



Published in final edited form as:

J Cell Biochem. 2013 October ; 114(10): 2375–2383. doi:10.1002/jcb.24586.

HOX antisense lincRNA HOXA-AS2 is an apoptosis repressor in all trans retinoic acid treated NB4 promyelocytic leukemia cells

Hang Zhao¹, Xueqing Zhang¹, Josias Brito Frazão^{1,2}, Antonio Condino-Neto², and Peter E. Newburger^{1,3,*}

¹Department of Pediatrics, University of Massachusetts Medical School, Worcester, MA, USA

²Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP, Brazil

³Department of Cancer Biology, University of Massachusetts Medical School, Worcester, MA, USA

Abstract

HOXA cluster antisense RNA 2 (HOXA-AS2) is a long non-coding RNA located between the HOXA3 and HOXA4 genes in the HOXA cluster. Its transcript is expressed in NB4 promyelocytic leukemia cells and human peripheral blood neutrophils, and expression is increased in NB4 cells treated with all trans retinoic acid (ATRA). Knockdown of HOXA-AS2 expression by transduced shRNA decreases the number of viable cells and increases the proportion of apoptotic cells, measured by annexin V binding and by activity and cleavage of caspases-3, -8, and -9. The increase in death of HOXA-AS2 knockdown cells was accompanied by an elevated TNF-related apoptosis-inducing ligand (TRAIL) levels, but ATRA-induced NB4 cells treated with TRAIL did show an increase in HOXA-AS2 expression. These results demonstrate that ATRA induction of HOXA-AS2 suppresses ATRA-induced apoptosis, possibly through a TRAIL-mediated pathway. HOXA-AS2-mediated negative regulation thus contributes to the fine-tuning of apoptosis during ATRA-induced myeloid differentiation in NB4 cells.

Keywords

myeloid; apoptosis; long intergenic non-coding RNA (lincRNA); non-coding RNA; Hox gene cluster

Long intergenic non-coding RNAs (lincRNAs) are non-coding transcripts longer than 200 nucleotides, transcribed from loci between coding genes and often from the opposite DNA strand (Cabili et al., 2011; Esteller, 2011). Most well-characterized lincRNAs have a functional role in the regulation of gene expression, typically at the transcriptional level, targeting genes that are genomically local (cis-regulation) or distant (trans-regulation) (Prensner and Chinnaiyan, 2011). In addition to long-recognized roles in X-inactivation and genetic imprinting, lincRNAs have recently been recognized to function in development and

*Corresponding author: Peter E. Newburger, Department of Pediatrics, University of Massachusetts Medical School, Worcester, MA 01655. Tel. (508) 856-4225; Fax (508) 856-4282, peter.newburger@umassmed.edu.

differentiation (Guttman et al., 2011; Rinn et al., 2008), including hematopoiesis (Hu et al., 2011; Zhang et al., 2009).

lincRNAs are particularly common within the four paralogous clusters of HOX genes (Derrien et al., 2012; Mainguy et al., 2007; Rinn et al., 2007), a set of highly homologous transcription factors that control not only embryological development (Duboule, 2007) but also hematopoietic lineage and differentiation (Eklund, 2011). Three functional lincRNAs have been identified in the HOXA cluster: HOTAIRM1, MISTRAL, and HOTTIP (Bertani et al., 2011; Wang et al., 2011; Zhang et al., 2009). HOTAIRM1, located at the 3' end of the cluster, is a myeloid-specific lincRNA that appears to influence expression of both local and distant genes (Zhang et al., 2009). HOTTIP, located at the 5' end, enhances expression of neighboring HOXA genes, most prominently HOXA13 (Wang et al., 2011). These observations distinguish HOTAIRM1 and HOTTIP, as most other lincRNAs have been associated with gene repression.

Acute promyelocytic leukemia (APL) is unique among myeloid leukemias because of its sensitivity to all trans retinoic acid (ATRA), due to the pathogenic t(15; 17) translocation that creates a fusion of the retinoic acid receptor α (RAR α) to the promyelocytic leukemia (PML) proteins (Grimwade et al., 2010; Sanz and Lo-Coco, 2011). ATRA induces both cell cycle arrest and the maturation of APL cells which leads to the phenotype of terminally differentiated granulocyte-like cells characterized by limited lifespan and spontaneous, caspase-dependent apoptosis (Gianni et al., 2000). ATRA-induced apoptosis in NB4 cells, a human promyelocytic leukemia cell line derived from a patient with APL, is associated with the activation of various caspase proteases that lead to the execution phase of apoptosis (Gianni et al., 2000).

The current studies examined the expression and function in NB4 cells and peripheral blood neutrophils of HOXA cluster antisense RNA 2 (HOXA-AS2), a lincRNA located between and antisense to the human HOXA3 and HOXA4 genes. We identified and chose to study this RNA because of its proximity and similarity to the nearby lincRNA HOTAIRM1, (Zhang et al., 2009) and initially hypothesized that its function would be similar: that is, to regulate myeloid gene expression (Zhang et al., 2009) and cell cycle (unpublished data). The current results, however, indicate that HOXA-AS2 instead plays a role in regulation cell survival through suppression of apoptosis.

Materials and Methods

The BCA protein assay kit was purchased from Thermo Scientific; antibodies for cleaved caspase-3, cleaved caspase-8, cleaved caspase-9 and Bax, from Cell Signaling; TRAIL antibody, from BD Biosciences; and recombinant human TRAIL, from Invitrogen and BioVision.

Cell Culture

NB4, NB4R2 and K562 cell lines were obtained from American Type Culture Collection. NB4 cells, established from a patient with relapsed APL, terminally differentiates in response to ATRA; (Idres et al., 2001; Lanotte et al., 1991) whereas the derivative line

NB4R2 is ATRA resistant.(Duprez et al., 2000) K562 is an ATRA non-responsive Philadelphia chromosome-positive chronic myelogenous leukemia cell line.(Lozzio and Lozzio, 1975)

Cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin 100 U/ml, and streptomycin 100 µg/ml. Human peripheral blood neutrophils were isolated from heparinized (10 U/ml) blood of healthy individuals by Ficoll-Paque PLUS gradient solution (GE Healthcare Bioscience AB, 17-1440-02). The purity of the neutrophils was determined by Wright-Giemsa solution staining and viable cells were counted by trypan blue dye exclusion. The neutrophils were washed three times with RPMI 1640 medium (GIBCO) and resuspended at 10⁶ cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin 100 U/ml, and streptomycin 100 µg/ml. After culture, cell viability was 99 % by trypan blue exclusion. Cultured neutrophils were treated with or without IFN-γ (1000U/ml) or TNF-α (1000U/ml) for 30 or 120 min.

High throughput RNA sequencing and data visualization

Cells were lysed in Tri-Reagent (Molecular Research Center, Inc.) and total RNA isolated as instructed by manufacturer and treated with TurboDNase (Ambion). The integrity of the RNA was evaluated on an Agilent Bioanalyzer, and samples with sufficient RNA integrity were used to create libraries for deep sequencing. Samples were analyzed by paired-end sequencing on the Genome Analyzer Iix (Illumina) at the Yale Center for Genomics and Proteomics. Base calling and paired-end short reads (FASTQ format) were generated by Genome Analyzer Pipeline software at the sequencing facility. The resulting sequence reads were aligned to the human reference genome of Hg19 February 2009 GRCh37 build with bowtie and followed by exon junction detection with tophat (Trapnell et al., 2012). Reads coverage and detected junction tracks were visualized with the R package 'ggbio' of Bioconductor Project (Gentleman et al., 2004; Yin et al., 2012).

qRT-PCR

Total RNA was isolated by TRIzol (Invitrogen) reagent. First-strand cDNAs were reverse transcribed from total RNA using SuperScript III Reverse Transcriptase (Invitrogen). qRT-PCR assays using SYBR Green Master Mix (Applied Biosystems) were performed on an Applied Biosystems 7300 thermal cycler (Foster City, CA). Quantification was performed using the relative comparative CT Method and all measurements were performed in triplicate. Primer sequences are listed in the Supplementary Table 1.

HOXA-AS2 shRNA

Three MISSION® lentiviral particles that encode for shRNA against HOXA-AS2 were purchased from Sigma-Aldrich. The complementary target sequences on three different regions of human HOXA-AS2 genomic DNA (GenBank: AK311383) are presented in Supplementary Table 2. NB4 cells were transduced with MISSION lentiviral particles following the manufacturer's protocol, at final multiplicities of infection of 0.1, 0.5, 1.0, 5.0, or 10. Control NB4 cells were transduced with a "scramble" shRNA (Addgene plasmid 1864) in the same vector. Transduced cells were selected in culture medium containing puromycin 10 µg/ml. Visible clones were picked from the 96-well plates and expanded in

24-well tissue culture plates. The knockdown of HOXA-AS2 RNA was determined by quantitative (q)RT-PCR.

Annexin V binding

Cell apoptosis was analyzed on an LSR II flow cytometer (BD Biosciences) using an annexin V-FITC and propidium iodide apoptosis detection kit (BD Biosciences) according to the manufacturer's instructions. Data were analyzed using FlowJo software (Tree Star).

Caspase activity

Caspase activity assay kits (BioVision Research Products, Mountain View, CA) based on colorimetric detection of cleaved para-nitroaniline-labeled substrates specific for caspase-3 (acetyl-AspGlu-Val-Asp-*p*-nitroanilide), caspase-8 (acetyl-Ile-Glu-Thr-Asp-*p*-nitroanilide), and caspase-9 (acetyl-Leu-Glu-His-Asp-*p*-nitroanilide) were used to measure respective activities, according to manufacturer instructions, as sample absorbance at 405 nm in a spectrophotometer (NanoDrop).

Western blot analysis

Cells were lysed in chilled RIPA buffer (Cell Signaling) with 1 mM PMSF and boiled for 5 minutes. Protein was electrophoresed on 4–20% gradient SDS-PAGE gels (Invitrogen) and electro-transferred to PVDF membranes (Millipore). The blocked membranes were incubated with the primary antibody in TBST buffer containing 5% nonfat milk at 4°C overnight, followed by incubation with the species-appropriate HRP-conjugated secondary antibody according to manufacturer's instructions. Membranes were developed with Super-Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

Statistics

Descriptive statistics were performed and the results represented by bar graphs showing mean \pm standard deviation (SD) values. ANOVA and Tukey's Multiple Comparison test were used for comparisons among groups; a *p* value <0.05 was considered significant.

Results

ATRA selectively induces HOXA-AS2 expression

lincRNAs are relatively common in the HOXA cluster (Derrien et al., 2012;Mainguy et al., 2007;Rinn et al., 2007), where they play regulatory roles in development and differentiation, including in myeloid cells (Zhang et al., 2009). In this study, we focus on the role of HOXA-AS2, a large intergenic lincRNA, on proliferation and apoptosis of ATRA-treated NB4 cells. HOXA-AS2 is located between HOXA3 and HOXA4 in the HOXA cluster in the human genome, and transcribed in the opposite direction (Fig. 1A). It is widely, but not universally, expressed and spliced into several isoforms, varying from 339 to 2045 nt (NCBI UniGene, 2012;Ensembl, 2012). As shown in Fig.1A, HOXA-AS2 is expressed and responsive to ATRA induction in NB4 cells and neutrophils as assessed by RNA deep-sequencing. Splice junction analysis by Tophat (green bars) demonstrates splicing and confirms transcription in both NB4 cells and primary neutrophils. The signal is not sufficiently precise in alignment to

permit identification of which isoforms are expressed in these cells, perhaps because of a mix of isoforms and the known inefficiency of lincRNA splicing (Tilgner et al., 2012). qRT-PCR analysis confirmed progressive up-regulation in ATRA-treated NB4 cells (Fig. 1B). In contrast, ATRA had no significant effect on HOXA-AS2 expression in ATRA-resistant NB4R2 and K562 cells (Fig. 1B).

HOXA-AS2 expression is induced by IFN- γ and TNF- α in neutrophils

As all other experiments were performed in the NB4 cell line, we sought to verify regulated expression of HOXA-AS2 in primary peripheral blood neutrophils as well. Gene expression was analyzed by qRT-PCR for early (30 min) and late (2 hr) responses (Fig. 2). HOXA-AS2 expression in response to IFN- γ was higher at the 30 min time point while TNF- α induced a higher level of gene expression at 2 hr.

HOXA-AS2 knockdown decreases cell viability

To investigate the role of HOXA-AS2 on myeloid cell proliferation and apoptosis, three different shRNA constructs targeting HOXA-AS2 were transduced into NB4 cells to inhibit its expression. Four stable cell lines of NB4 were established: one with each targeting shRNA and one with the scramble control. These cell lines exhibited vigorous growth and microscopic appearance identical to the parental NB4 cells (data not shown). The efficacy of HOXA-AS2 knockdown by the shRNAs was analyzed by qRT-PCR. All three shRNA constructs caused a reduction of HOXA-AS2 RNA expression when compared with scramble (Fig. 3A). shRNA constructs A and C exhibited more efficient knockdown (clone A 76%, clone C 73%) and were therefore used in all other experiments. To determine the effect of HOXA-AS2 on proliferation and apoptosis, NB4 transductant clones were incubated with ATRA (1 μ M) and the number of viable cells determined at days 1, 2, 3, 4 and 5. The HOXA-AS2 knockdown cells treated with ATRA showed progressive relative decreases in cell number (Fig. 3B) and viability (Fig. 3C) compared to scramble-expressing cells; whereas HOX3AS knockdown did not affect proliferation or viability of uninduced NB4 cells. The scramble control showed a smaller, non-specific effect on proliferation and viability.

ATRA-induced apoptosis in knockdown cells

Since decreased cell counts and viability can be attributed to increased apoptosis, we investigated the effect of HOXA-AS2 on ATRA-induced apoptosis in NB4 cells. Cells were stained with FITC-labeled annexin V and analyzed by flow cytometry at 3 days after ATRA (1 μ M) treatment. The percentage of annexin V positive cells markedly increased in HOXA-AS2 knockdown cells compared to scramble (Fig. 4A,B), indicating enhanced apoptosis in HOXA-AS2 knockdown cells. Consistent with these results, colorimetrically-determined caspase-3 activity (Fig. 4C) and levels of cleaved caspase-3 on western blot (Fig. 4D) also increased in the knockdown clones. Thus, HOXA-AS2 plays a negative regulatory role in caspase-3-dependent apoptosis in ATRA-induced NB4 cells.

Extrinsic and intrinsic pathways in apoptosis

In TRAIL-mediated apoptosis (Bodmer et al., 2000), death-inducing signaling complex activity is known to mediate the processing of caspase-8. In addition to direct activation of executor caspases, caspase-8 activation of the initiator caspase-9 also triggers activation of executor caspases (Adams and Cory, 2007; Garrido et al., 2006). To determine the involvement of caspases-8 and -9 in the effects of HOXA-AS2 knockdown on NB4 cell apoptosis, we measured intracellular caspase-8 and -9 activity in native NB4 cells, HOXA-AS2 knockdown clones, and scramble controls, with or without 4 days of treatment with ATRA (1 μ M). Both caspase activities were increased in the ATRA-induced HOXA-AS2 knockdown cells, compared to controls (Fig. 5A,B). Consistent with these results, western blot analysis confirmed that cleaved products of caspase-8 and -9 in HOXA-AS2 knockdown cells were elevated over native NB4 and scramble controls (Fig. 5C). Western blot analysis also showed that HOXA-AS2 knockdown cells contain increased amounts of BAX (Fig. 5C), a proapoptotic protein of the BCL2 protein family (Gavathiotis et al., 2010). Thus, ATRA induces apoptosis in HOXA-AS2 knockdown cells through activation of both caspase-8 and caspase-9 pathways.

TRAIL levels in HOXA-AS2 knockdown cells

ATRA-induced cell death in APL and NB4 cells is linked to paracrine production of TRAIL (Altucci et al., 2001; Johnstone et al., 2008), a member of the tumor necrosis factor (TNF) superfamily that induces apoptosis via the extrinsic death receptor pathway and activation of caspases -3, -8, and -9. We therefore assessed the role of HOXA-AS2 on TRAIL induction in ATRA-treated NB4 cells. TRAIL protein (Fig. 6A) and mRNA (Fig. 6B) expression was markedly increased in HOXA-AS2 knockdown cells after 3 day of ATRA (1 μ M) treatment. These results indicate that HOXA-AS2 negatively regulates ATRA-induced TRAIL production. The elevation of caspase-8 and -9 activities in HOXA-AS2 knockdown cells occurred, at least in part, in response to the paracrine effects of TRAIL, as the addition of TRAIL-neutralizing antibody partially suppressed the levels of cleaved caspases-8 and -9 in ATRA-treated knockdown cells (Fig. 6C).

HOXA-AS2 is not a target of TRAIL

As shown in Fig. 1B, HOXA-AS2 expression was selectively increased in ATRA-sensitive NB4 cells but not in the TRAIL-resistant K562 cell line (Nimmanapalli et al., 2001), raising the possibility that ATRA-mediated induction of HOXA-AS2 itself may be secondary to a paracrine function of TRAIL. We therefore added recombinant human TRAIL to NB4 cell cultures and evaluated cleaved caspase-8 level and HOXA-AS2 expression. TRAIL exposure did not result in up-regulation of HOXA-AS2 RNA expression (Fig. 7A), indicating that its induction is not TRAIL-mediated. Exogenous TRAIL was active in these cells, as evident in the dose-dependent increases in cleaved caspase-8 (Fig. 7B), as in previous studies (Altucci et al., 2001; Sakoe et al., 2010).

Discussion

Myeloid cell development critically relies on control mechanisms that maintain the balance between proliferation and apoptosis, and defective regulation of apoptosis during

hematopoiesis may promote leukemogenesis or neutropenia. (de Thé and Chen, 2010;Grenda et al., 2007) In the current studies, we have shown that lincRNA HOXA-AS2 is expressed in both human peripheral blood PMN and in the human promyelocytic leukemia cell line NB4. In PMN, its expression shows rapid up-regulation by the inflammatory cytokine IFN- γ and a later response to TNF α , with a synergistic response to the combined cytokines. HOXA-AS2 is dramatically upregulated during ATRA-induced granulocytic differentiation of NB4 cells.

HOXA-AS2 knockdowns by shRNAs demonstrate that the lincRNA negatively regulates ATRA-induced NB4 cell apoptosis, with a mechanism that involves the key pro-apoptotic mediator, TRAIL. HOXA-AS2 appears to repress TRAIL mRNA and protein expression, as knockdown of HOXA-AS2 by transduced shRNAs result in increased TRAIL levels. Thus, HOXA-AS2-mediated regulation could contribute to the fine-tuning of apoptosis during induced myeloid differentiation. The anti-apoptotic effect of HOXA-AS2 reveals new layer of modulation of the tightly controlled cell death program that is required for the proper generation of mature myeloid cells. The lincRNA may also be involved in development of other tissues, as it is similarly up-regulated by ATRA in human embryonic stem cells (Colleoni et al., 2011;EMBL-EBI, 2012).

The precise events downstream of ATRA that result in HOXA-AS2 induction are not known. Up-regulation of HOXA-AS2 by ATRA may reflect either the engagement of regulatory networks following the formation of ATRA-PML-RAR α complexes or regulation by other early biochemical cellular events induced by ATRA. HOXA-AS2 may contribute to a graded response that optimizes TRAIL output, in concert with other known mediators of ATRA-induced TRAIL-mediated apoptosis. ATRA induces interferon regulatory factor (IRF)-1-dependent TRAIL expression and subsequently triggers apoptosis in leukemia cells through a paracrine action of TRAIL (Clarke et al., 2004;Altucci et al., 2001). ATRA-induced STAT1 and p48/IRF-9 play a key role in the interferon response and induce the synthesis and the secretion of interferons (Gianni et al., 2000;Matikainen et al., 1997). FOXO3A also plays an important role in ATRA-induced TRAIL-mediated apoptosis in NB4 cells (Sakoe et al., 2010). More studies are required to determine whether any of these pathways mediate TRAIL repression by HOXA-AS2.

HOXA-AS2 may also directly affect transcription of the TRAIL gene, distantly located at chromosome 3q26. The archetypical HOX region lincRNA, HOTAIR, regulates target genes in trans: HOTAIR generated from the HOXC locus on chromosome 12 represses transcription across 40-kb of the HOXD locus on chromosome 2, by recruitment of polycomb repressive complex 2 (Rinn et al., 2007).

Emerging evidence suggests that lincRNAs are an important component of tumor biology. Inappropriate expression of lincRNAs in HOX loci has been implicated in the development of both hematopoietic malignancies and solid tumors (Gibb et al., 2011;Prensner and Chinnaiyan, 2011). HOTAIR is upregulated in breast and hepatocellular carcinomas and on pancreatic cancer (Gibb et al., 2011;Kim et al., 2012). Overexpression of HOTAIR in breast cancer is an independent predictor of overall survival and the progression of free survival (Gupta et al., 2010). Moreover, HOXA genes particularly HOXA9 are broadly known to be

important for many malignancies, including acute myeloid leukemias (Thorsteinsdottir et al., 2001).

In conclusion, HOXA-AS2 functions as an apoptosis repressor in ATRA-treated NB4 cells, through mechanisms mediated in part by TRAIL. As targeted therapy of APL by ATRA and by arsenic compounds depends upon induction of apoptosis, further investigation of HOXA-AS2 in TRAIL-mediated apoptosis could provide insight into further therapeutic target for APL and possibly other myeloid malignancies. The lincRNA could also be important in the mediation of inflammatory disorders, where apoptosis plays a major role in the resolution of inflammation and prevention of tissue damage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Current grant sponsors: NIH R01DK054369 (PEN), Fundação de Amparo à Pesquisa do Estado de São Paulo (ACN).

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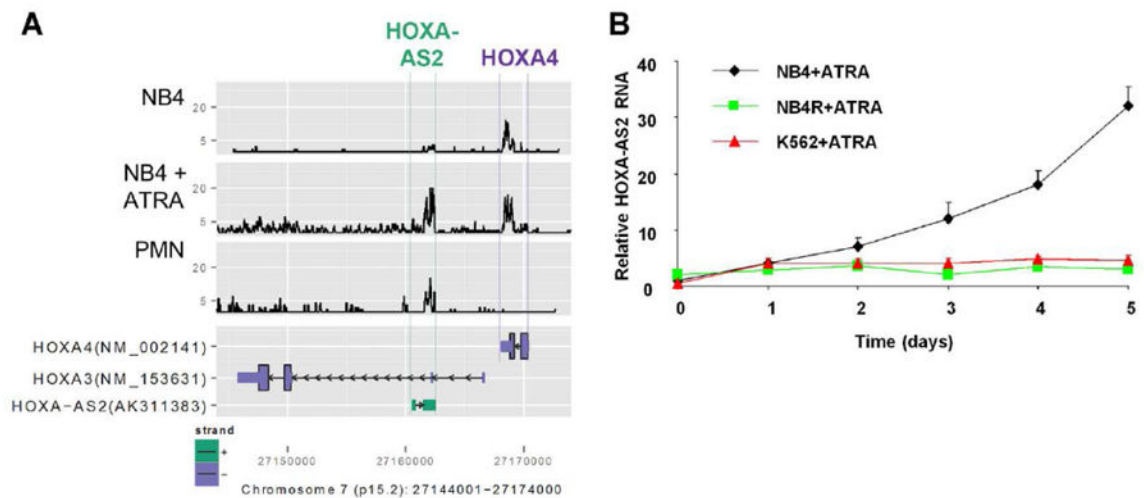


Figure 1.

ATRA induction of HOXA-AS2 expression in NB4 cells. (A) lower panel: UCSC Genome Browser map of expressed sequences in the region between HOXA3 and HOXA4 in the HOXA cluster on chromosome 7. Upper panel: RNA-Seq signal from this region in RNA from NB4 cells with and without ATRA induction (1 μ M for 5 days) and normal peripheral blood polymorphonuclear leukocytes (PMN). (B) HOXA-AS2 transcripts measured by qRT-PCR in NB4, NB4R2 (ATRA-resistant) or K562 cells were incubated with ATRA (1 μ M) for the indicated number of days. Point and error bars represent the mean \pm SD of four independent experiments.

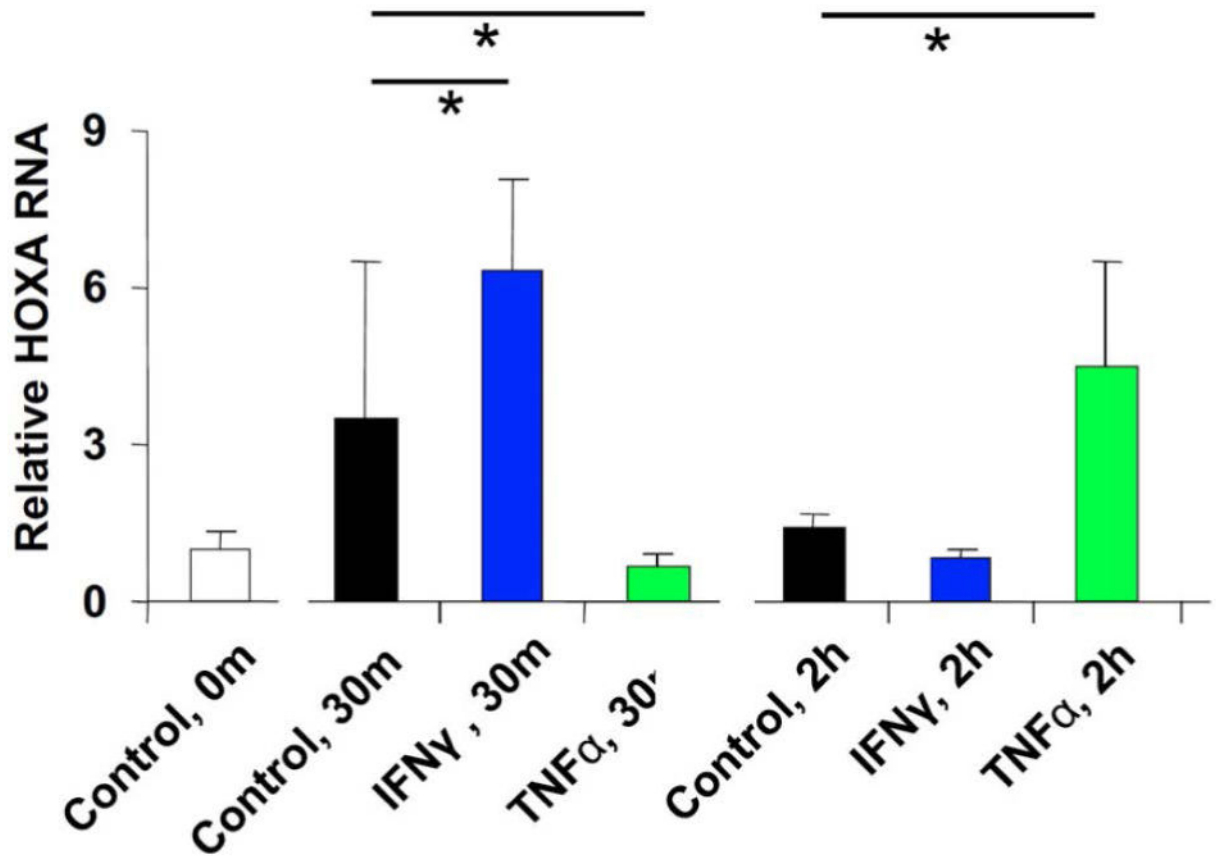


Figure 2.

Effect of IFN γ and TNF α on HOXA-AS2 gene expression in PMN. Relative expression of (A) HOXA-AS2 and (B) TRAIL in normal PMN incubated with or without IFN- γ (1000U/ml), TNF- α (1000U/ml), or IFN- γ plus TNF- α for 30 min or 2 hours.

Bars and error bars represent the mean \pm SD of three measurements in a representative experiment, of three independent experiments with PMN from two donors. Horizontal bars with asterisks indicate comparisons that reached statistical significance at $p < 0.05$.

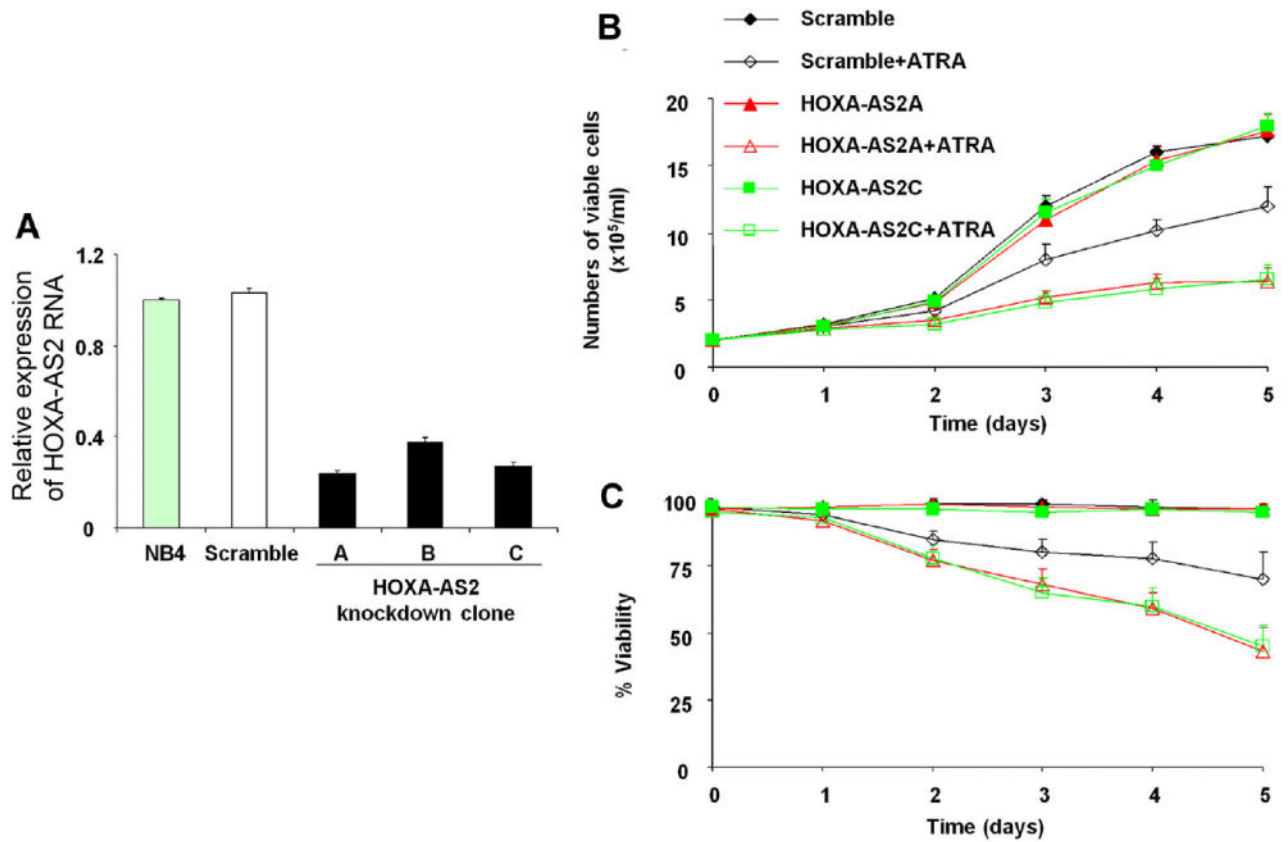


Figure 3.

Effect of HOXA-AS2 knockdown on NB4 cell proliferation. (A) HOXA-AS2 gene expression, measured by qRT-PCR, in NB4 cells transfected with three HOXA-AS2 shRNAs. (B,C) NB4 cells transfected with the indicated constructs, with or without ATRA induction, as represented by the colors and symbols in the insert, were seeded at 2×10^5 /mL and cell number (B) and viability (C) determined at the indicated numbers of days in culture. Points and error bars represent the mean \pm SD of three independent experiments.

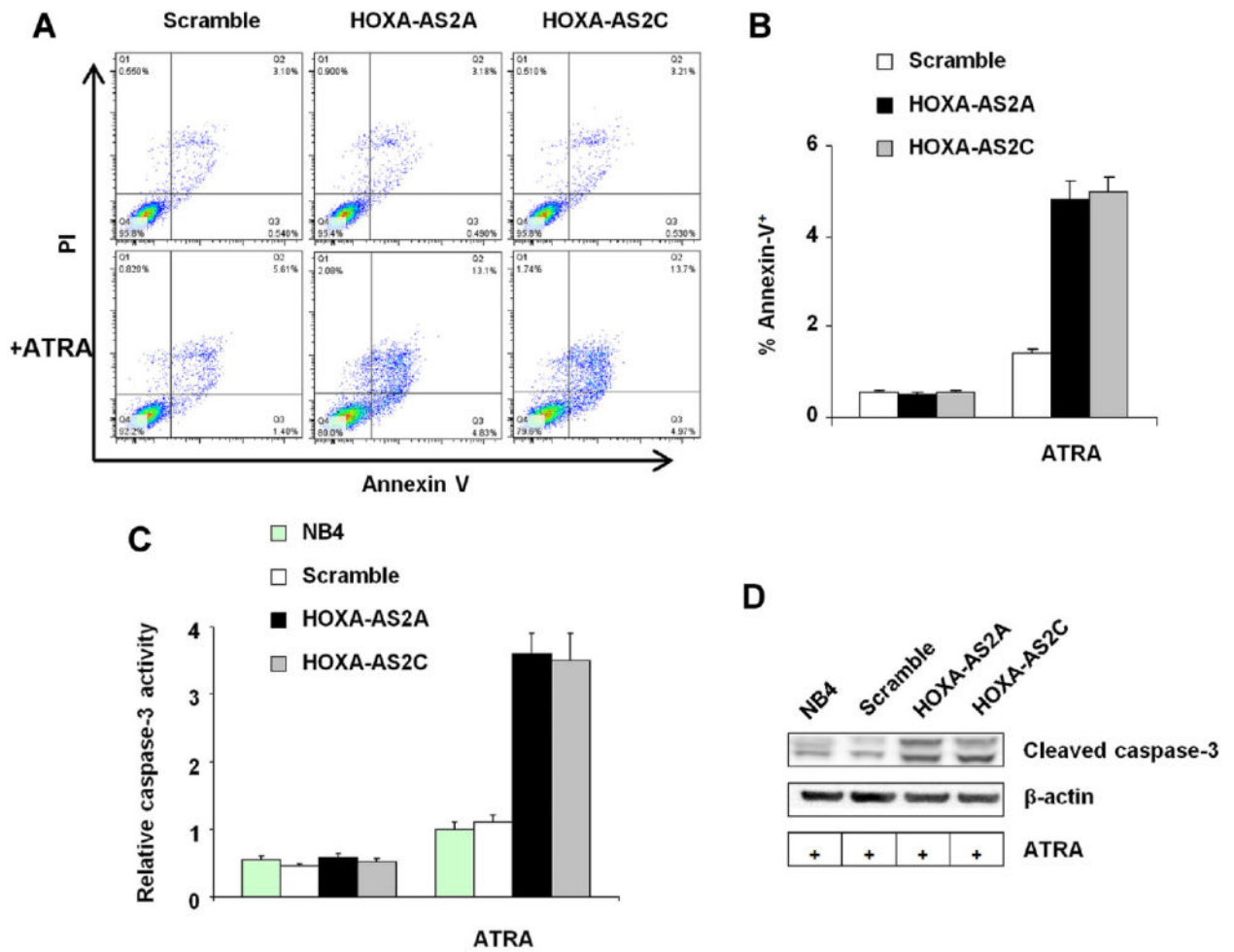


Figure 4.

Effect of HOXA-AS2 knockdown on NB4 cell apoptosis. Annexin V staining of NB4 cells transfected with scramble shRNA or the indicated HOXA-AS2 targeting shRNAs, incubated with or without ATRA (1 μ M for 3 days) as indicated. (A) Scatter plots; (B) Bar plot, with bars and error brackets representing mean \pm SD of four independent experiments; $p < 0.001$ for differences between scramble and knockdown clones A and C. Caspase-3 activity was measured (C) colorimetrically with substrate acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide, bars and error brackets representing mean \pm SD of four independent experiments, $p < 0.001$ for differences between scramble and knockdown clones A and C; and (D) by western blot detection of cleaved caspase-3 protein; the blot is representative of three experiments.

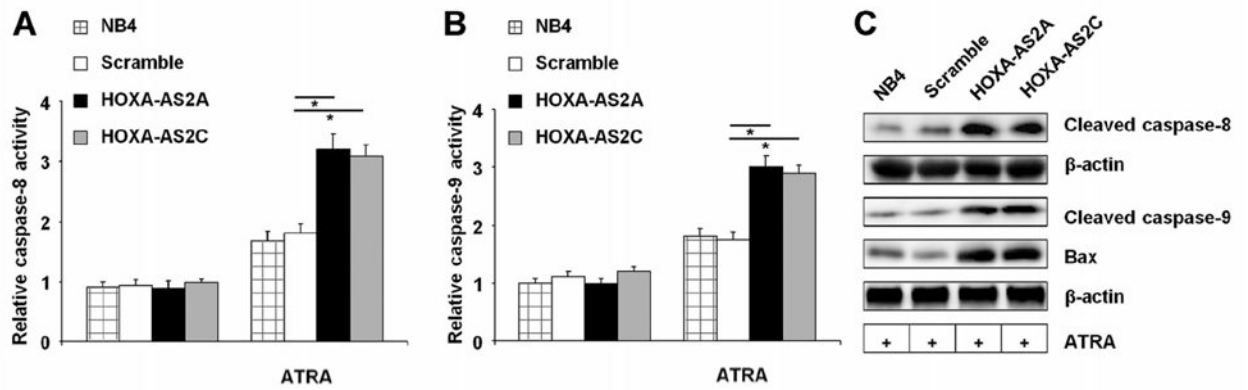


Figure 5.

Effect of HOXA-AS2 on ATRA-induced extrinsic apoptosis pathways. (A) Caspase-8 and (B) caspase-9 activity, measured respectively as acetyl-Ile-Glu-Thr-Asp-*p*-nitroanilide-cleaving and acetyl-Leu-Glu-His-Asp-*p*-nitroanilide-cleaving activity in NB4 cells with or without treatment with ATRA (1 μ M for 4 days). Bars and error brackets represent mean \pm SD of four independent experiments. Horizontal bars with asterisks indicate comparisons that reached statistical significance at $p < 0.05$. (C) Western blot of cleaved caspases-8 and -9, Bax, and β -actin in the indicated cell lysates; the blot is representative of three experiments.

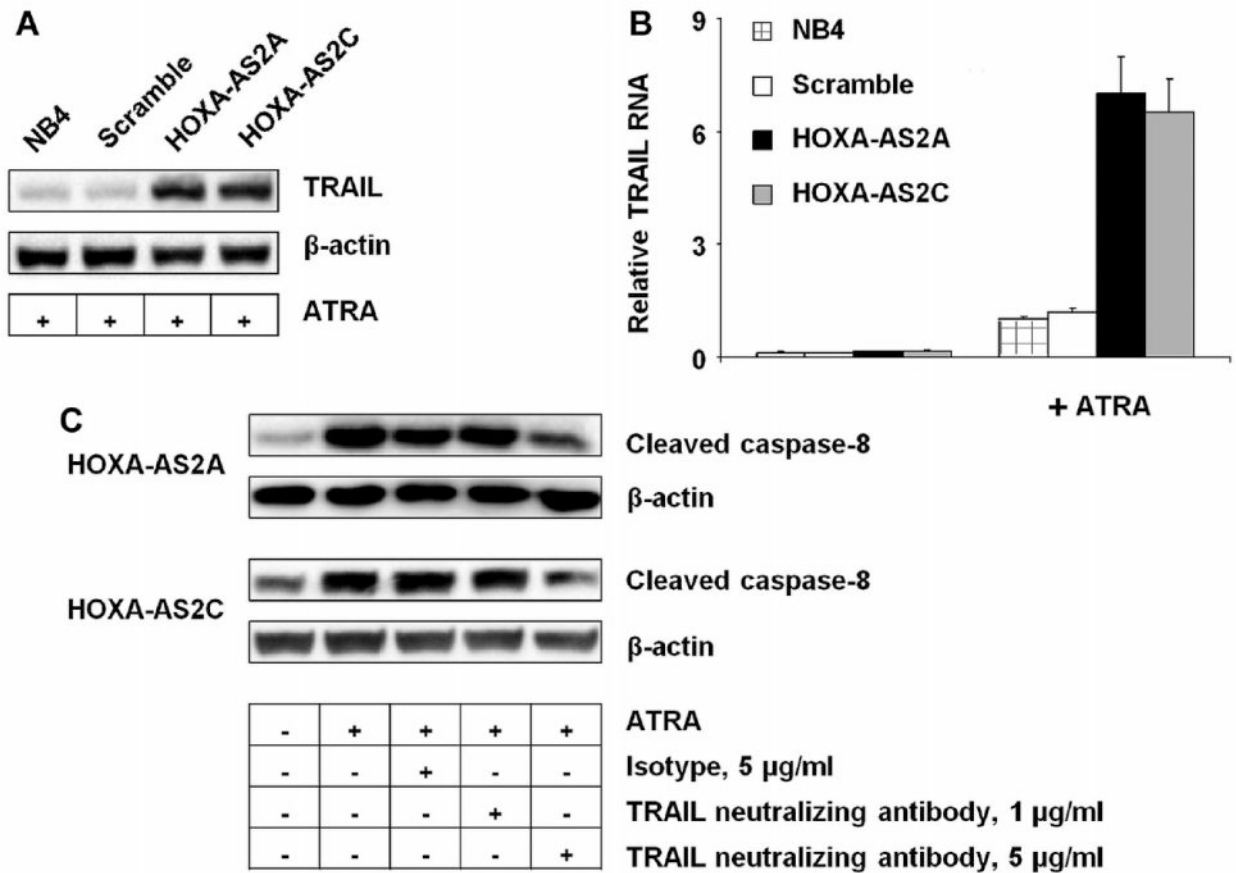


Figure 6.

Effect of HOXA-AS2 on TRAIL expression and function. NB4 cells transfected with the indicated knockdown constructs were cultured with or without ATRA (1 μ M for 3 days). (A) Western blot of TRAIL and β -actin in the indicated cell lysates; blot representative of three experiments. (B) TRAIL transcripts measured by qRT-PCR. Bars and error brackets represent mean \pm SD of three independent experiments; $p < 0.01$ for difference between scramble and knockdown clone A, and $p < 0.01$ for difference between scramble and knockdown clone C. (C) Western blot of cleaved caspases-8 and 9, and β -actin, in NB4 cell lysates with or without ATRA (1 μ M for 3 days) and with anti-TRAIL or isotype control antibodies as indicated; the blot is representative of three experiments.

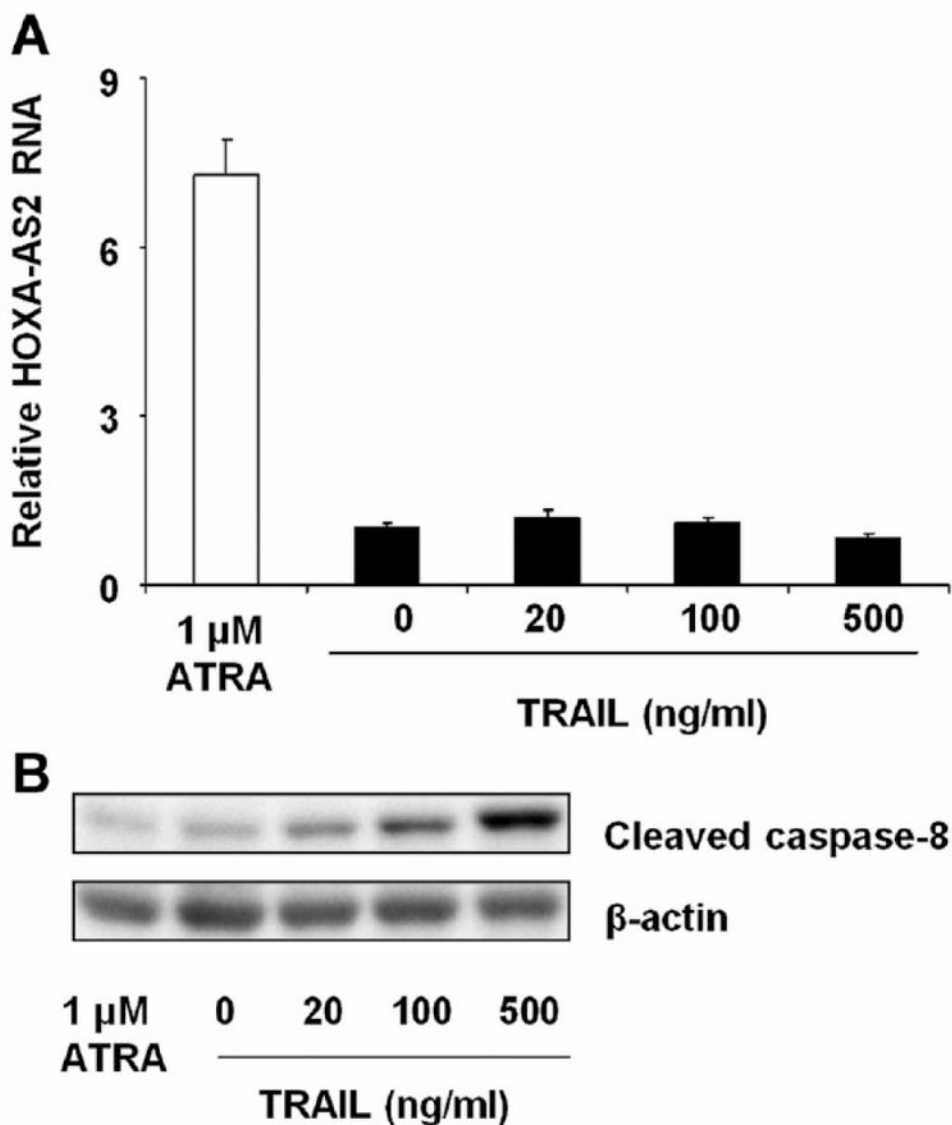


Figure 7.

Effect of TRAIL on HOXA-AS2 expression. NB4 cells were incubated with ATRA (1 μ M) or with the indicated concentrations of recombinant human TRAIL for 2 days. (A) HOXA-AS2 expression was analyzed by qRT-PCR. Bars and error brackets represent mean \pm SD of three independent experiments. (B) Western blot analysis of cleaved caspase-8 and β -actin protein; the blot is representative of three experiments.