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PLAG1 Fusion Oncogenes in Lipoblastoma[1]

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ABSTRACT

Lipoblastomas are pediatric neoplasms resulting from transformation of adipocytes. These benign tumors are typically composed of adipose cells in different stages of maturation within a variably myxoid matrix, and they contain clonal rearrangements of chromosome band 8q12. Because lipoblastomas resemble embryonic adipose tissue, characterization of their transforming mechanisms might reveal biological pathways in physiological adipogenesis. Herein, we demonstrate that lipoblastoma chromosome 8q12 rearrangements bring about promoter-swapping events in the *PLAG1* oncogene. We show that the hyaluronic acid synthase 2 (*HAS2*) or collagen 1 α 2 (*COL1A2*) gene promoter regions are fused to the entire *PLAG1* coding sequence in each of four lipoblastomas. *PLAG1* is a developmentally regulated zinc finger gene whose tumorigenic function has been shown previously only in epithelial salivary gland cells. Our findings reveal that *PLAG1* activation, presumably resulting from transcriptional up-regulation, is a central oncogenic event in lipoblastoma.

INTRODUCTION

Lipoblastomas are benign adipose tumors that are circumscribed (lipoblastoma) or diffuse and infiltrative (lipoblastomatosis). They are usually diagnosed in children under the age of 5 years and are thought to arise from lipoblastic cells resembling those in the embryonic and fetal stages of development (1–3). However, unlike the benign lipomas of adulthood, they can grow rapidly and may recur after resection. Most lipoblastomas arise in the soft tissues of the extremities and are more prevalent in males (1–3). They are distinctive histologically and are classically composed of lobulated tissue containing a variable number of lipoblasts and stellate mesenchymal cells as well as immature and mature adipocytes. Other typical findings include a plexiform capillary network and a prominent myxoid matrix consisting primarily of mucopolysaccharides (1). Although the pathogenesis of lipoblastoma is uncertain, it is believed to be more closely related to developing white fat than brown fat (4–7). Given the resemblance of lipoblastoma cells to those in embryological and fetal fat, it has been suggested that the biological pathways responsible for lipoblastoma proliferation might be related to those involved in adipose tissue development (8). Hence, characterization of oncogenic mechanisms in lipoblastoma might shed light on biological pathways that regulate proliferation and maturation of developing adipose tissue.

All human lipoblastomas reported to date contain clonal chromosomal rearrangements involving the 8q11–13 region (9–15). In contrast, 8q11–13 rearrangements are uncommon in other types of adipose tumors, including lipoma and liposarcoma. The lipoblastoma 8q11–13 rearrangements are generally balanced, involving no net loss or gain of 8q material as judged at the cytogenetic level of resolution. The balanced nature of these rearrangements is most consistent with an oncogenic activating mechanism in which one or more genes in the 8q11–13 region are rearranged and/or transcriptionally up-regulated. Notably, there appear to be varied mechanisms by which the putative lipoblastoma 8q oncogene is activated. Reported lipoblastoma 8q rearrangements include fusions with the telomeric aspect of the same chromosome arm (10) and translocations with a number of different partner chromosomes (9, 11–15). Therefore, the cytogenetic evidence implicates an 8q11–13 oncogene in the neoplastic transformation of immature fat cells. However, the nature of that gene and of the various 8q11–13 partner genes is unknown.

To characterize molecular mechanisms of adipose cell transformation, we mapped and cloned the oncogenes associated with lipoblastoma chromosome 8q rearrangements. Structurally similar *PLAG1* fusion genes were identified in each of four lipoblastomas. These studies reveal a major oncogenic mechanism in lipoblastoma and suggest biological pathways that might be important in adipose tissue development.

MATERIALS AND METHODS

YAC[3] and BAC Clone Identification and DNA Isolation. YAC clones were obtained from Research Genetics (Huntsville, AL) or CEPH (Paris, France). YAC DNA was isolated as described previously (16), except that YAC cultures were grown in bulk and harvested directly. BAC clones were isolated from the Research Genetics CITB Human BAC DNA Pools Release IV using primers PLAG 1650-E5-F (CCCTAGATGATGGTGCAGGAGACCTC) and PLAG 1873-E5-R (CTGAAGATCCTGTGTTTGTGTGG). BAC DNA was isolated using an established protocol (4), with minor modifications. Bacterial cell pellets were frozen at −80°C, thawed, and then digested with 25 mM Tris-HCl (pH 8.0), 50 mM glucose, 10 mM EDTA, 2.5–5.0 mg/ml lysozyme, and 100–200 μg/ml RNase. DNA was precipitated by the addition of 0.1 volume of 3 M NaOAc and 2 volumes of 95% ethanol. The presence of PLAG1 in BAC227K20 was confirmed by PCR amplification using primers PLAG 17-E1-F (CAATGGCTGCTGGAAAGAGG) and PLAG 133-E1-R (CCCGTCCGCCG CCTCTACACC).

FISH Mapping. Metaphase harvest and slide preparation were performed as described previously (17), with minor modifications. Metaphase cell preparations were applied to glass slides, which were then stored at room temperature for up to 2 weeks and pretreated by incubation in 2× SSC for 1 h at 37°C before hybridization. YAC and BAC clones were biotin or digoxigenin labeled by random octamer priming using the BioPrime DNA Labeling System (Life Technologies, Inc., Rockville, MD). The labeled products were purified by S-200HR spin column chromatography (Pharmacia, Uppsala, Sweden), coprecipitated with 5× Cot-1 DNA and 1 μg of herring sperm DNA, and then resuspended in hybridization buffer (50% formamide, 10% dextran sulfate, and 2× SSC). Dual-color FISH with BAC, YAC, and centromeric probes was performed as described previously (16), except that washing steps were performed using PBS-T (PBS with 0.1% Tween 20). Probe detection was performed in a stepwise manner as follows: slides were incubated with FITC antidigoxigenin and Texas Red-streptavidin, then incubated with anti-FITC rabbit IgG, then incubated with biotinylated antistreptavidin, and, finally, incubated with FITC antirabbit and Texas Red-strepavidin (Zymed Laboratories, San Francisco, CA). All detection incubations were for 30 min at 37°C,

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[3] The abbreviations used are: YAC, yeast artificial chromosome; FISH, fluorescence *in situ* hybridization; BAC, bacterial artificial chromosome; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-PCR; CEPH, Centre d'Etude du Polymorphisme Humain; IGF, insulin-like growth factor.

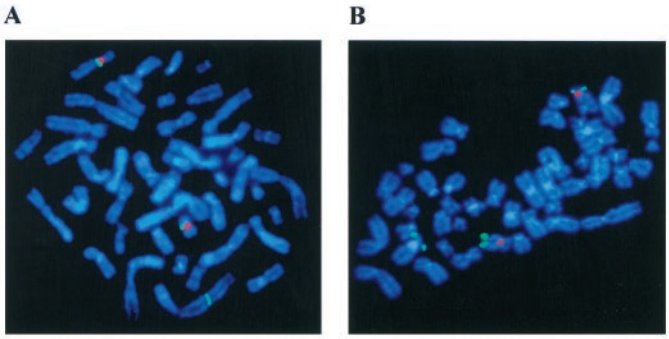

Fig. 1. Dual-color FISH mapping of 8q12 and 8q24.1 chromosomal breakpoints in lipoblastoma case 1, which has a complex rearrangement involving chromosomes 1, 7, and 8, as reported previously (10). *A*, metaphase cell hybridized with *PLAG1* BAC 227k20 (*green*) and chromosome 8 pericentromeric probe *D8Z2* (*red*). Nonrearranged chromosome 8 is at the *top*, derivative chromosome 8 with a partial BAC signal is in the *middle*, and derivative chromosome 7 with a partial BAC signal is at the *bottom*. *B*, metaphase cell hybridized with *HAS2* YAC 947h7 (*green*) and *D8Z2* (*red*). Nonrearranged chromosome 8 is in the *middle*, derivative chromosome 8 with a partial YAC signal is at the *top*, and derivative chromosome 1 with a partial YAC signal is at the *left*.

and slides were washed three times in PBS-T after each incubation. Images were obtained using a cooled charge-coupled device camera (Photometrics).

RNA Isolation and 5′ RACE. Total RNA was isolated using Trizol (Life Technologies, Inc.) according to the supplier's protocol after homogenization of tissues by extensive vortexing. 5′ RACE was performed using the Marathon cDNA amplification kit (Clontech Laboratories, Inc., Palo Alto, CA) according to the manufacturer's instructions, with minor modifications as described below. First-strand cDNAs were synthesized in a 3-h reaction from 5 μg of total RNA. Amplification of 5′ cDNA ends was performed using adapter primer AP1 and *PLAG1* exon 5 primer MV5 (18). Nested PCR amplification was performed using adapter primer AP2 and *PLAG1* exon 5 primer MV6 (18). All amplifications were performed using Advantage 2 Polymerase Mix (Clontech Laboratories, Inc.). Gel-purified nested PCR products were cycle sequenced by incorporation of ABI PRISM Big Dye Terminators (Perkin-Elmer) and analyzed on an ABI 377 automated sequencer.

RT-PCR. RT-PCR was performed using the Gene Amp RNA PCR Kit (Perkin-Elmer) with first-strand synthesis using either an oligodeoxythymidylic acid primer or the *PLAG1* primer MV5 (18). *HAS2-PLAG1* fusion cDNA was then amplified by nested PCR using Advantaq Plus (Clontech Laboratories, Inc.). First-round primers were HAS 420-E1-F (GTCGTCTCAAATTCATCTGATCTC) and PLAG 433-E4-R (TCTTGTTGG ACACTTGGGAAC), and second-round primers were HAS 502-E1-F (CTGAGGACGACTTTATGACCAG) and PLAG 415-E4-R (CTTTAGGTGGCTTCTCAAGTTTC). The *COL1A2-PLAG1* fusion was amplified using primers COL1A2 E1+112F (AAGGAGTCTGCATGTCTAAGTGCTA) and PLAG 415-E4-R.

Northern Blot Analysis. Total RNA was isolated from cultured cells using Trizol (Life Technologies, Inc.), separated by electrophoresis through a formaldehyde-containing gel, and then transferred to a Hybond-N membrane (Amersham Pharmacia Biotech). The blot was hybridized with a PLAG1 cDNA probe (2074 bp) obtained by nested PCR using primers PLAG 947-E5-F (TGCAAACTTTTGAAAGCACG) and PLAG 3928-E5-R (ATGAAGTGCGGTATGTGTGC), followed by PLAG 1265-E5-F (ATAAAAGACGAGCTCCTTCCG) and PLAG 3338-E5-R (CAGAGATGCATGAAA GTGGG). The blot was then stripped and rehybridized with a HAS2 cDNA probe (785 bp) obtained using primers HAS 49-E1-F (CCCATTGAACCAGAGACTTGAAA) and HAS 833-E2-R (GTTCAACTTTATGGGGGTTTCTA). All cDNA probes were labeled using a Prime-It II Random Primer Labeling Kit (Stratagene, La Jolla, CA) and then purified by S-200HR spin column chromatography (Pharmacia).

RESULTS

Localization of 8q Chromosomal Breakpoints in Lipoblastoma. Metaphase cell FISH mapping revealed that CEPH YAC 166f4 spanned the 8q12 breakpoints in lipoblastoma cases 1–4. YAC 166f4 is known to contain the *PENK* (preproenkephalin), *MOS*, and *PLAG1* genes. Notably, *PLAG1* is an oncogene targeted by chromosomal translocations in salivary gland tumors (18). To evaluate *PLAG1* as a potential target of the lipoblastoma 8q12 breakpoint, we next identified a BAC clone containing sequences from the entire *PLAG1* coding sequence. This clone, 227k20, spanned the lipoblastoma 8q12 breakpoints (Fig. 1*A*). We then performed FISH mapping to identify a CEPH YAC clone, 947h7, spanning the 8q24 breakpoint in lipoblastoma case 1 (Fig. 1*B*). This 1400-kb YAC contains the *HAS2* gene encoding hyaluronan synthase type 2 and also contains the Langer-Gideon critical region, including the loci for *TRPS1* (tricho-rhino-phalangeal syndrome 1) and *EXT1* (exostoses 1).

Identification of *PLAG1* Fusion Partners. Because FISH mapping implicated *PLAG1* as a strong candidate gene, we performed RACE to identify potential *PLAG1* fusion partners. In case 1, PCR amplification of the 5′ end of the *PLAG1* transcripts gave 950- and 1050-bp products. Sequence analysis of these two products revealed an identical ectopic sequence fused to *PLAG1* exon 3 (GenBank accession number AF221548) or exon 2 (GenBank accession number AF221549), respectively. This finding indicates a genomic fusion breakpoint in *PLAG1* intron 1 with alternative splicing of exon 2 and is consistent with a known splice variant of *PLAG1* (18). BLAST analysis identified the ectopic sequence as exon 1 of the hyaluronic acid synthase gene, *HAS2* (Fig. 2*A*). *HAS2* maps to chromosome 8q24.1 (19) and to YAC clone 947h7 that spans the 8q24.1 breakpoint in lipoblastoma case 1. In addition, Southern blotting analyses revealed genomic *PLAG1* and *HAS2* rearrangements in lipoblastoma case 1 (data not shown). Hence, both genomic and mRNA analyses in lipoblastoma case 1 support *HAS2-PLAG1* fusion as the oncogenic consequence of the cytogenetic 8q12:8q24.1 fusion event.

RACE analysis of lipoblastoma case 4 revealed a second *PLAG1* fusion partner. This lipoblastoma contained an apparently balanced translocation, t(7;8). PCR amplification of the *PLAG1* 5′ end gave a 617-bp product, and sequence analysis revealed a fusion transcript in which exon 1 of the *COL1A2* gene was joined to exon 3 of *PLAG1* (GenBank accession number AF221550; Fig. 2*A*). *COL1A2* maps to chromosome band 7q22.1, and its fusion with *PLAG1* is consistent with the cytogenetic mechanism in lipoblastoma case 4.

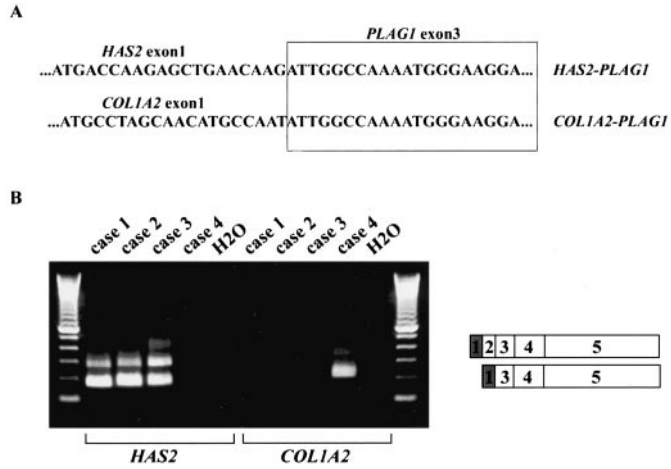

Fig. 2. *HAS2-PLAG1* and *COL1A2-PLAG1* fusion transcripts in lipoblastoma. *A*, cDNA sequence of *HAS2-PLAG1* and *COL1A2-PLAG1* fusion regions as demonstrated by 5′ RACE. *B*, RT-PCR analyses of lipoblastomas using *HAS2-PLAG1* (*left*) and *COL1A2-PLAG1* (*right*) primers. The adjacent schematic (to the *right* of *B*) shows the exon structure of the two major fusion transcripts resulting from alternate splicing of *PLAG1* exon 2 that are seen in each tumor. *PLAG1* exons are shown in *white*, whereas the ectopic first exon is *shaded*. An uncharacterized third fusion transcript also resulting presumably from alternate splicing is seen in lipoblastoma case 3 using *HAS2-PLAG1* primers.

Table 1 *Clinical, cytogenetic, and molecular data for four lipoblastomas*

Case no.	Age (yr)	Sex	Chromosome 8 rearrangement	Gene fusion product
1	1.3	F	der(8)(8pter→8q12::8q24.1→8qter)[a]	*HAS2-PLAG1*
2	2.5	M	der(8)(8pter→8q12::8q24.1→8qter)[a]	*HAS2-PLAG1*
3	6.0	M	ring chromosome 8	*HAS2-PLAG1*
4	1.8	M	t(7;8)	*COL1A2-PLAG1*

[a] Ref. 10.

***HAS2-PLAG1* Fusion Is a Recurrent Mechanism in Lipoblastoma.** RT-PCR analyses in the group of four lipoblastomas demonstrated *HAS2-PLAG1* fusions in cases 1–3, whereas *COL1A2-PLAG1* fusion was found only in case 4 (Fig. 2*B*). Each of the cases with *HAS2-PLAG1* fusions had intrachromosomal 8q rearrangements, which were 8q12:8q24.1 fusions in cases 1 and 2 and a ring chromosome 8 in case 3 (Table 1). Two *HAS2-PLAG1* fusion products (293 and 188 bp) were identified in cases 1–3, and sequence analysis revealed that these products were alternative splicing variants that either included or lacked *PLAG1* exon 2, respectively. Similarly, *COL1A2-PLAG1* fusion products (354 and 249 bp) were alternative splicing variants that differed only in the inclusion of *PLAG1* exon 2. Therefore, the genomic translocation breakpoints are within *PLAG1* intron 1 in each of the four lipoblastomas (Fig. 2*B*).

Expression of *PLAG1* and *HAS2* in Mesenchymal Tumors. *PLAG1* expression was evaluated by Northern blot analysis in a *HAS2-PLAG1* lipoblastoma cell line (established from case 1) and in cell lines established from other benign and malignant mesenchymal tumors (Fig. 3*A*). A 7.5-kb transcript similar in size to the known wild-type *PLAG1* transcript was expressed strongly in the lipoblastoma. Rehybridization with a 785-bp probe containing *HAS2* exons 1 and 2 confirmed the fusion nature of the lipoblastoma 7.5-kb *PLAG1* transcript. *HAS2* transcripts of 3.2 and 4.4 kb were demonstrated in all tumors, whereas an additional 7.5-kb transcript was detected exclusively in the lipoblastoma (Fig. 3*B*). The 7.5-kb *HAS2-PLAG1* fusion transcript was not detected when the blot was rehybridized with a 1389-bp *HAS2* cDNA probe lacking exon 1 (data not shown).

DISCUSSION

In previous studies, we characterized two lipoblastomas (Table 1, cases 1 and 2,) that had 8q intrachromosomal rearrangements joining band 8q12 with 8q24.1 (10). The intervening material, from chromosome band 8q12 to 8q24.1, was inserted into another chromosome in each case. We hypothesized that the 8q12::q24.1 fusions were associated with juxtaposition of genes from those two regions. In this study we demonstrate that *PLAG1*, a developmentally regulated zinc finger gene, is the oncogene target in lipoblastoma chromosome band 8q12 rearrangements. *PLAG1*, which encodes a transcription factor comprised of seven NH_2-terminal C_2H_2 zinc fingers and a COOH-terminal serine-rich region, was discovered recently by virtue of its oncogenic role in pleomorphic adenomas of the salivary gland (20). We now report novel *PLAG1* fusion mechanisms involving the *HAS2* gene at chromosome band 8q24.1 and the *COL1A2* gene at chromosome band 7q22 in lipoblastoma. Additionally, we show that *PLAG1* is expressed more strongly in *HAS2-PLAG1* lipoblastoma cells than in several other mesenchymal tumors.

The mechanism of *PLAG1* oncogenic activation in pleomorphic salivary adenomas differs from that in most fusion oncogenes. Pleomorphic adenoma translocation breakpoints generally involve the 5′ untranslated regions of both *PLAG1* and a fusion partner gene. Consequently, the entire *PLAG1* coding sequence, which begins in exon 4, is placed under the transcriptional control of an active and ectopic promoter region. These "promoter-swapping" events lead to high-level *PLAG1* expression in salivary gland cells, where *PLAG1* transcripts are normally undetectable. The most frequent *PLAG1* fusion in pleomorphic adenomas involves promoter swapping with the constitutively expressed β-catenin gene, *CTNNB1* (18). Alternate tumorigenic mechanisms involve *PLAG1* promoter swapping with the constitutively expressed genes for leukocyte inhibitory factor receptor (*LIFR*; Ref. 21) and transcription elongation factor A 1 (*TCEA1*; Ref. 22).

Here we implicate *PLAG1* promoter swapping as a critical event in lipoblastoma tumorigenesis. Notably, the general mechanism of *PLAG1* activation in lipoblastoma is similar to that in pleomorphic adenomas. Most pleomorphic adenomas, like the lipoblastomas reported herein, have chromosomal breakpoints involving *PLAG1* intron 1 and resulting in loss of *PLAG1* exon 1. A hypothetical explanation for the recurrent *PLAG1* intron 1 breakpoints is that upstream sequences might contain transcriptional repressor binding sites. If so, the intron 1 breakpoints would enable PLAG1 transcriptional upregulation by introducing active, ectopic promoter regions and by eliminating negative control elements.

Although *PLAG1* mechanisms in pleomorphic adenomas and lipoblastomas are likely related, the specific *PLAG1* fusion partners differ in these two tumors. We identified *HAS2-PLAG1* or *COL1A2-PLAG1* fusion genes in each of four lipoblastomas, whereas those fusions have never been reported in pleomorphic adenoma. In addition, published reports do not reveal lipoblastomas having the specific translocations, including t(3;8) and t(5;8), that frequently activate *PLAG1* in pleomorphic adenomas (19, 21, 22).

The genomic *HAS2-PLAG1* fusion breakpoints are in *HAS2* intron 1, whereas the *HAS2* coding sequence begins with the first codon of exon 2. Therefore, the *HAS2-PLAG1* fusion genes contain the entire *HAS2* 5′ untranslated region. There are no known transcription factor binding sites in the *HAS2* coding sequences, and it is therefore likely that *HAS2-PLAG1* is under the transcriptional control of an intact *HAS2* promoter. Histological correlations suggest that the *HAS2* promoter might be particularly active in lipoblastoma cells. HAS2 belongs to a multiprotein family that controls hyaluronic acid production at the level of either synthesis or transport from the cell (23, 24). Hyaluronic acid is a glycosaminoglycan that influences various biological activities, including cellular maturation, migration, and metabolism. Hyaluronic acid synthesis and metabolism are tightly regulated (25), and lipoblastomas have a variably myxoid stroma containing extracellular hyaluronic acid (1, 26). These observations

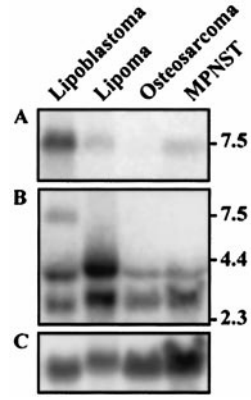

Fig. 3. Northern blot analysis of benign (*HAS2-PLAG1* lipoblastoma and lipoma) and malignant (osteosarcoma and malignant peripheral nerve sheath tumor) mesenchymal tumor cell lines. The RNA blot was hybridized with a 2074-bp *PLAG1* cDNA (*A*) and then rehybridized sequentially with 785-bp *HAS2* exon 1-containing cDNA (*B*) and β-actin cDNA (*C*). *PLAG1* expression was substantially stronger in the lipoblastoma than in the other tumors (*A*), and the 7.5-kb *HAS2-PLAG1* fusion transcript was seen only in the lipoblastoma (*B*).

are consistent with the notion that HAS2 is expressed at relatively high levels in lipoblastoma cells. If so, lipoblastoma cell *HAS2* promoter swapping might be a particularly effective mechanism for accomplishing *PLAG1* up-regulation.

In addition to the *HAS2-PLAG1* fusion, we have characterized a second lipoblastoma fusion involving the *PLAG1* and *COL1A2* genes. The *COL1A2-PLAG1* fusion gene retains a short portion of the *COL1A2* coding sequence in exon 1 that is joined with either *PLAG1* exon 2 or exon 3 in the fusion transcripts. Consequently, *COL1A2-PLAG1* encodes a full-length PLAG1 protein and a short, COOH-terminal-truncated, COL1A2 protein. The truncated COL1A2 protein is comprised of the first 23 amino acids of COL1A2, followed by 25 or 22 additional amino acids encoded by *PLAG1* exons 2 or 3, respectively. However, it is unknown whether this predicted COL1A2-PLAG1 fusion protein is functional or stably expressed. *COL1A2*, a member of the collagen gene family, encodes a protein that coils at a 1:2 ratio with collagen 1 α 1 to form the collagen fibers that make up bones, tendons, and connective tissues (27). Notably, lipoblastomas are often arranged in nodules separated by collagenous septae (26). It is possible that the lipoblastoma cells are directly responsible for production of the collagenous architectural features. Hence, *COL1A2* might be an especially strong promoter in lipoblastoma cells.

Our findings in lipoblastoma provide the first example of *PLAG1* rearrangement in mesenchymal neoplasia. *PLAG1* expression has been demonstrated previously in certain mesenchymal tumors, particularly those of smooth muscle cell origin (22), but *PLAG1* rearrangements have been found only in pleomorphic adenomas, which are believed to be epithelial neoplasms. Here we demonstrate stronger *PLAG1* expression in *HAS2-PLAG1* lipoblastoma cells than in several other benign or malignant mesenchymal tumors. Lipoblastoma *PLAG1* expression was 4- and 6-fold greater than that in lipoma and malignant peripheral nerve sheath tumor, respectively, whereas *PLAG1* transcripts were undetectable in an osteosarcoma. These findings provide further evidence that tumor chromosomal rearrangements affect *PLAG1* transcriptional up-regulation. Most lipoblastomas have cytogenetic rearrangement of the *PLAG1* (chromosome band 8q12) region, and it is likely that *PLAG1* activation is a pivotal oncogenic event in lipoblastoma.

Notably, PLAG1 transforming activity in lipoblastoma may be related to its recently described function as a transcriptional activator of the *IGF2* gene. IGF2 is up-regulated by PLAG1 interactions at an *IGF2* promoter 3 consensus binding site, and IGF2 is expressed abundantly in salivary gland adenomas containing *PLAG1* oncogenes (28). IGF2 stimulates proliferation of adipocyte progenitor cells (29), and it is therefore likely that *IGF2* transcriptional up-regulation contributes to neoplastic proliferation in *PLAG1*-mutant lipoblastoma cells.

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