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Knockdown of T-cell intracellular antigens triggers cell proliferation, invasion and tumor growth

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Synopsis

T-cell intracellular antigen (TIA) proteins function as DNA/RNA trans-acting regulators to expand transcriptome and proteome diversity in mammals. We hereby report that stable silencing of TIA1 and/or TIAR/TIAL1 expression in HeLa cells enhances cell proliferation, anchorage-dependent and -independent growth and invasion. HeLa cells lacking TIA1 and/or TIAR generate larger and faster-growing epithelial tumors with high rates of proliferation and angiogenesis in nude mice xenografts. Protein array analysis of a collection of human tumors shows that TIA1 and TIAR protein expression is downregulated in a subset of epithelial tumors relative to normal tissues. Our data suggest a link between the epigenetic control exerted by TIA proteins on transcriptional and post-transcriptional regulation of a subset of specific gene involved in tumor progression. Taken together, these results are consistent with a role for TIA proteins as growth/tumor suppressor genes.

Short title: Depletion of T-cell intracellular antigens accelerates oncogenesis.

Keywords: TIA1, TIAR, tumor progression, gene expression control.

Abbreviations used: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HE, hematoxylin and eosin staining; hnRNPs, heterogeneous ribonucleoproteins; HuR/ELAVL1, Hu antigen R/embryonic lethal, abnormal vision, *Drosophila*-like (ELAVL); MTT, methyl thiazolyl tetrazolium; MYC, v-myc myelocytomatosis viral oncogene homolog (avian); NF1, neurofibromatosis 1 gene; PTB/hnRNP I, polypyrimidine tract binding protein; RNAi, RNA interference; siRNA, small interfering RNA; shRNA, short hairpin RNA; TIA1, T-cell intracellular antigen 1; TIAR/TIAL1, TIA1 related/like protein; VEGF, vascular endothelial growth factor A; WT1, Wilm's tumor-suppressor gene.

Introduction

T-cell intracellular antigen 1 (TIA1) and TIA1 related/like (TIAR/TIAL1) proteins are DNA/RNA binding proteins involved in transcriptional and post-transcriptional regulation of eukaryotic gene expression [1]. These proteins are structurally similar to heterogeneous ribonucleoproteins (hnRNPs) and encompass three RNA recognition motifs and one C-terminal glutamine-rich domain [2,3]. TIA proteins have been shown to control nuclear and cytoplasmic regulatory events including gene transcription, alternative pre-mRNA splicing and turnover and translation of cellular mRNAs. In the nucleus, they modulate the rates of gene transcription by interacting with DNA [4,5] and RNA polymerase II [6]. TIA proteins also regulate alternative pre-mRNA splicing of approximately 15% of alternative cassette human exons, as well as 5' splice-site selection of Alu exons, through binding to U-rich intronic stretches and facilitating atypical 5' splice site recognition by U1 small nuclear ribonucleoprotein [7-12]. In the cytoplasm, TIA proteins regulate the stability and translation of some mRNAs through their binding to A-, U- and C-rich sequences located in 3' untranslated regions [13-19].

Consequently, TIA proteins have a pleiotropic role in the control of cell physiology. Indeed, it has been described a role for TIA proteins in the regulation of

apoptosis [20], cell death caused by viruses [21], viral replication [22], inflammation [23] and cell response to metabolic stress [24]. Furthermore, mice lacking either TIA1 or TIAR show higher rates of embryonic lethality [14,25].

In this report, we show that TIA proteins control several pathways involved in different aspects of tumorigenesis. Downregulation of TIA proteins expression results in increased cell proliferation, tumor growth and invasion. Furthermore, we show that these proteins are downregulated in a variety of tumors of epithelial origin. These results highlight the role of TIA proteins as tumor suppressors.

Experimental

Cell culture, transfection, RNA interference and Western blot analyses. Adherent HeLa cells were cultured under standard conditions [1,10]. The TIA1 and TIAR shRNA-pSUPER constructs were generated with the pSUPER RNA interference (RNAi) System (OligoEngine, USA) using specific sequences as described [1,10,26-28]. In fact, we had previously tested three different siRNAs against different regions of the genes for each of the two proteins (see Figure S1 in [10]), with similar silencing effects. Further, we had thereby generated mutant versions of the proteins resistant to the silencing siRNAs to exclude off-target RNAi effects (one mutant for one of the siRNAs for each protein as described in Figures 1D and S1C in [10]). These specific sequences against TIA1 and TIAR mRNAs are hereby used. Briefly, to knock-down TIA1 and TIAR, we have followed previously described methods (OligoEngine, USA) to generate TIA1 and TIAR short hairpin RNA (shRNA) pSUPER or empty pSUPER constructs from pSUPER.retro vectors. Sequences coding for short hairpin RNAs were inserted as double-stranded oligos followed by a short spacer, the reverse complement of the sense strand and five thymidines as an RNA polymerase III transcriptional stop signal into pSUPER vectors using the BglII and XhoI sites. The target sequences for the TIA1 and TIAR genes were 5'-AAGCTCTAATTCTGCAACTCT-3' and 5'-AACCATGGAATCAACAAGGAT-3', respectively, as described previously (see Figure S1 in [10]). To recapitulate the results of the silencing of TIA proteins on cell proliferation and growth, two new siRNAs against TIA1 (5'-AACAACTAATGCGTCAGACTTTT-3') and TIAR (5'-AAGTCCTTATACTTCAGTTGTTC-3') mRNAs were used (Figure S1). The configuration of the constructs was verified by DNA sequencing. The TIA1 and TIAR shRNA pSUPER constructs and the empty pSUPER vector as control were transfected into the cell line HeLa (1 µg of DNA/1×10⁶ cells). Stable cell lines expressing empty or TIA1 and/or TIAR shRNA-pSUPER constructs were selected by plating for 3 weeks according to the manufacturer's instructions (OligoEngine, USA). Clonal lines were isolated with cloning cylinders and verified by Western blotting.

Proliferation rates, cell-cycle analyses, anchorage-dependent and -independent growth and invasion assays. Measurement of cell proliferation by direct count of cell number for 4/6 days and methyl thiazolyl tetrazolium (MTT) cell proliferation assays were carried out as described previously [1]. Cell-cycle progression was analyzed by flow cytometry and propidium iodide staining, and colony formation was assessed on plastic and in soft agar as described previously [1]. In vitro invasiveness was evaluated using Matrigel-coated invasion chambers with an 8-µm pore size (BD Biosciences, USA). Briefly, 1×10⁴ cells per 100 µl were allowed to migrate for 24 h through the Matrigel-coated membranes using 20% fetal bovine serum as chemoattractant. Cells on the upper

surface of the membrane were scraped and the cells that reached the lower surface of the membrane were stained with 0.1% crystal violet, counted and photographed under a light microscope at 100x magnification.

Xenograft tumor development in nude mice, hematoxylin-eosin staining and immunohistochemistry. Subconfluent HeLa cells stably transfected with TIA1 and/or TIAR shRNAs or with empty vector ($1-2 \times 10^6$ cells per site in 200 μ l) were injected s.c. into each rear flank in female nude mice (Harlam Ibérica nu/nu, Spain). Tumor growth was monitored and mice were sacrificed. Eight mice were used per experimental condition. Formalin-fixed, paraffin-embedded tumor samples were stained with hematoxylin and eosin as described previously [29]. For immunohistochemical analyses, the tumor samples were incubated with anti-MIB1 (Master Diagnostica SL, Spain), dilution 1:80; anti-E-Cadherin (Master Diagnostica SL, Spain), dilution 1:20; anti-VEGF (Santa Cruz Biotechnology, USA), dilution 1:400; anti-TIA1, anti-TIAR and anti-HuR (Santa Cruz Biotechnology, USA), dilution 1:200, antibodies as described elsewhere [29]. Samples were visualized by light microscopy and photographed at 100x magnification. All experiments were performed with 8- to 12-wk-old female mice and conducted according to the guidelines of the Committee on Animal Experimentation of the Centro de Biología Molecular Severo Ochoa and Universidad Autónoma de Madrid.

Protein expression profiling using human tumor array. A protein array (BioChain, USA) consisting of 47 pairs of tumor samples and their respective normal tissues spotted as duplicates was probed with anti-TIA1, anti-TIAR, anti-HuR and anti-GAPDH (Santa Cruz Biotechnology, USA) and anti-PTB/hnRNP I (BB7, generous gift of Prof. C.W. Smith) antibodies. The blots were processed using the ECL luminescence technique (GE Healthcare, UK).

Results

Knockdown of TIA proteins increases cell proliferation, growth and invasion.

Knockdown of TIA1 and TIAR was performed using short hairpin RNA (shRNA) pSUPER constructs and verified by Western blot (see Experimental section and Figure 1A). Comparison of control clones (transfected with empty vector) with three independent stably transfected clones for each condition revealed 60-90% depletions of both TIA1 and TIAR proteins in single and double knockdown experiments (Figure 1A-C and Figure S1). α -Tubulin protein levels used as a control for shRNA specificity were not significantly affected. These results agree with previous TIA1/TIAR small interfering RNAs (siRNA) mediated depletion experiments in HeLa cells [1,10,26-28].

Transient depletion of TIA1 and/or TIAR proteins in HeLa cells increases cell proliferation and growth [1 and Figure S1]. To confirm these observations in stably transfected cell lines, we analyzed total cell number and metabolic activity. Reduced TIA1 and/or TIAR protein levels were correlated with proliferation in independent clones (Figure 1A-C). An increase in the proportion of cells in G2/M phases correlated with TIA1 and/or TIAR depletion (Figure 1D), thus supporting a role for these proteins in the control of cell proliferation. It is also noteworthy that depletion of TIA1 and TIAR significantly reduced the percentage of apoptotic cells (identified by the sub G1 peak) (Figure 1D). For subsequent experiments, the cell clones with TIA1 and/or TIAR stable knockdown referred in Figure 1 (panels A-D) as (1) were chosen.

Features of many transformed cells are their capacity to grow in an anchorage-dependent and -independent fashion and their invasive ability. To address the effect on invasiveness of TIA1 and/or TIAR depletion, the colony-forming capacity in both plastic and soft agar plates was performed. As shown in Figure 2, TIA1-, TIAR- and TIA1/R-depletion significantly increased the colony-forming capacity of the cells compared with the corresponding controls (Figure 2A and B). Invasiveness was also tested in the chemoinvasion assay, in which cells move through a reconstituted basement membrane of Matrigel coated on top of Transwell filters. The invasion potential of stable TIA1-, TIAR- and TIA1/R-depleted clones was significantly higher than that of control cells (Figure 2C). These results strongly support a role for TIA proteins in anchorage-dependent and -independent cell growth and invasion.

Depletion of TIA proteins in HeLa cells triggers tumor growth potential in vivo.

Tumor growth following TIA-protein knockdown was analyzed in nude mice xenografts. TIA1-, TIAR- or TIA1/R-depleted cells and corresponding controls were injected into the left and right hind leg, respectively, of female nude mice. Palpable tumors developed within 1-2 wk, at which time animals were euthanized and the tumors analyzed (Figure 3). Comparison with the corresponding control tumors revealed a clear increase in tumor size in TIAR (7 of 8) and TIA1 plus TIAR (8 of 8) clones, whereas tumor size was more heterogeneous for TIA1-depleted clone, showing only an evident increase in tumor size in 5 out of 8 mice (Figure 3A). Interestingly, depletion of both TIA1 and TIAR caused maximum tumor growth, thus suggesting certain redundancy in the functions of both genes in the control of cell homeostasis.

Knockdown persistence was verified by immunohistochemistry of the isolated tumors using antibodies against TIA1 (Figure 4A) and TIAR (Figure 4B) or by reverse-transcription polymerase chain reaction (data not shown). As expected, tumors from nude mice inoculated with TIA1-, TIAR- or TIA1/R-depleted HeLa cells had lower expression levels of the corresponding TIA1 or TIAR proteins compared to control tumors, which had focal and cytoplasmic expression of both proteins (Figure 4A and B; compare panels A with B and D). Formalin-fixed, paraffin-embedded tumors were stained with hematoxylin and eosin or analyzed by immunohistochemistry for MIB1 (to evaluate proliferation index), E-Cadherin (to test epithelial nature and cell adhesion), VEGF (to assess angiogenesis) or HuR (to evaluate another RNA-binding protein). The histological features of all the tumors pointed to poorly differentiated carcinomas. The epithelial cells of the tumors had large nucleoli and nuclei and displayed abundant events of mitosis (Figure 4C and Figure S2 and S3, panels A and B). Cells with reduced TIA1 and/or TIAR expression gave rise to large tumors with a higher number of mitoses and a greater degree of necrosis than the control group and only featured tumor cells around blood vessels. Furthermore, immunohistochemistry revealed that these tumor cells were highly proliferative, with higher MIB1 expression, undetectable E-Cadherin expression, and an extensive expression of VEGF, whereas control tumor cells had a lower proliferation index (MIB1), a lower expression of VEGF, and focally maintained E-Cadherin expression in the cell membrane (Figure 4C and Figure S2 and S3, panels C-H). The nuclear expression of HuR was similar in the tumors from the different experimental groups (Figure 4C and Figure S2 and S3, panels I and J). These findings suggest that the reduction in TIA1 and/or TIAR expression increases the *in vivo* tumorigenicity of HeLa cells.

Downregulation of TIA proteins in human cancers. To further assess the role of TIA proteins in tumorigenesis, we examined the expression of TIA proteins and other RNA-binding proteins in a panel of normal and tumor-derived tissues (Figure 5 and Figure S4). TIA1 and/or TIAR proteins were downregulated in many tumor types, including adrenal gland, lung, ovary, pancreas, parotid gland, skin, small intestine, stomach, thymus, thyroid gland and uterus for TIA1 and brain, breast, colon, duodenum, lung, lymphoma, ovary, pancreas, skin, thymus, thyroid gland, ureter and uterus for TIAR (Figure 5A and B and Figure S4). A subset of these tumors also showed variable expression of HuR and PTB, two closely related RNA-binding proteins, while the GAPDH metabolic protein showed characteristic expression levels according to the glycolytic potential of the different tumors. These results suggest that expression of TIA proteins can be specifically downregulated in several human cancers of epithelial origin.

Discussion

Association of TIA proteins with the repression of malignant cell phenotypes. Our results show that stable knockdown of TIA1 and/or TIAR in HeLa cells leads to increased cell proliferation, invasiveness and tumor growth. These clones produced larger and faster-growing tumors following injection in nude mice. In contrast, cells that stably overexpress TIA1 (10 of 10 clones) or TIAR (9 of 10 clones) are not viable and undergo apoptosis (data not shown and [2]). We also showed that a subset of human tumors had reduced expression of TIA1 and/or TIAR. Previous results showed that mutant mice lacking either TIA1 or TIAR have a hyperinflammatory phenotype due to overexpression of cytokines [13,14,23]. Epidemiological and experimental data suggest a close connection between inflammation and cancer [30,31]. Thus, many inflammatory mediators are important for growth and survival of pre-malignant cells and activate oncogenic transcription factors such as NF- κ B [32] or oncogenes such as Myc, which can also initiate the inflammatory response [30]. Interestingly, these oncogenic factors can be regulated by TIA proteins at the transcriptional and translational levels, respectively [1,33]. Tumor-associated inflammation can suppress anti-tumor immune responses and cause tumor-specific immune cells to become tumorigenic [30]. The inflammatory response can also stimulate tumor angiogenesis, invasion and metastasis [31]. Mice with a disruption in the *tiar* gene develop ovarian sex cord stromal tumors [25]. Furthermore, TIA proteins modulate alternative splicing of Wilm's tumor-suppressor gene WT1 [34] and the neurofibromatosis 1 gene NF1 [35], whose protein isoforms have been implicated in the development of childhood kidney cancer and fibrosarcoma, respectively. In the same vein, TIA1 expression in tumors correlates strongly with responsiveness to immunotherapy for melanoma patients [36]. Thus, downregulation of TIA proteins could play a role in oncogenesis by modulating genes involved in neoplastic and malignant transformation processes to evade the immune system and to enhance the growth and survival of cancer cells [37,38,39].

To our knowledge, TIA1 proteins have never been reported to be mutated in cancer. Human cancers are caused by both genetic and epigenetic alterations. Progressive acquisition of mutations in oncogenes (gain-of-function mutations) or tumor-suppressor genes (loss-of-function mutations) might act in concert with epigenetic events such as functional downregulation of TIA proteins to provide transformed cell clones with a competitive growth advantage. TIA proteins regulate the transcription, alternative splicing, stability and/or translation of many human genes with

potential roles in tumor development and progression (Figure S5 and references included). Thus, tumor suppressor role of TIA proteins could influence mechanisms such as the control of cell proliferation, apoptosis, angiogenesis, invasion and metastasis, and evasion of immune recognition (Figure S5). Further studies on the anti-oncogenic role of TIA-proteins are warranted.

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References

1. Reyes, R., Alcalde, J. and Izquierdo, J. M. (2009) Depletion of T-cell intracellular antigen (TIA)-proteins promotes cell proliferation. *Genome Biol.* **10**, R87
2. Tian, Q., Streuli, M., Saito, H., Schlossman, S. F. and Anderson, P. (1991) A polyadenylate binding protein localized to the granules of cytolytic lymphocytes induces DNA fragmentation in target cells. *Cell* **67**, 629-639
3. Dember, L. M., Kim, N. D., Liu, K. Q. and Anderson P. (1996) Individual RNA recognition motifs of TIA1 and TIAR have different RNA binding specificities. *J. Biol. Chem.* **271**, 2783-2788
4. Suswam, E. A., Li, Y. Y., Mahtani, H. and King, P. H. (2005) Novel DNA-binding properties of the RNA-binding protein TIAR. *Nucleic Acids Res.* **33**, 4507-4518
5. McAlinden, A., Liang, L., Mukudai, Y., Imamura, T. and Sandell, L. J. (2007) Nuclear protein TIA1 regulates COL2A1 alternative splicing and interacts with precursor mRNA and genomic DNA. *J. Biol. Chem.* **282**, 24444-24454
6. Das, R., Yu, J., Zhang, Z., Gygi, M. P., Krainer, A. R., Gygi, S. P. and Reed, R. (2007) SR proteins function in coupling RNAP II transcription to pre-mRNA splicing. *Mol. Cell* **26**, 867-881
7. Del Gatto-Konczak, F., Bourgeois, C. F., Le Guiner, C., Kister, L., Gesnel, M. C., Stevenin, J. and Breathnach, R. (2000) The RNA-binding protein TIA1 is a novel mammalian splicing regulator acting through intron sequences adjacent to a 5' splice site. *Mol. Cell. Biol.* **20**, 6287-6299
8. Förch, P., Puig, O., Kedersha, N., Martínez, C., Granneman, S., Séraphin, B., Anderson, P. and Valcárcel, J. (2000) The apoptosis-promoting factor TIA1 is a regulator of alternative pre-mRNA splicing. *Mol. Cell* **6**, 1089-1098
9. Förch, P., Puig, O., Martínez, C., Séraphin, B. and Valcárcel, J. (2002) The splicing regulator TIA1 interacts with U1-C to promote U1 snRNP recruitment to 5' splice sites. *EMBO J.* **21**, 6882-6892
10. Izquierdo, J. M., Majós, N., Bonnal, S., Martínez, C., Castelo, R., Guigó, R., Bilbao, D. and Valcárcel, J. (2005) Regulation of Fas alternative splicing by antagonistic effects of TIA1 and PTB on exon definition. *Mol. Cell* **19**, 475-484
11. Aznarez, I., Barash, Y., Shai, O., He, D., Zielenski, J., Tsui, L. C., Parkinson, J., Frey, B. J., Rommens, J. M. and Blencowe, B. J. (2008) A systematic analysis of

- intronic sequences downstream of 5' splice sites reveals a widespread role for U-rich motifs and TIA1/TIAL1 proteins alternative splicing regulation. *Genome Res.* **18**, 1247-1258
12. Gal-Mark, N., Schwartz, S., Ram, O., Eyras, E. and Ast, G. (2009) The pivotal roles of TIA proteins in 5' splice-site selection of Alu exons and across evolution. *PLoS Genet.* **5**(11), e1000717.doi:10.1371/journal.pgen.1000717
 13. Gueydan, C., Droogmans, L., Chalon, P., Huez, G., Caput, D. and Kruys, V. (1999) Identification of TIAR as a protein binding to the translational regulatory AU-rich element of tumor necrosis factor alpha mRNA. *J. Biol. Chem.* **274**, 2322-2326
 14. Piecyk, M., Wax, S., Beck, A. R., Kedersha, N., Gupta, M., Maritim, B., Chen, S., Gueydan, C., Kruys, V., Streuli, M. and Anderson, P. (2009) TIA1 is a translational silencer that selectively regulates the expression of TNF-alpha. *EMBO J.* **19**, 4154-4163
 15. López de Silanes, I., Galbán, S., Martindale, J. L., Yang, X., Mazan-Mamczarz, K., Indig, F. E., Falco, G., Zhan, M. and Gorospe, M. (2005) Identification and functional outcome of mRNAs associated with RNA-binding protein TIA1. *Mol. Cell. Biol.* **25**, 9520-9531
 16. Mazan-Mamczarz, K., Lal, A., Martindale, J. L., Kawai, T. and Gorospe, M. (2006) Translational repression by RNA-binding protein TIAR. *Mol. Cell. Biol.* **26**, 2716-2727
 17. Yamasaki, S., Stoecklin, G., Kedersha, N., Simarro, M. and Anderson, P. (2007) T-cell intracellular antigen-1 (TIA1)-induced translational silencing promotes the decay of selected mRNAs. *J. Biol. Chem.* **282**, 30070-30077
 18. Pullmann, R. Jr., Kim, H. H., Abdelmohsen, K., Lal, A., Martindale, J. L., Yang, X. and Gorospe, M. (2007) Analysis of turnover and translation regulatory RNA-binding protein expression through binding to cognate mRNAs. *Mol. Cell. Biol.* **27**, 6265-6278
 19. Kim, H. S., Kuwano, Y., Zhan, M., Pullmann, R. Jr., Mazan-Mamczarz, K., Li, H., Kedersha, N., Anderson, P., Wilce, M. C., Gorospe, M. and Wilce, J. A. (2007) Elucidation of a C-rich signature motif in target mRNAs of RNA-binding protein TIAR. *Mol. Cell. Biol.* **27**, 6806-6817
 20. Kawakami, A., Tian, Q., Duan, X., Streuli, M., Schlossman, S. F. and Anderson, P. (1992) Identification and functional characterization of a TIA1-related nucleolysin. *Proc. Natl. Acad. Sci.* **89**, 8681-8685
 21. Iseni, F., Garcin, D., Nishio, M., Kedersha, N., Anderson, P. and Kolakofsky, D. (2002) Sendai virus trailer RNA binds TIAR, a cellular protein involved in virus-induced apoptosis. *EMBO J.* **21**, 5141-5150
 22. Li, W., Li, Y., Kedersha, N., Anderson, P., Emara, M., Swiderek, K. M., Moreno, G. T. and Brinton, M. A. (2002) Cell proteins TIA1 and TIAR interact with the 3' stem-loop of the West Nile virus complementary minus-strand RNA and facilitate virus replication. *J. Virol.* **76**, 11989-20000
 23. Phillips, K., Kedersha, N., Shen, L., Blackshear, P. J. and Anderson, P. (2004) Arthritis suppressor genes TIA1 and TTP dampen the expression of tumor necrosis factor alpha, cyclooxygenase 2 and inflammatory arthritis. *Proc. Natl. Acad. Sci.* **101**, 2011-2016
 24. Kedersha, N. and Anderson, P. (2002) Stress granules: sites of mRNA triage that regulate mRNA stability and translatability. *Biochem. Soc. Trans.* **30**, 963-969
 25. Beck, A. R., Miller, J. J., Anderson, P. and Streuli, M. (1998) RNA-binding protein TIAR is essential for primordial germ cell development. *Proc. Natl. Acad. Sci.* **95**, 2331-2336

26. Izquierdo, J. M. and Valcárcel, J. (2007) Two isoforms of the T-cell intracellular antigen 1 (TIA1) splicing factors display distinct splicing regulation activities. Control of TIA1 isoform ratio by TIA1 related protein. *J. Biol. Chem.* **282**, 19410-19417
27. Izquierdo, J. M. (2008) Hu antigen R (HuR) functions as an alternative pre-mRNA splicing regulator of Fas apoptosis-promoting receptor on exon definition. *J. Biol. Chem.* **283**, 19077-19084
28. Izquierdo, J. M. (2008) Fas splicing regulation during early apoptosis is linked to caspase-mediated cleavage of U2AF65. *Mol. Biol. Cell.* **19**, 3299-3307
29. López-Malpartida, A. V., Ludeña, M. D., Varela, G. and García-Pichel, J. (2009) Differential ErbB receptor expression and intracellular signalling activity in lung adenocarcinomas and squamous cell carcinomas. *Lung Cancer* **65**, 25-33
30. Mantovani, A., Allavena, P., Sica, A. and Balkwill, F. (2008) Cancer-related inflammation. *Nature* **454**, 436-444
31. Grivennikov, S. I. and Karin, M. (2010) Inflammation and oncogenesis: a vicious connection. *Curr. Opin. Genet. Dev.* **20**, 65-71
32. Karin, M. (2006) Nuclear factor-kappaB in cancer development and progression. *Nature* **441**, 431-436
33. Liao, B., Hu, Y. and Brewer, G. (2007) Competitive binding of AUF1 and TIAR to MYC mRNA controls its translation. *Nat. Struct. Mol. Biol.* **14**, 511-518
34. Yang, C. and Romaniuk, P. J. (2008) The ratio of +/-KTS splice variants of the Wilms' tumour suppressor protein WT1 mRNA is determined by an intronic enhancer. *Biochem. Cell. Biol.* **86**, 312-321
35. Zhu, H., Hinman, M. N., Hasman, R. A., Mehta, P. and Lou, H. (2008) Regulation of neuron-specific alternative splicing of neurofibromatosis type 1 pre-mRNA. *Mol. Cell. Biol.* **28**, 1240-1251
36. Wang, E., Miller, L. D., Ohnmacht, G. A., Mocellin, S., Perez-Diez, A., Petersen, D., Zhao, Y., Simon, R., Powell, J.I., Asaki, E., Alexander, H. R., Duray, P. H., Herlyn M., Restifo, N. P., Liu, E. T., Rosenberg, S. A. and Marincola, F. M. (2002) Prospective molecular profiling of melanoma metastases suggests classifiers of immune responsiveness. *Cancer Res.* **62**, 3581-3586
37. David, C. J. and Manley, J. L. (2010) Alternative pre-mRNA splicing regulation in cancer: pathways and programs unhinged. *Genes Dev* **24**, 2343-2364
38. Izquierdo, J.M. (2010) Cell-specific regulation of Fas exon 6 splicing mediated by Hu antigen R. *Biochem. Biophys. Res. Commun.* **402**, 324-328
39. Izquierdo, J.M. (2010) Heterogeneous ribonucleoprotein C displays a repressor activity mediated by T-cell intracellular antigen-1-related/like protein to modulate Fas exon 6 splicing through a mechanism involving Hu antigen R. *Nucleic Acids Res.* **38**, 8001-8014

Figure legends

Figure 1. Knockdown of TIA proteins activates cell proliferation. (A-C, left panels) Western blot analyses of total HeLa cell extracts (5 µg (1/5) or 25 µg, lanes 1 and 2-6, respectively) prepared from cells stably transfected with shRNAs against control (panels A-C, lanes 1-3), TIA1 (panel A, lanes 4-6), TIAR (panel B, lanes 4-6) or TIA1 plus TIAR (panel C, lanes 4-6). The blots were probed with specific antibodies against

TIA1, TIAR and α -Tubulin proteins, as indicated. Molecular weight markers and the identities of protein bands are shown (**A-C, middle panels**) HeLa cells transfected with shRNAs against control (diamond and square), TIA1 (panel A, circle, triangle and blade), TIAR (panel B, circle, triangle and blade) or TIA1 plus TIAR (panel C, circle, triangle and blade) were seeded in 6-well plates and the total number was counted daily for 6 days. Each time point represents the mean \pm SEM of duplicate experiments (n=2 per experiment; * P <0.001; ** P <0.01; *** P <0.05). (**A-C, right panels**) Cells grown for 6 days were monitored by MTT assay. The represented values were normalized and expressed relative to control (identified as C1), which was assigned an arbitrarily fixed value of 100, and are shown as means \pm SEM of duplicate experiments (n=2 per experiment; * P <0.001; ** P <0.01). (**D**) Flow cytometry after propidium iodide staining. The data are means \pm SEM of duplicate experiments (n=2 per experiment; * P < 0.01; ** P <0.05). (**A-D**) For subsequent experiments, the cell clones with TIA1 and/or TIAR stable knockdown corresponding to the category identified as (1) were chosen.

Figure 2. Depletion of TIA proteins triggers anchorage-dependent and -independent growth and invasion. (**A**) Representative micrographs of colonies generated from stably transfected HeLa cells. One week after seeding 2,000 cells in a 6-well plate, the cells were fixed in paraformaldehyde (5%), stained with crystal violet (0.01%) and quantified by light microscopy. Values are shown as means \pm SEM from two independent experiments performed in duplicate for at least 15 different counted fields (* P <0.001). (**B**) Soft-agar colony formation. In each 6-well plate, 5,000 transfected HeLa cells were seeded into soft-agar matrix and incubated for 14 days. The cell number was counted under a light microscope after staining with crystal violet. Values are shown as means \pm SEM for two independent experiments performed in duplicate for at least 15 counted fields (* P <0.001). (**C**) Invasion assays. Cells (5×10^4 per well) were seeded onto Transwell filters coated with Matrigel on the upper surface (BD Biosciences, USA). After 24 h, cell invasion through the Matrigel was quantified by crystal violet staining. The values are means \pm SEM for three independent experiments performed in duplicate for at least 11-17 counted fields (* P <0.001). Scale bars represent 100 μ m.

Figure 3. Silencing of TIA proteins supports tumor cell growth in vivo. (**A**) Cells (2×10^6) were injected s.c. into the hind legs of female nude mice. In all cases, pairs of control and TIA-protein-depleted cells were injected. Eight mice were used for each cell pair. Animals were sacrificed 2 wk after injection of the cells. (**B**) Representative photographs are shown for tumors arising in the same mice. (**C**) Progression of tumor size after inoculation. Tumor size is shown as the mean \pm SEM (n=5-8) volume in mm³ (* P <0.01; ** P <0.05).

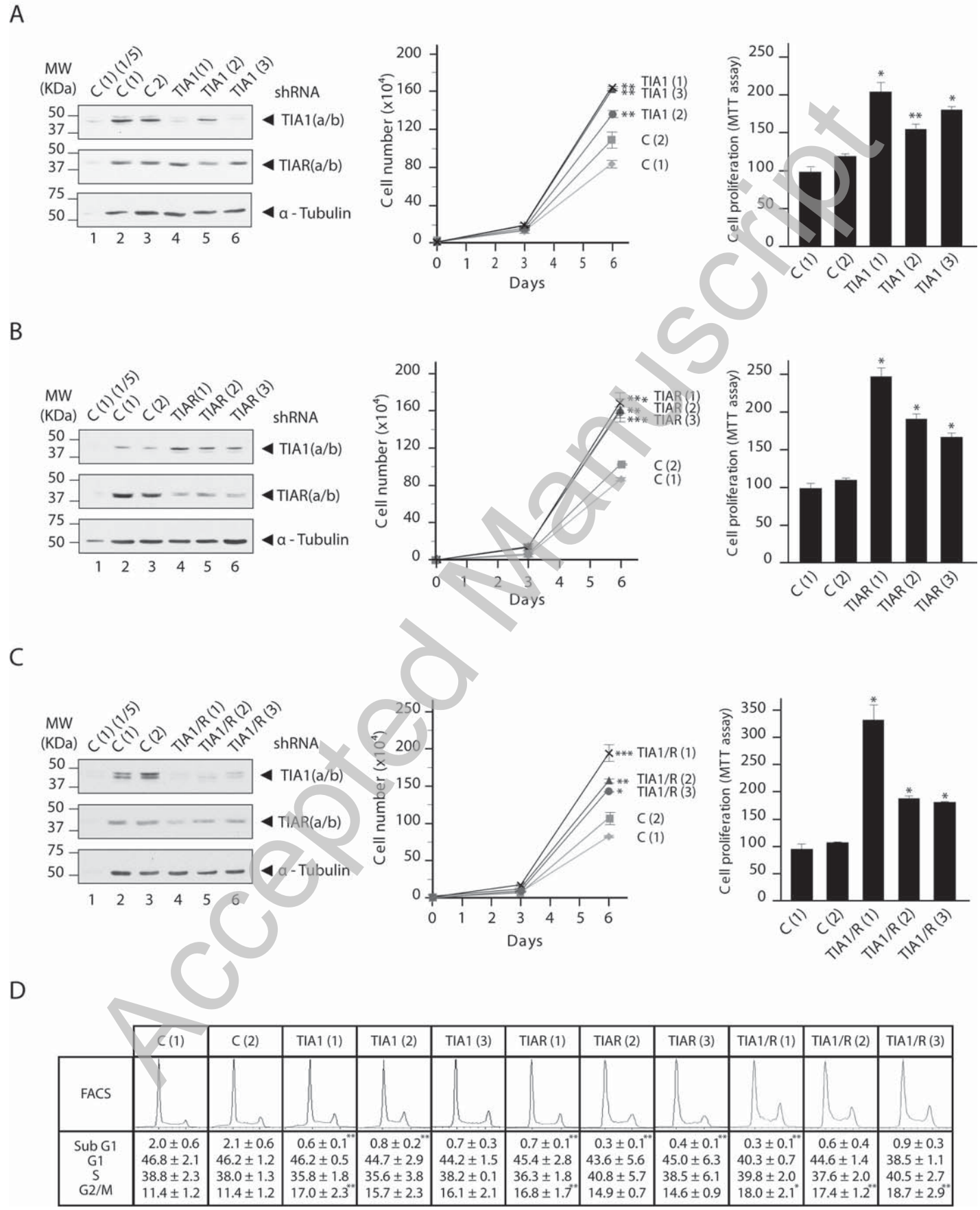
Figure 4. Immunohistochemical characterization of xenograft tumors derived from TIA1- and/or TIAR-depleted HeLa cells. (**A** and **B**) Light micrographs of formalin-fixed, paraffin-embedded tissue sections of tumors derived from HeLa cells following knockdown of TIA1 and/or TIAR and their corresponding controls. Sections were immunostained with anti-TIA1 (**A**) or anti-TIAR (**B**) antibodies. (**C**) Light micrographs of sections of tumors derived from control HeLa cells (panels A, C, E, G and I) and HeLa cells following knockdown of TIA proteins (panels B, D, F, H, and J). Sections were stained with hematoxylin and eosin (HE, panels A and B) or immunostained with anti-MIB1 (panels C and D), anti-E-Cadherin (panels E and F), anti-VEGF (panels G

and H) and anti-HuR (panels I and J) antibodies. Representative photomicrographs are shown. Scale bar represents 100 μm .

Figure 5. Downregulation of TIA proteins in human tumors. (A and B) A protein microarray (BioChain) consisting of 47 pairs of human tumor samples (T) and their respective normal tissues (N), spotted as duplicates, was probed with antibodies against TIA1, TIAR, HuR, PTB and GAPDH proteins. Tumor samples in which TIA1 (A) and TIAR (B) proteins are downregulated are shown with their corresponding controls.

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Figure 1



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Figure 2

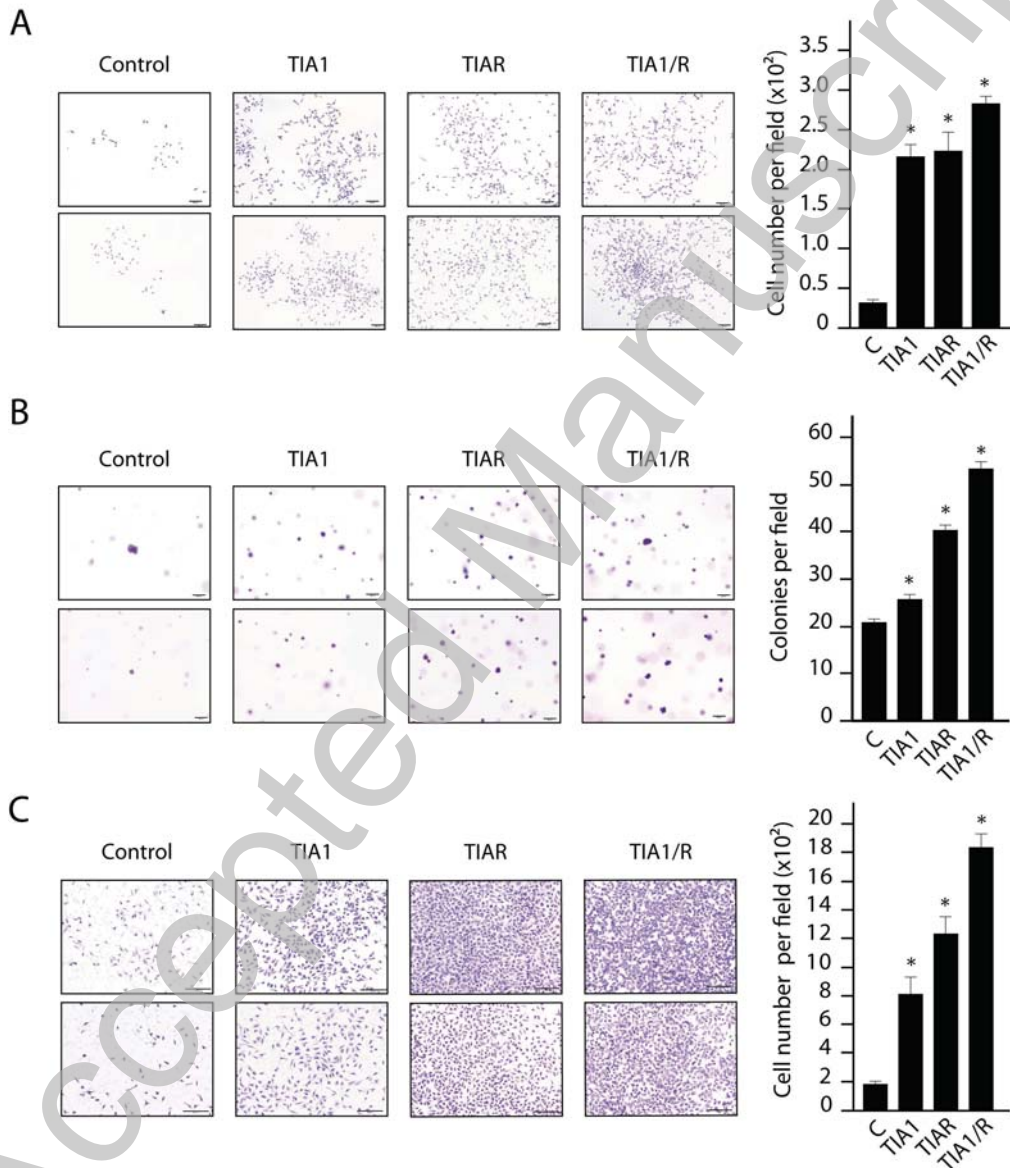
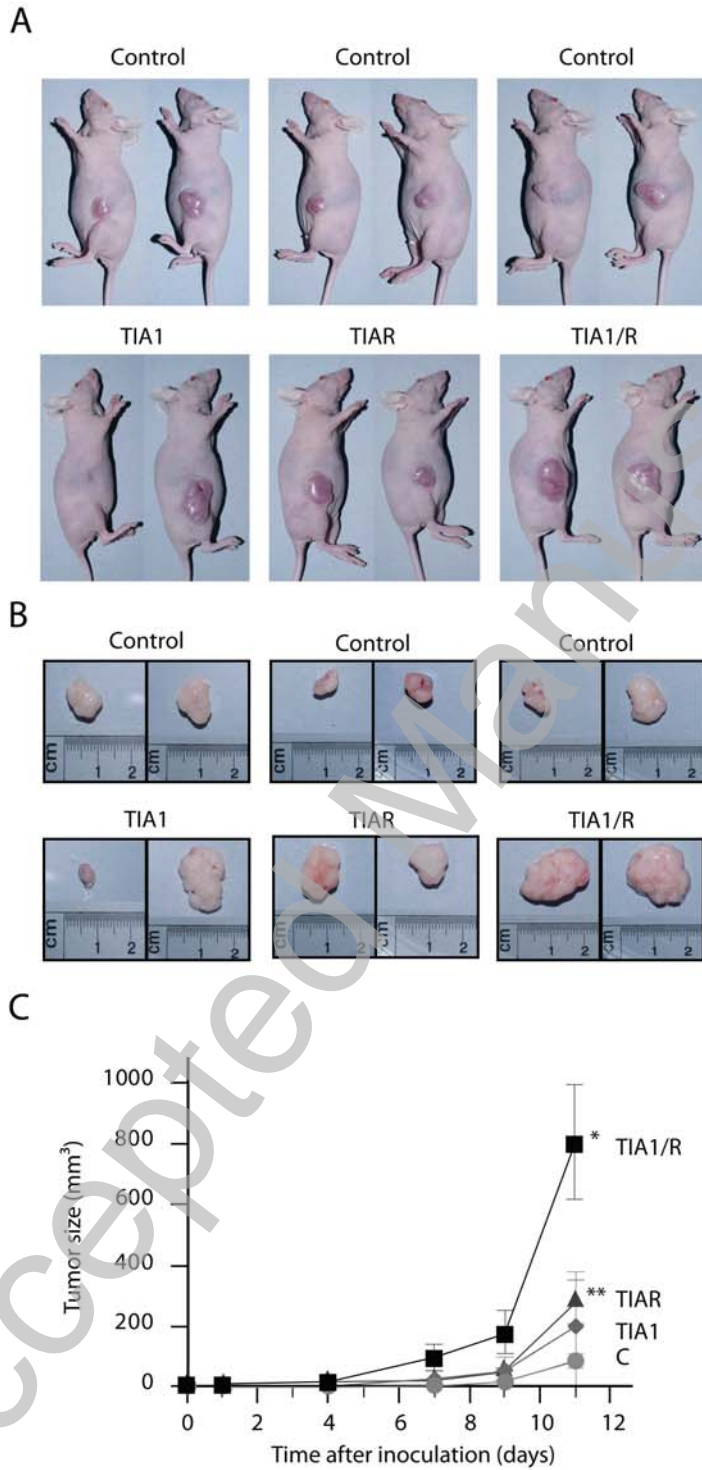
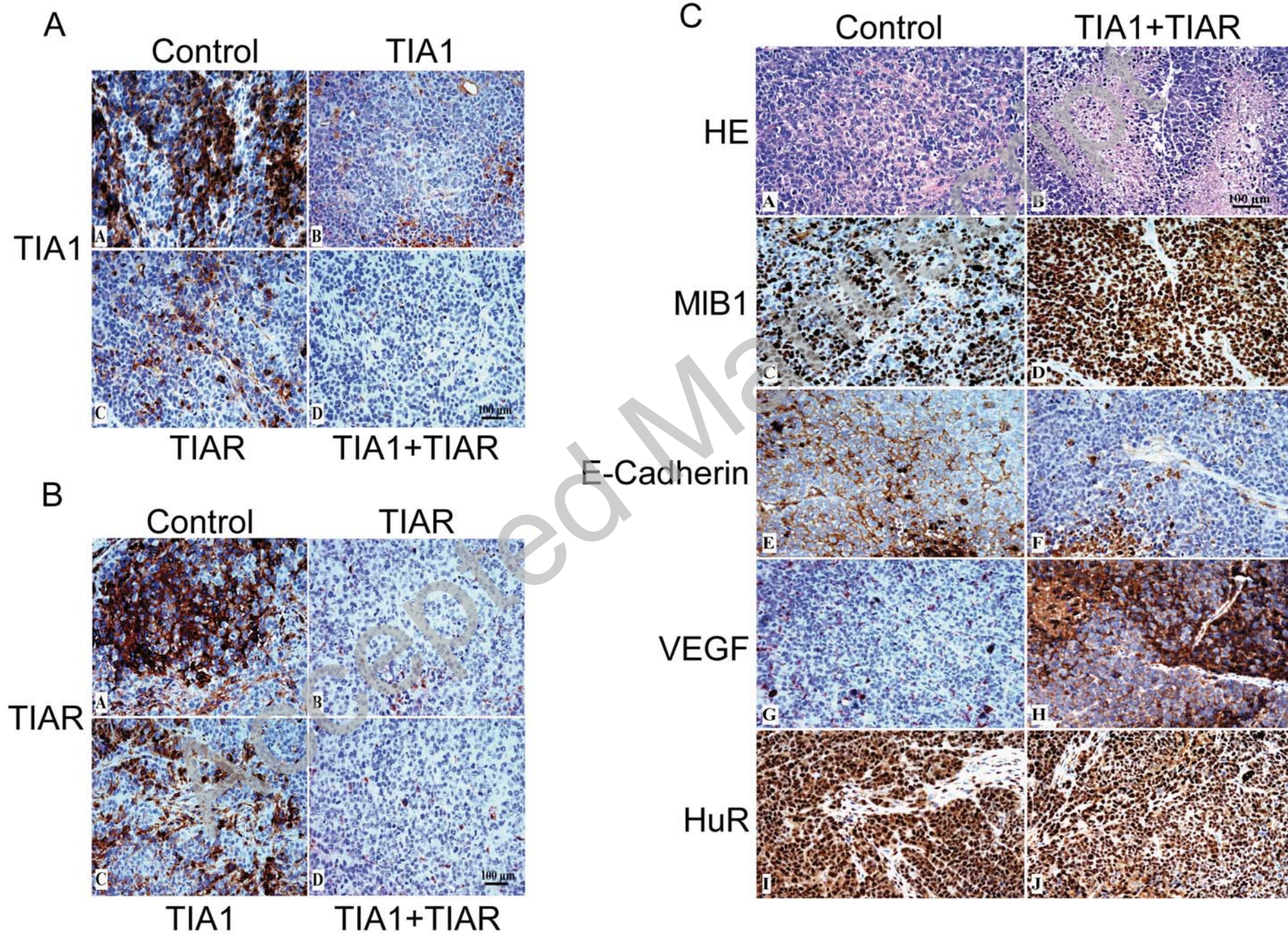


Figure 3



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Figure 4



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Figure 5

