The t(14,15) in Mouse Strain *CBA/CaH-T(14;15)6Ca/J* Causes a Break in the *ADAMTS12* Gene

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The mouse strain *CBA/CaH-T(14;15)6Ca/J* carries a homozygous balanced reciprocal translocation between mouse chromosomes 14 and 15, but the break points of this translocation have not previously been examined in detail. Using fluorescent in situ hybridization, we assigned the break point in 14qE3 to a 200-kb region devoid of any known gene. We similarly defined the break point in 15qA1 to a 27-kb region containing involving *ADAMTS12*. The chromosomal break likely is between exons 2 and 3 of *ADAMTS12*. This gene encodes a disintegrin and metalloproteinase with thrombospondin motifs, and this product plays crucial roles in both vascularization and cancer progression and has been implicated in the development of arthritis. The *CBA/CaH-T(14;15)6Ca/J* mouse strain likely is a suitable model for further examination of the influences of defective ADAMTS12 in various pathologic processes.

Abbreviations: BAC, bacterial artificial chromosome; DAPI, 4′-6-diamidino-2-phenylindole; DEAC, diethylaminocoumarin; FISH, fluorescent in situ hybridization.

CBA/CaH-T(14;15)6Ca/J is an inbred, primary repository mouse strain maintained through sibling crosses (stock no. 000655, Jackson Laboratories, Bar Harbor, ME). This strain has a homozygous balanced reciprocal translocation. Analysis of linkage groups located the rearrangement between unknown chromosomes.[4,5] Jackson Laboratories report in their web presentation about nondisjunctions occur at frequencies of 4.4% in male mice and 22.2% in female.[17] Approximately half of the gametes produced by heterozygotes carry characteristic physical abnormalities, leading to zygotes that die in utero. One gene located in the breakpoint region that has the potential to cause this effect is *ADAMTS12*, at 15A1. ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) belongs to a family of proteolytic enzymes involved in collagen maturation, organogenesis, angiogenesis, reproduction, and inflammation.[11] To delve further into the roles of ADAMTS in these processes, a gene-deficient animal model is of high interest. Here we provide evidence that *CBA/CaH-T(14;15)6Ca/J* mice carry interruptions in both *ADAMTS12* alleles due to translocation breakpoints between exons 2 and 3, which correspond to the N-terminal metalloprotein and disintegrin domains.

Materials and Methods

Breeding. All procedures involving animals were performed according to guidelines contained in German law for the protection of animals (Tierschutz–Gesetz)[6] and approved by the Representative of Animal Protection of Christian Albrechts University–Kiel. To define the break point, we used chromosome preparations prepared from fibroblasts of heterozygous B6D2F1 × *CBA/CaH-T(14;15)6Ca/J*

and *C57BL/6 × CBA/CaHT(14;15)6Ca/J* mice and homozygous *CBA/CaH-T(14;15)6Ca/J* mice.

To obtain mice heterozygous for the target translocation, *B6D2F1* female mice (age, 5 to 12 wk) were paired with *CBA/CaHT(14;15)6Ca/J* male mice (age, 8 to 18 wk) and *CBA/CaH-T(14;15)6Ca/J* female mice were paired with *C57BL/6* male mice (age, 6 to 18 wk). Mice were housed together for term of 1 wk; female mice then were moved to individual cages and monitored until delivery at 20 to 22 d after breeding. Pups remained with their dams for 3 to 4 wk, after which the progeny were segregated by sex into new cages. In addition, in the context of another study (reported elsewhere), which examines the segregation of the translocation products in murine embryos, pregnant mice were euthanized weekly from 5 to 25 wk of age.

Culture and preparation of mouse skin fibroblasts. To obtain fibroblasts, samples of abdominal skin were rinsed with mouse embryonic fibroblast medium (435 mL DMEM, Sigma, St Louis, MO; 50 mL FCS, Biochrom, Cambridge, UK; 5 mL L-glutamine, Biochrom; 5 mL nonessential amino acids, 100×, PAA, Pasching, Austria; 5 mL penicillin–streptomycin, 100×, Biochrom; and amphotericin B, final concentration, 2.5 µg/mL, Sigma) and placed into sterile culture dishes. Skins were minced into 1-mm² pieces; 9 or 10 skin pieces were placed into a T75 flask (Sarstedt, Nümbrecht, Germany) and rinsed with FCS. Skin pieces were arranged so that the nonhaired (peritoneal) side was fixed to the flask surface away at a distance from the other pieces. Skin pieces were kept in FCS for approximately 10 min, after which 800 µL mouse embryonic fibroblast medium was added. Flasks were incubated at 37 °C, 5% CO_2 for approximately 15 d until the cell layer was at least 90% confluent.

Preparation of metaphase chromosome spreads. Growing mouse fibroblasts were synchronized by incubating them overnight in amethopterin (methotrexate) in HBSS (final concentration, 55 ng/mL); the following morning the cells were washed

with PBS (Ca^{2+}- and Mg^{2+}-free) to stop the cell-cycle block. Cells were incubated 7 h in thymidine (Sigma) in HBSS (final concentration, 2 µg/mL), washed again with PBS (Ca^{2+}- and Mg^{2+}-free), and incubated overnight in HBSS containing 10 µg/mL colcemid to collect cells at metaphase stage. Cells were washed once with PBS (Ca^{2+}- and Mg^{2+}-free).

To harvest cells, 3 to 5 mL trypsin solution was incubated to each flask and incubated at room temperature for 10 min; 3 to 10 mL cell culture medium containing 10% FCS was added to stop the reaction. Cell-containing medium was transferred to tubes and centrifuged at $300 \times g$ for 10 min; the supernatant was discarded. Prewarmed (37 °C) 75 mM KCl (7 mL) was slowly added to the cell pellet, which was resuspended carefully and then incubated in a 37 °C water bath for 20 to 30 min. After incubation tubes were centrifuged at $300 \times g$ for 10 min, the supernatant was discarded and the cell pellet slowly mixed into 7 mL Carnoy fixation solution (3:1 methanol:glacial acetic acid, freshly prepared, –20 °C). This suspension was incubated on ice for 30 min, and then cells were pelleted and washed twice in fresh Carnoy fixation solution as previously. Finally the pellet was resuspended in 2 to 3 mL fixative to prepare cells for adhering to slides.

Fluorescence in situ hybridization (FISH). FISH was performed according to standard procedures to analyze the position of the break points by using bacterial artificial chromosomes (BAC) transformed into DH10B cells. Purified BAC DNA, isolated by using BacMax (Epicentre, Madison, WI), was amplified and labeled by PCR using degenerate primers.[16]

Amplification DNA and labeling of the probes. Probes were generated by PCR using degenerate primers[16] (5' CCG ATG CGA GNN NNN NAT GTG G 3') with template DNA prepared from various BAC (ImaGenes, Berlin, Germany). The procedure carried out in a thermocycler (PTC 200, MJ Research, Waltham, MA).

The procedures we used were modified slightly from those used previously.[16,19] Briefly, for amplification we used 1 µL BAC DNA probe as template. The multiplex probes (Figure 1) were labeled as follows: RP23-203G21 and RP23-220O14 with Texas Red-conjugated dUTP; RP2398B21 and RP23-462M9 with Spectrum Orange-conjugated dUTP; RP23-51F3 with diethylaminocoumarin-5-dUTP (DEAC); and RP23-399K3 with fluorescein-12-dUTP. The product was precipitated and solved in hybridization buffer ($2\times$ SSC [$20\times$: 3M NaCl, 0.3 M sodium citrate; pH 7], 50% formamide, 10% dextran sulfate, 1% Tween 20; pH 7). The cut-off value of the probe has been calculated as 71.6% (mean – 2 SD). The cells are genetically unobtrusive if at least 71.6% of nuclei show signal pattern to be expected in normal mice. The cut-off value was determined by counting 3 samples of 50 normal mouse fibroblasts. Normal signal distribution was found in 86% ± 7.2% of cells on average.

Denaturation and hybridization. One drop (approximately 10 to 12 µL) of fibroblast cell suspension was placed onto a clean slide and checked in an inverted microscope for the presence of metaphase. For denaturation, fibroblast cell metaphases were washed with PBS, dehydrated in an ascending ethanol series (70%, 95%, and absolute) at room temperature for 2 min at each concentration, and incubated for 2 to 3 h at 60 °C.[1] Slides were denatured in denaturation solution (70% formamide, $2\times$ SSC) in a water bath at 72 °C, dehydrated by incubating in an ascending ethanol series (70%, 95%, and absolute) at room temperature for 3 min at each concentration, and air-dried.
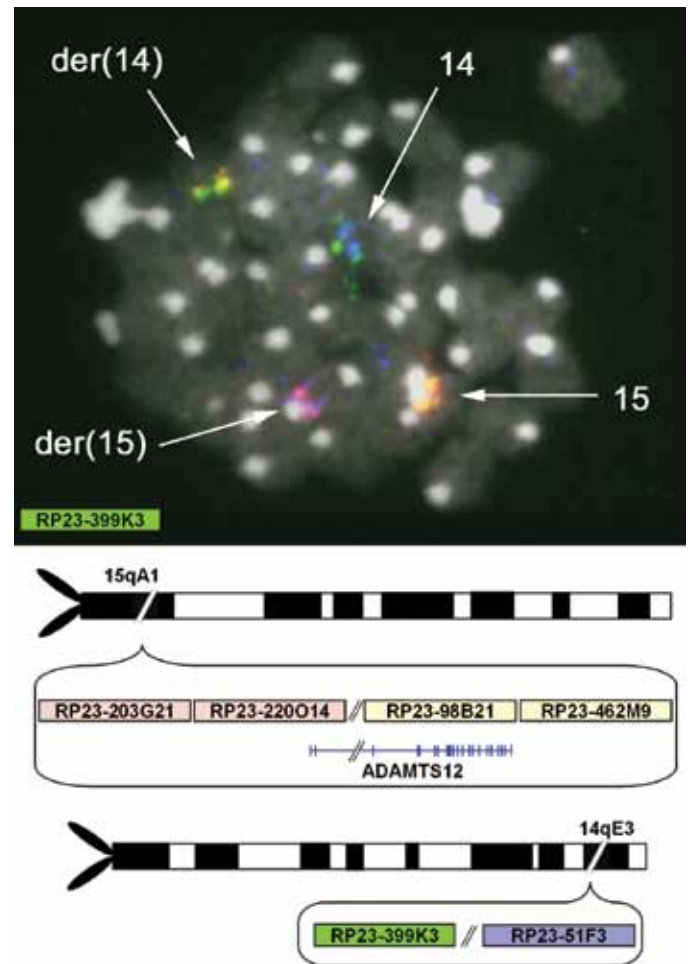

Figure 1. Top: FISH of heterozygotic B6D2F1 × *CBA/CaH-T(14;15)6Ca/J* mice. On translocated derivative (der) chromosomes, the signals from BAC probes RP23-220O14 (red, chromosome 15) and RP23-51F3 (blue, chromosome 14) recombine, as do those from RP2398B21 (yellow, chromosome 15) and RP23-399K3 (green, chromosome 14). Bottom: Diagrams of normal mouse chromosomes 15 and 14, indicating the positions and dye-labels of BAC clones used for FISH experiments. Double bar, break point.

Probe DNA, generated from BAC, was denatured at 75 °C for 5 min, chilled briefly, and pre hybridized at 37 °C for 30 min before adding to metaphase cells. For hybridization, 2 µL probe was added to each slide and coverslipped (7×7 mm). Cover slips were sealed with rubber cement and the slides placed at 37 °C overnight in a moist chamber.

Posthybridization washing and detection. After overnight hybridization, cover slips were removed, and the slides were washed in $0.5\times$ SSC for 5 min in a water bath at 72 °C. The slides then were washed twice in $4\times$ SSC, 0.05% Tween 20 (pH 7) for 2 min, once in PBS for 1 min, and briefly in distilled water at room temperature. Cells were counterstained with 4'-6-diamidino-2-phenylindole (DAPI) and signals were visualized by fluorescent microscopy (Axioplan 2, Carl Zeiss, Oberkochen, Germany) with

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optical filter combinations for DAPI, FITC, Texas Red, Spectrum Orange, and DEAC. Images were recorded by using a charge-coupled camera and analyzed (ISIS version 3, Metasystems, Altlussheim, Germany).

RT-PCR. We used RT-PCR to determine whether the *ADAMTS12* gene is expressed in normal and translocated mice. RNA (50 ng) isolated from mouse lung tissue was transcribed to cDNA (50 ng). For each tissue-specific cDNA, master mixes containing 12.5 μL iQ SYBR Green Supermix, 2.5 μL QuantiTect Primer Assay primer pair (Qiagen, Hilden, Germany), 6 μL MgCl$_2$, 2.0 μL H$_2$O, and 2 μL cDNA. Reactions were performed in an I-cycler (Bio-Rad, Hercules, CA) and the primers used were those for mouse *ADAMTS12* (QT00148239, Qiagen) and (for a control) 18S rRNA (QT00199367, Qiagen), which amplifies both human and mouse samples. The *ADAMTS12* amplicon generated was 116 bp in length and spanned exons 5 and 6. The PCR protocol was 95.0 °C for 3 min; 40 cycles of 95.0 °C for 30 s, 55.0 °C for 30 s, and 72.0 °C for 30 s; and 95.0 °C for 30 s. Finally, melt curve data collection were analysed by increase temperature of 0.5ºC per cycle starting at 55.0 °C for 80 cycles, 10 s each. The amplification products were separated on a 1% agarose gel.

Western analysis. Total protein lysate was prepared from mouse lung tissue. To this end, 500 μL lysis buffer (1 mM EDTA, 0.5% Triton X100, 5 mM NaF, 1 M urea, 1% protease inhibitor cocktail II, Sigma; and 1% phosphatase inhibitor, Sigma) was added to 0.5 g tissue. Samples were placed in reaction tubes (Lysing Matrix A, MP Biomedicals, Solon, OH), homogenized by using three 5-s pulses in a tissue disrupter (Fast Prep FP120, Savant Instruments, Holbrook, NY), and allowed to sit on ice for 1 h. Afterward lysates were centrifuged at $20,160 \times g$ for 5 min (Universal 16R, Hettich Centrifuge, Hettich, Beverly, MA) and supernatants transferred to clean reaction tubes. Sample protein concentration was quantified by using a total protein assay (Bio-Rad). Equal amounts of protein were loaded into the wells of a denaturing gel, separated by SDS–PAGE, and electrotransferred onto a polyvinylidene fluoride membrane (GE Healthcare, Piscataway, NJ). Membranes were blocked and incubated at 4 °C overnight with a rabbit polyclonal antibody against *ADAMTS12* (ab45041, Abcam, Cambridge, MA) according to the manufacturer's recommendations, followed by incubation at room temperature for 30 min with a horseradish-peroxidase–conjugated donkey antirabbit secondary antibody (NA934V, GE Healthcare). Proteins were visualized by using enhanced chemiluminescence (ECL Plus, GE Healthcare) and X-ray film (Hyperfilm, GE Healthcare).

Results

Breakpoint localization. The known breakpoint regions in cells of mouse strain *CBA/CaH-T(14;15)6Ca/J* are located on chromosome 15 in cytoband A2 and chromosome 14 in cytoband E3.[18] Here we delimited both breakpoint regions by using FISH probes that were generated from BAC and that spanned an 8-Mb region. On normal mouse chromosomes of heterozygote mice, BAC clones RP23-220O14 (red) and RP23-98B21 (yellow), which lie adjacent to the breakpoint, colocalize on chromosome 15 as do neighboring BAC RP23-399k3 (green) and RP23-51F3 (blue) on chromosome 14. On translocated chromosomes, the probe signals recombine (Figure 1). Among 12 euthanized heterozygous mice, 40 of the 47 metaphases evaluated showed a signal distribution identical to that in Figure 1. Among the 5 euthanized homozygous *CBA/CaH-T(14;15)6Ca/J* mice, all 10 metaphase spreads examined

showed only the recombinant signal pattern. We then repeated multiplex hybridization on homozygous *CBA/CaH-T(14;15)6Ca/J* mice but used a different green marker, a BAC clone on chromosome 15 (RP23-406N13) that overlaps the putative break point (Figure 2). Accordingly, the green signal was split between both translocation products in homozygous *CBA/CaH-T(14;15)6Ca/J* metaphase spreads. In contrast, BAC clone RP23-290G19, which is slightly more distal than RP23-406N13, was not split between the 2 translocation products in any of the 6 metaphases analyzed. Therefore, the break point on chromosome 15qA1 occurs between RP23-98B21 and RP23-220O14, a distance of 26,699 bp, in the second intron of *ADAMTS12*.

Transcription of *ADAMTS12* mRNA. To confirm the presence of *ADAMTS12* mRNA transcripts, we used isolate RNA from the lungs of both translocated and normal mice and used it as a template for cDNA in RT-PCR. Samples from normal mice all yielded the expected 116-bp amplicon (Figure 3), but mice homozygous for the t(14;15)(qE3;qA1) translocation lacked this band. In addition, Western blotting confirmed the presence of a 178-kDa prepeptide from *ADAMTS12* in the lungs of normal but not homozygous *CBA/CaH-T(14;15)6Ca/J* mice (Figure 3).
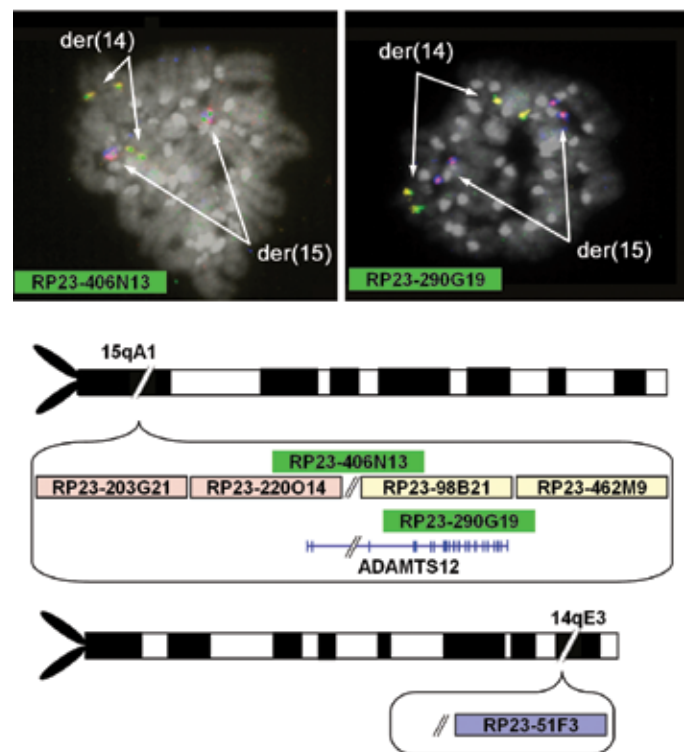

Figure 2. FISH of homozygotic cells of *CBA/CaH-T(14;15)6Ca/J*. Whereas the red- (RP23-220O14, chromosome 15), blue- (RP23-51F3, chromosome 14), and yellow- (RP2398B21, chromosome 15) labeled probes are the same as those in Figure 1, here 2 different green-labeled probes are used. Left: Here the RP23-406N13 BAC clone was labeled in green. Because the probe sequence overlapped the putative break point on chromosome 15, the green signal was split between the 2 translocation products. Right: The BAC clone RP23-290G19 was labeled in green. This sequence is distal to PR23-406N13, and its associated signal did not split between derivative (der) chromosomes but was translocated in its entirety to derivative chromosome 14. Below: Diagrams of normal mouse chromosomes 15 and 14, indicating the position and dye-labels of the BAC clones used, the position of *ADAMTS12*, and the locations of break points (double bar).
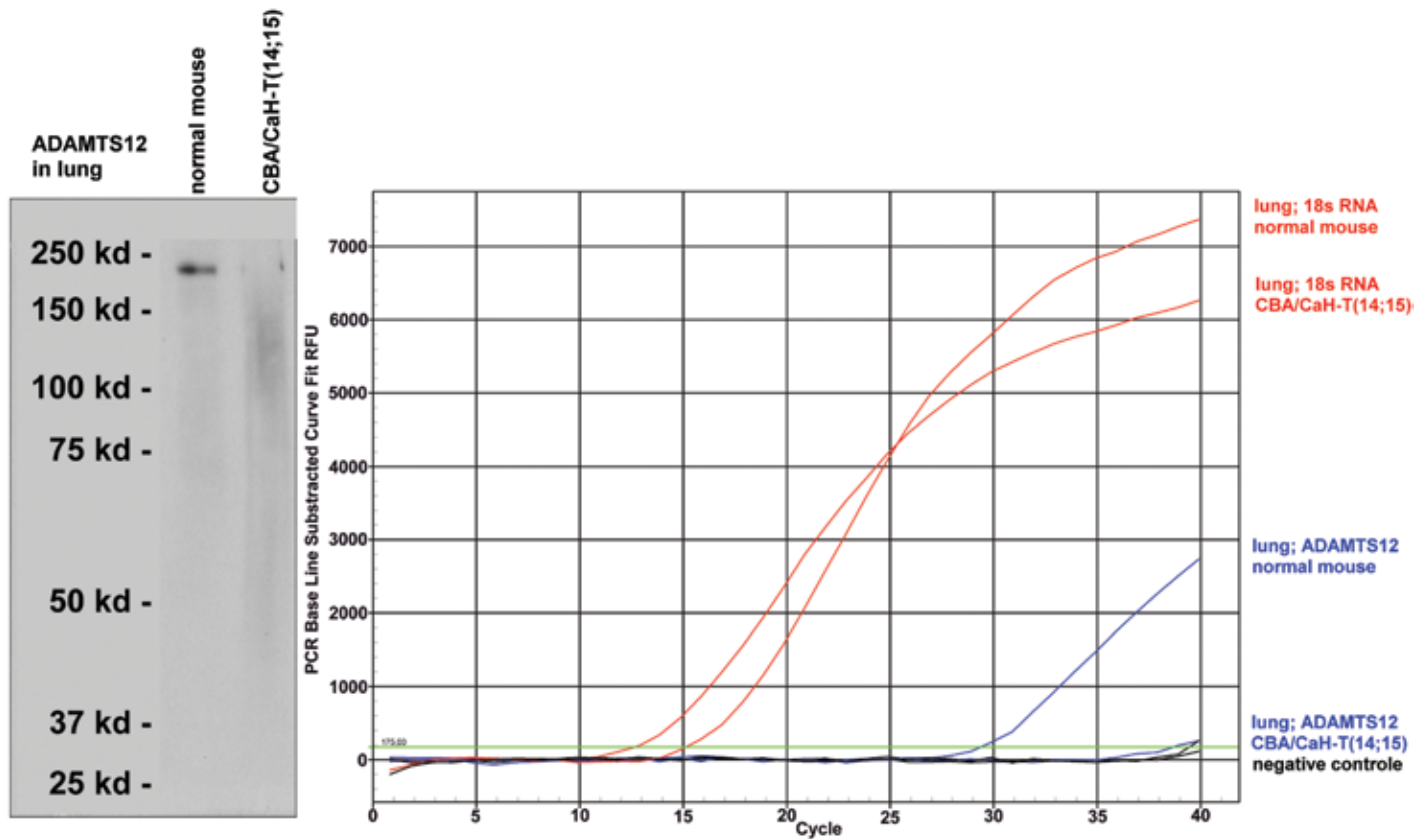
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Figure 3. Expression of ADAMTS12 in *CBA/CaH-T(14;15)6Ca/J* and normal mouse lung. Right: *ADAMTS12* mRNA transcripts (blue lines) were present in the lung tissue of normal mice but not in those homozygous for translocation chromosomes t(14;15)(qE3;qA1). The expression of 18S rRNA (control) is shown as red lines. Left: Western blotting revealed a 178-kD product in lung of normal mice but not in those homozygous for translocation chromosomes t(14;15)(qE3;qA1).

Discussion

ADAMTS12 is synthesized as a precursor molecule that is activated by furin-mediated cleavage of the 178-kDa prodomain, resulting in 2 components. The 120-kDa N-terminal ADAMTS12 element involves the metalloproteinase and disintegrin domains, and the 83-kDa C-terminal domain primarily contains TS1 thrombospondin repeats.[3,15] In vivo human ADAMTS12 protein can be found in fetal lung tissue only.[3] Our data show *ADAMTS12* RNA transcripts in the lung tissue of normal but not homozygous *CBA/CaH-T(14;15)6Ca/J* mice. In addition, Western blotting detected a 178-kDa protein product of *ADAMTS12* in lung tissue from normal but not homozygous *CBA/CaHT(14;15)6Ca/J* mice.

The protein product of *ADAMTS12* is involved in the initiation and progression of the arthritic process,[12] by contributing to the degradation of cartilage oligomeric matrix protein.[2,10] The C-terminal metalloproteinase domain is important for binding substrates and determining enzyme selectivity;[13] that the C-terminal thrombospondin 1 repeats of *ADAMTS12* are required and sufficient for binding to cartilage oligomeric matrix protein supports this function.[10] The ADAMTS12 protein also may play a role in asthma.[8] Arthritis and asthma often are associated with advancing age, but whether homozygous *CBA/CaH-T(14;15)6Ca/J* show increased incidence of these illnesses is unknown because they were euthanized at 25 wk of age. Certainly none of the mice showed any signs of arthritis or asthma during the study period.

Although the exact function of ADAMTS12 in arthritis[7] and asthma[8] remains unclear, deficiencies of this factor contribute to faulty vascularization and perhaps the suggested role of ADAMTS12 in cancer proliferation depends on failure of this function.[3] Human endothelial cells with multiple copies of *ADAMTS12* lose their ability to form tubules or spindle-shaped extensions under influence of vascular endothelial growth factor.[11] The thrombospondin motif domain is required for this effect.[11] These cells have diminished levels of the active phosphorylated form of the signaling factor ERK, which is necessary for vascular development in tumors and during the epithelial–mesenchymal transition.[9,14]

The cited data indicate that ADAMTS12 influences the Ras–MAP kinase signaling pathway. Its expression in Madin–Darby canine kidney cells prevents the tumorigenic effects of hepatocyte growth factor by blocking the activation of the Ras–MAPK signaling pathway through the thrombospondin domains of the metalloproteinase.[11] Furthermore, sustained activation of ERK seems to be crucial for cell scattering.[9] Cells of the nonsmall-cell lung cancer cell line A549 engineered to overexpress *ADAMTS12* produce smaller subcutaneous tumors in immunodeficient SCID mice than do cells lacking the construct.[9] Therefore, *ADAMTS12* apparently unites various functions to lead to tumor suppression. However, studies of tumor development in animal models lacking *ADAMTS12* are unavailable. Loss of *ADAMTS12* does not

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seem to be highly pathogen, because illnesses are not overt before 25 wk of age.

In contrast with its restricted expression in normal tissues, ADAMSTS12 is highly expressed in gastric carcinoma and other tumors. Furthermore, fibroblasts producing TGFβ can induce the expression of ADAMSTS12 in tumor cell lines.[2] However ADAMTS12 expression may delay the tumor growth of cells that produce this proteolytic enzyme.[11] Use of the *CBA/CaH-T(14;15)6Ca/J* mouse strain may help elucidate the regulatory roles of ADAMTS12 in angiogenesis and metastasis.

Whether the gene defect we defined here yields a useful knockout mouse model of *ADAMTS12* must be examined more thoroughly. An ADAMTS12 protein that lacks the thrombospondin domains, as we observed for homozygous *CBA/CaH-T(14;15)6Ca/J* mice, likely will not inhibit the effects induced by hepatocyte growth factor and thus will fail to ameliorate cancer progression. Whether homozygous *CBA/CaH-T(14;15)6Ca/J* mice are healthy and whether other gene products of this multifunction gene family can compensate for the loss of the thrombospondin domains is unclear as yet. Sequencing the breakpoint regions we defined may reveal the influence of other genes on the phenotype of homozygous *CBA/CaH-T(14;15)6Ca/J* mice. Further study of various mouse strains deficient in ADAMTS12 likely will clarify the relevance of this enzyme in tumor-associated processes, asthma, arthritis, organogenesis, angiogenesis, and reproduction.

Acknowledgments

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