism of action in angiogenesis.

KEY WORDS: zebrafish, tsga10, angiogenesis, development

The TSGA10 gene, predominantly expressed in testis, has an important role in spermatogenesis. It is mapped to chromosome 2q11.2, consists of 19 exons and is transcribed into a 3 kb RNA that is subsequently translated into a 698-amino acid protein (Behnam et al., 2006, Modarressi et al., 2004, Modarressi et al., 2001).

TSGA10 is overexpressed in various types of cancers, including solid tumors and blood malignancies (Behnam et al., 2009, Mobasher et al., 2007, Mobasher et al., 2006, Reimand et al., 2008, Smith et al., 2011).

It has been shown that the C-terminus fragment of TSGA10 protein, which co-localizes with hypoxia-inducible factor (HIF-1α) in the midpiece of spermatozoa, impairs the nuclear localization of HIF-1α (Hagele et al., 2006). HIF-1α activates the transcription of genes involved in critical aspects of cancer development, such as angiogenesis and cell survival (Semenza, 2003). A recent study has shown that TSGA10 overexpression decreases HIF-1α transcriptional activity and inhibits angiogenesis, cell growth, and invasion of HeLa cells (Mansouri et al., 2016). Although these studies support the concept that TSGA10 has a repressive role in angiogenesis, these are still insufficient to explain the precise role of TSGA10 particularly in a living organism. In another study using whole-genome chromosomal microarray analysis, TSGA10 deletions at 2q11.2 have been found in patients with developmental disabilities and congenital anomalies (Kaminsky et al., 2011).

Zebrafish is an attractive model for the study of gene function and organogenesis (Lieschke and Currie, 2007). As a model organism, zebrafish has unique advantages for studying vasculogenesis,
as well as angiogenesis, in vivo (McKinney and Weinstein, 2008). Seventy percent of human genes, including TSGA10, have at least one orthologue in zebrafish (Howe et al., 2013). The small size, optical transparency, high number of offspring, and external fast embryonic development make zebrafish an excellent model system for forward-genetic analysis (Gore et al., 2012). Moreover, the early development of the cardiovascular system in zebrafish embryos provides a unique opportunity for direct blood circulation observation. Due to the availability of zebrafish transgenic lines with endothelial expression of fluorescent proteins mediated by a vascular gene promoter, such as Tg(flk:EGFP) and Tg(fli1:EGFP), vascular and blood development can be visualized and analyzed in great detail. Finally, genetic studies have uncovered the preservation of molecular pathways between mammals and fish, making vasculogenesis research in zebrafish doubtlessly applicable for human health (Chavez et al., 2016).

Given the emerging evidence of tsga10 overexpression role in cell growth and angiogenesis, we employed a zebrafish-based in vivo functional study to characterize the zebrafish tsga10 and to analyze the mRNA overexpression of tsga10 in developmental and aberrant angiogenesis.

Results

Identification and in silico analysis of zebrafish tsga10

NCBI database analysis indicates the presence of one orthologue of the human TSGA10 in zebrafish, namely, tsga10, which is mapped to chromosome 1. The Danio rerio tsga10 gene (NC_007112.6, Ensembl Id: ENSDARG00000052512) has 18 exons from which, similar to its human counterpart, 16 exons are protein-coding exons (Fig. 1A). It is expressed mainly as a 2.5 kb transcript isoform encoding a 710-amino acid protein (NP_001070757.2) with 43% identity to the human TSGA10 protein (Fig. 1B). A single tsga10 gene is present in other species (data not shown). UniGene analysis of zebrafish tsga10, represented by 47 Expressed Sequence Tags (ESTs) from cDNA libraries, shows a restricted expression pattern of the gene in the reproductive system from hatching to adult stages; in addition to this expression pattern, our study finds tsga10 expression in other developmental stages and tissues.

The comparison of zebrafish Tsga10 protein with its orthologues in human, mouse, chimpanzee, clawed frog, dog, and brown rat indicates that the COG1196 domain (Fig. 1C, D) has been highly conserved across different species. This domain, with a coiled-coiled structure, is a member of the SMC (structural maintenance of chromosomes) superfamily of proteins, involved in the control of cell cycle, cell division, chromosome segregation, regulation and repair of the gene (Hirano, 2006).

Analysis of the EPD database reveals that zebrafish tsga10 has one promoter, (Chromosome \[NC_007112.5\]; Strand [+]; Fig. 1. Analysis of the tsga10 transcript and protein. Number of exons, transcript length in basepairs, and translation length (number of amino acids in the protein) (A). Multiple sequence alignment of Human, zebrafish, and mouse Tsga10 protein using the T-Coffee algorithm (B). Schematic representation of the position of COG1196 (SMC_N superfamily member), the conserved domain of zebrafish Tsga10 (C) and human TSGA10 (D) proteins.)
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Temporal and spatial expression pattern of tsga10 during zebrafish development

The mRNA levels of tsga10 were investigated in zebrafish embryos at different developmental stages, and in adult tissues using RT-PCR. Our results showed that tsga10 expression is enriched during early development, detectable as early as the one-cell stage and 18 hpf, but gradually subsides between 24 and 72 hpf with no expression at 72 hpf. There was no detectable transcript at the larval stages (Fig. 2A). In the adult stage, tsga10 was highly expressed in testis and unfertilized egg and at low levels in brain, eye, and muscle. The gene was not expressed in adult caudal fin and heart. (Fig. 2B).

To study the spatial and tissue-specific expression pattern of tsga10, whole mount in situ hybridization was conducted on zebrafish embryos from 0.2 (one-cell) to 48 hpf. A sense probe was used in parallel control experiments with embryos of the same stages, which gave no detectable signal (Supplementary Fig. S1 in supplementary materials). We detected a ubiquitous signal in zygote (one-cell stage) and in the early stages of development showing that tsga10 promoter at the CCAAT box region and activates gene expression. NF-Y also has different target genes in human, mouse, and rat.

Overexpression of tsga10 causes zebrafish angiogenic development and morphological defects

To investigate the role of tsga10 in zebrafish angiogenesis, we overexpressed the protein by injecting tsga10 mRNA into one-cell stage flk-GFP zebrafish embryos (n=50), which resulted in embryos with angiogenic and morphological phenotypes (56% n=28), normal embryos (24% n=12), and dead embryos (20% n=10). The presence of the injected mRNA was confirmed by RT-PCR using the T1 and T2 primers (Supp. Fig. S2). The embryos injected with tsga10 mRNA showed a decrease in the number and length of ISVs compared to control embryos injected with nuclease-free water (Fig. 4A, B, and C). At 24 hpf, mRNA-injected embryos had an average number of 7 angiogenic sprouts in tail, compared to 14 angiogenic sprouts in control embryos (Fig. 4B and E, p<0.001, n=28). In addition, the caudal vein plexus was not completely formed in mRNA-injected embryos (Fig. 4B).

At 48 hpf, the analysis of complete ISVs showed that mRNA-injected embryos had an average number of 6 complete ISVs and 5 angiogenic sprouts in tail, compared to 20 fully extended ISVs in control embryos (Fig. 4C and F p<0.001, n=28). Injected embryos also had less developed blood vessels in the brain compared to control embryos (Fig. 4H). Neither angiogenic defects nor morphological phenotypes were detected at 72 hpf (Fig. 4D and G). Our data suggest that reducing the concentration and activity of tsga10 mRNA, between 48 and 72 hpf, may probably lead to the ISV recovery and the rescue of flk-GFP mRNA-injected embryos. In addition to angiogenic defects, the overexpression of tsga10mRNA resulted in a range of morphological defects in 48 hpf embryos, including a small head, head edema, and an abnormally bent fin (Fig. 4H, p<0.001, n=28). All control injected zebrafish embryos developed normally (Fig. 4H).

Discussion

TSGA10 gene in human localizes to region q11.2 of chromosome 2 and is mainly expressed in testis and some tumors (Behnam et al., 2009, Behnam et al., 2006, Mobasher et al., 2007, Modarressi et al., 2004, Modarressi et al., 2001). In recent years, intense efforts to identify the function of TSGA10 led to the discovery of the inhibitory effect of TSGA10 on tumor angiogenesis (Mansouri et al., 2016). Previous studies have shown that TSGA10 is expressed during spermatogenesis (in testis), embryogenesis (undifferentiated embryonic stem cells), brain development, and in different types of cancer (Behnam et al., 2009, Behnam et al., 2006, Miryounesi et al., 2014, Mobasher et al., 2007, Mobasher et al., 2006, Reimand et al., 2008, Smith et al., 2011). In this study, we characterize the zebrafish homologue of TSGA10 and analyze its expression pattern and the effect of its overexpression in zebrafish angiogenic.
development. We show that zebrafish \textit{tsga10} mRNA is present as a maternal transcript during early developmental stages, from the newly fertilized egg until 48 hpf, indicating a transient functional role during embryogenesis. Furthermore, \textit{tsga10} in the adult stage is expressed in the reproductive organs, brain, eye, and muscle, which is similar to the TSGA10 expression pattern in human. These data suggest that \textit{tsga10} might have a related biological role in different cell types and tissues. We also report the \textit{tsga10} expression pattern in the head, intersomitic regions, and different parts of the brain in zebrafish embryos. These results are in line with data obtained from previous studies in which TSGA10 is expressed in a broad range of tissues (Behnam \textit{et al.}, 2006, Dianatpour \textit{et al.}, 2012, Fratta \textit{et al.}, 2011, Mobasher \textit{et al.}, 2007, Mobasheiri \textit{et al.}, 2006) and suggest that \textit{tsga10} may have a role in head and axis development. There is a TSGA10 orthologue in mouse, chimpanzee, clawed frog, dog, and brown rat (Church \textit{et al.}, 2011, Gibbs \textit{et al.}, 2004, Hellsten \textit{et al.}, 2010, Lindblad-Toh \textit{et al.}, 2005, McConkey, 2004). By comparing Tsga10 protein domains, we found that the COG1196 domain (a member of the superfamily SMC: structural maintenance of chromosomes) is conserved in human, zebrafish, and all the animals mentioned above. Our bioinformatics analysis also shows that the nuclear transcription factor Y (NF-Y) binds to the CCAAT box region of \textit{tsga10} promoter and acts as a transcriptional activator. Given the central role of SMC proteins as ATPases involved in cell cycle control, chromosome segregation in mitosis and meiosis, gene regulation, and recombinational DNA repair (Hirano, 2006), and the fact that NF-Y target genes are mostly cell-cycle-related and related to human disease such as cancer (Ly \textit{et al.}, 2013), \textit{tsga10} could have an important role in cell survival in different diseases and cancer. Further investigation is necessary to better understand and to confirm these findings.

A previous study of TSGA10 found that tumor cell characteristics such as migration, proliferation, invasion, and angiogenic activity are remarkably affected by TSGA10 overexpression. According to this study, which was done on HeLa cells, TSGA10 overexpression has an inhibitory role in angiogenesis for tumor cells (Mansouri \textit{et al.}, 2016). In another study, TSGA10 has been identified as a tumor suppressor gene (Jakhesara \textit{et al.}, 2013, Yuan \textit{et al.}, 2013). Interestingly, our results support the published data showing the inhibitory role of \textit{tsga10} on angiogenesis, as evidenced by deficiencies in ISV, brain vasculature, and caudal vein plexus formation at 24 and 48 hpf after \textit{tsga10} overexpression by \textit{tsga10} mRNA injection. The mechanisms that regulate angiogenic sprouting and extension of zebrafish ISVs are different than the de novo vasculogenic formation of the posterior cardinal vein and the dorsal aorta (Herbert \textit{et al.}, 2009). This could explain the effect of \textit{tsga10} overexpression on zebrafish ISV sprouting and extension but not on the main vasculature. The effect of \textit{tsga10} on ISV angiogenesis is probably indirect as we found that the expression of \textit{tsga10} is mainly located in the head, trunk, and intersomitic boundaries; however, more experimental evidence is needed to support these conclusions.

The obtention of different morphological phenotypes after \textit{tsga10} overexpression such as heart edema, small head, and abnormal...
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fin, is similar to the results of a human study in which TSGA10 deletions at 2q11.2, were found by whole-genome chromosomal microarray analysis in patients with developmental and congenital anomalies (Kaminsky et al., 2011). These findings suggest additional roles for tsga10 in zebrafish development, which need to be further investigated.

Zebrafish specific biology allows easy access to all developmental stages, and the optical transparency of embryos and larvae make real-time imaging of developing pathologies possible (Lieschke and Currie, 2007). Zebrafish has also become a valuable vertebrate model to study the process of vasculogenesis and angiogenesis among various in vitro and in vivo models (Chavez et al., 2016, Thisse and Zon, 2002). Zebrafish have a closed circulatory system, and the anatomy of the vasculature, the vessel congregation process, and the molecular mechanisms of vasculogenesis are highly similar to those in humans (Isogai et al., 2001). The external and fast embryonic development and the straightforward access to the developing vasculature due to available fluorophore-tagged strains, made zebrafish an ideal in vivo model to study the effects of gene modification on angiogenesis and tumor neovascularization (Gore et al., 2012, Santoro, 2012).

Our study is the first to identify zebrafish tsga10 and to generate in vivo data that demonstrate the role of tsga10 overexpression in zebrafish angiogenesis at the early stages of development. There is no evidence yet that our results regarding the gene angiogenic effects could be the same in the human pathology; therefore, investigating the role of tsga10 in mammalian experimental models of angiogenesis could be of interest. We are not aware of any mechanism linking tsga10 overexpression and reduced angiogenesis, so our findings may open a new perspective to investigate...

**Fig. 4. tsga10 overexpression causes angiogenic defects and morphological phenotypes.** Tg(flk1-EGFP) embryos were injected at one-cell stage with 150 ng/µl tsga10 mRNA (final concentration of 0.3 per embryo). Overexpression of tsga10 resulted in a decrease in the number of angiogenic sprouts in tail; see asterisks in (A,B) and incomplete formation of the caudal vein plexus; see arrow in (B) at 24 hpf (A,B,E). a decrease in the number of complete ISVs in tail; see arrowheads in (C) at 48 hpf (C,F), fewer brain vessels, a small head, heart edema, and an abnormally bent fin at 48 hpf; see arrows in (H). Defective angiogenesis was recovered at 72 hpf (D,G).
those mechanisms. Knowing that zebrafish is a valuable model organism to study the tsga10 function, and given the emerging evidence that TSGA10 has an important role in tumor angiogenesis and metastasis (Mansouri et al., 2016), further studies using tumor cell xenograft in zebrafish are required to confirm the angiogenic inhibitory role of tsga10 in cancer.

Materials and Methods

**Zebrafish husbandry**

All zebrafish experiments were performed under St. Michael’s Hospital Animal Care Committee approved protocol ACC403. Zebrafish were maintained on a 14 h light/10 h dark cycle at 28 °C in the Li Ka Shing Knowledge Institute Research vivarium. Embryos were collected and raised in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, and 0.33 mM MgSO4), and treated with 0.2 mM 1-phenyl-2-thiourea (PTU) at 24 hpf to minimize pigmentation. The following genetic strains were used: wild-type Tulip Long fin (TL) (Zebrafish International Resource Center (ZIRC)) and transgenic Tg(flkl:EGFP) (Jin et al., 2005).

**Bioinformatics analysis**

Zebrafish genomic sequences were analyzed using the NCBI (National Center for Biotechnology Information) and the Ensembl zebrafish genome database. The Basic Local Alignment Search Tool (BLAST) was used to compare nucleotide and amino acid sequences to sequences from the NCBI database. tsga10 promoter and transcription factor analyses were achieved using EDP and TRANSFAC databases, respectively. Multiple protein sequence alignment was obtained using the T-Coffee algorithm.

**RT-PCR**

Total RNA of wild-type TL zebrafish embryos was isolated from different developmental stages (1-cell stage to 120 hpf, 30 embryos/stage) and from adult tissues, including testis, fin, heart, eyes, brain, muscle, and unfertilized egg (14 RNA groups), using an RNaseasy mini kit (Qiagen) and DNase treatment. For tissue dissection, adult fishes were anesthetized with 0.2% Tricaine and then euthanized by submersion in ice water for 15 minutes (Gupta and Mullins, 2010). Four dissected samples were used for each tissue RNA extraction. RNA was quantified using a NanoDrop Spectrophotometer and stored in aliquots at -80 °C.

Total testis RNA was extracted from adult zebrafish using an RNeasy mini kit (Qiagen) and treated with DNase (tissue dissection explained above). RNA was quantified using a NanoDrop Spectrophotometer and gel electrophoresis on a 1% TAE-agarose gel. Total cDNA was synthesized from 1 μg of total RNA using Superscript II reverse transcriptase kit (Invitrogen) and random hexamer primers following the manufacturer’s protocol. The full-length protein coding sequence of zebrafish tsga10 was PCR amplified using T1 primer, 5′-ATGTTGAGGCCCTGCGC-3′ (forward), and T2 primer, 5′-TCAGTCGTGAAGGCTCCCTA-3′ (reverse), starting at the start and stop codons of the tsga10 coding sequence, respectively. PCR was performed in a 25 μl volume using a Taq DNA polymerase (Qiagen), and consisted of an initial denaturation step (94 °C for 2 min); 40 cycles of denaturation (94 °C for 30 s), annealing (57 °C for 30 s), and extension (72 °C for 2 min); followed by a final extension step (72 °C for 10 min). The PCR product was gel-purified using the QiAquick Gel Extraction Kit (Qiagen) and cloned into the pCRII-TOPO vector (Invitrogen) and verified for the orientation and sequence of the insert. The resulting plasmid was used as a template for in vitro transcription to make a 450 bp tsga10 RNA probe. Antisense and sense riboprobes were synthesized by in vitro transcription using 10x Dig RNA labeling mix (Roche) and SP6 and T7 RNA polymerases, respectively (mMESSAGE mMACHINE in vitro transcription kit, Invitrogen). WISH images were taken with a fluorescent dissection microscope (Leica M205 FA).

**Generation of tsga10 expression construct and capped tsga10 mRNA**

Total testis RNA was extracted from adult zebrafish using an RNaseasy mini kit (Qiagen) and treated with DNase (tissue dissection explained above). RNA was quantified using a NanoDrop Spectrophotometer and gel electrophoresis on a 1% TAE-agarose gel. Total cDNA was synthesized from 1 μg of total RNA using Superscript II reverse transcriptase kit (Invitrogen) and random hexamer primers following the manufacturer’s protocol. The full-length protein coding sequence of zebrafish tsga10 was PCR amplified using T1 primer, 5′-ATGTTGAGGCCCTGCGC-3′ (forward), and T2 primer, 5′-TCAGTCGTGAAGGCTCCCTA-3′ (reverse), starting at the start and stop codons of the tsga10 coding sequence, respectively. PCR was performed in a 25 μl volume using a Taq DNA polymerase (Qiagen), and consisted of an initial denaturation step (94 °C for 2 min); 40 cycles of denaturation (94 °C for 30 s), annealing (57 °C for 30 s), and extension (72 °C for 2 min); followed by a final extension step (72 °C for 10 min). The PCR product was gel-purified using the QiAquick Gel Extraction Kit (Qiagen) and cloned into the pCR-II-TOPO vector (Invitrogen). Clones with sense orientation were confirmed by restriction digestion (SpeI and EcoRV restriction enzymes, FastDigest) and sequencing. Capped mRNA was transcribed in vitro using the mMESSAGE mMACHINE T7 kit (Invitrogen) and the Poly-A sequence added to the product of mMESSAGE mMACHINE T7 kit using Poly(A) tailing kit (Invitrogen), according to the manufacturer’s instruction. The mRNA was ethanol-precipitated, resuspended in nuclease-free water, and stored in aliquots at -80 °C.

**tsga10 mRNA microinjection and imaging**

We injected flk-GFP embryos with 150 ng/μl of tsga10 mRNA and final concentration of 0.3 per embryo (n=80, injection volume=2 nl/embryo) using an N2 drive microinjector 5171 (Eppendorf, USA) through the chorion into the cell at the one-cell stage. The dye tracer phenol red was added to the mRNA before injection (0.1% final concentration) to track the successful microinjection. We also injected control flk-GFP embryos with nuclease-free water (n=80). After microinjection, embryos were raised at 28 °C and imaged periodically using fluorescent dissection microscopy (Leica M205 FA) equipped with GFP fluorescence filter. To obtain the desired orientation, we anesthetized the embryos/larvae with 0.16 mg/ml of Tricaine and placed them in 2.5% methyl cellulose (Sigma). The mRNA microinjection was performed with three technical replicates.

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**Competing interests**

The authors declare that the research was conducted with no competing interests.

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