



The American Society for  
Bone and Mineral Research

# Meet Babatunde, an ASBMR member.

**The ASBMR helped Babatunde find a leadership role, allowing him to advance his career, contribute to the Society and make a lasting impact in the field.**

Did you know that more than 250 ASBMR members globally serve in volunteer roles with the Society? From the Council, committees, task forces, publications and the Annual Meeting, there are dozens of ways to get involved and make an impact throughout your career.

**We're here to help you, too. Find your scientific home with the American Society for Bone and Mineral Research.**

**Babatunde Oyajobi, M.D., Ph.D.**

*Professor, University of Texas Health Science Center*

*ASBMR member for 28 years*

**LEARN MORE ABOUT  
BABATUNDE'S STORY AT**

[www.asbmr.org/babatunde](http://www.asbmr.org/babatunde)

## ADAM12-S Stimulates Bone Growth in Transgenic Mice by Modulating Chondrocyte Proliferation and Maturation

Marie Kveiborg,<sup>1</sup> Reidar Albrechtsen,<sup>1</sup> Lise Rudkjær,<sup>1</sup> Guanmei Wen,<sup>1</sup> Karen Damgaard-Pedersen,<sup>2</sup> and Ulla M Wewer<sup>1</sup>

**ABSTRACT:** ADAM12-S transgenic mice exhibit a pronounced increase in the length of bones, such as femur, tibia, and vertebrae. The effect of ADAM12-S on longitudinal bone growth involves the modulation of chondrocyte proliferation and maturation, likely through proteolytic activities and altered cell–extracellular matrix interactions in the growth plate.

**Introduction:** The disintegrin and metalloprotease ADAM12 is expressed in both osteoblasts and osteoclasts, suggesting a regulatory role of ADAM12 in bone. However, thus far, no in vivo function of ADAM12 in the skeleton has been reported.

**Materials and Methods:** Transgenic mice expressing the secreted form of human ADAM12, ADAM12-S, or a truncated metalloprotease-deficient form of ADAM12-S in the circulation were used to study the effects of ADAM12 on the skeleton. In addition, murine chondrocyte cultures were used to study the effect of ADAM12-S on cell–extracellular matrix interactions.

**Results:** ADAM12-S transgenic mice exhibit increased longitudinal bone growth. The increased bone length is progressive and age dependent, with a maximum increase of 17% seen in the femur from 6-month-old transgenic mice. The effect is gene dose dependent, being more pronounced in mice expressing higher levels of the transgene than in a lower-expressing line. Histological analysis revealed no alterations in the growth plate organization, but mean growth plate width was increased. Both the cellular incorporation of bromodeoxyuridine and the width of the collagen type X–positive hypertrophic zone were increased in the growth plate of ADAM12-S transgenic mice. Importantly, mice expressing a truncated form of ADAM12-S that lacked the pro- and metalloprotease domains showed no alterations in bone length, suggesting that protease activity is required for the ADAM12-S effect. In vitro studies showed that ADAM12-S inhibits chondrocyte adhesion to fibronectin and collagen type II.

**Conclusions:** ADAM12-S stimulates bone growth in mice by modulating chondrocyte proliferation and maturation through mechanisms probably involving both metalloprotease and adhesion activities.

**J Bone Miner Res 2006;21:1288–1296. Published online on May 22, 2006; doi: 10.1359/JBMR.060502**

**Key words:** ADAM12, bone growth, chondrocytes, metalloprotease, integrins

### INTRODUCTION

ADAM12 BELONGS TO the family of ADAMs (a disintegrin and metalloproteases), which are multidomain transmembrane glycoproteins involved in a variety of biological processes.<sup>(1,2)</sup> Mouse ADAM12 (initially called meltrin  $\alpha$ ) is a prototype ADAM protein comprising extracellular pro-, metalloprotease, disintegrin, cysteine-rich, and epidermal growth factor (EGF)-like domains, as well as a transmembrane domain and a cytoplasmic tail. The human homolog exists in two splice variants: ADAM12-L, a transmembrane protein similar to mouse ADAM12, and ADAM12-S, a shorter secreted form that lacks the transmembrane and cytoplasmic domains, containing instead a

unique stretch of 33 amino acids at the C terminus.<sup>(3)</sup> Both the transmembrane and the secreted forms of ADAM12 are active metalloproteases. ADAM12-S can cleave IGF binding proteins (IGFBP)-3 and -5,<sup>(4,5)</sup> as well as the extracellular matrix proteins, gelatin, type IV collagen, and fibronectin.<sup>(6)</sup> ADAM12-L has been shown to shed heparin-binding EGF (HB-EGF)<sup>(7,8)</sup> and placental leucine aminopeptidase<sup>(9)</sup> from their membrane-anchored forms. In addition, both forms of ADAM12 exhibit cell adhesion activities through the disintegrin and cysteine-rich domains, which are known to interact with integrin and syndecan cell surface receptors.<sup>(10–12)</sup>

During mouse embryogenesis, ADAM12 (meltrin  $\alpha$ ) is primarily expressed in mesenchymal condensations in regions of muscle and bone formation, such as cranial membranous bones, ribs, and limbs, and in the bone marrow.<sup>(13)</sup>

The authors state that they have no conflicts of interest.

<sup>1</sup>Institute of Molecular Pathology, University of Copenhagen, Copenhagen, Denmark; <sup>2</sup>Department of Radiology, The Rigshospitalet University Hospital, Copenhagen, Denmark.



In addition, several studies have shown a role for ADAM12 in mesenchymal tissues.<sup>(8,14,15)</sup> ADAM12 (meltrin  $\alpha$ )-deficient mice exhibit 30% mortality within the first weeks after birth, but viable ADAM12 (meltrin  $\alpha$ )-deficient mice appear normal and are fertile. However, detailed analysis has revealed that homozygous mutant mice have impaired formation of neck and interscapular muscles<sup>(8)</sup> and exhibit moderate resistance to high-fat diet-induced obesity.<sup>(16)</sup> Moreover, transgenic mice overexpressing human ADAM12-S or ADAM12-L show increased adipogenesis<sup>(15)</sup> and improved pathology of dystrophic muscle when bred into the dystrophin-deficient *mdx* background.<sup>(17)</sup>

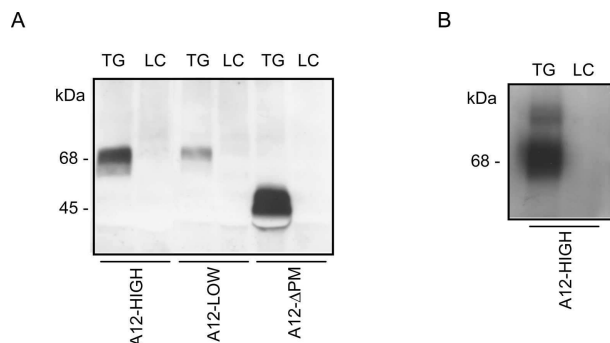
In agreement with the observed effects in mesenchymal tissues such as muscle and fat, several in vitro studies have shown a regulated expression of ADAM12 (meltrin  $\alpha$ ) in murine bone cells, including osteoblasts and osteoclasts.<sup>(18–22)</sup> Also, both ADAM12-S and ADAM12-L mRNA expression have been shown in human osteoblasts, whereas only the short form was detected in human osteoclasts.<sup>(23)</sup> In contrast, the expression of ADAM12 in chondrocytes has not been described. In terms of function, it has been suggested that ADAM12 plays a role in the fusion of multinuclear osteoclasts.<sup>(18,23)</sup> However, no skeletal abnormalities have been reported for ADAM12 (meltrin  $\alpha$ )-deficient mice, which may likely be caused by compensatory functions of other ADAM proteins.<sup>(24)</sup>

In certain situations characterized by excessive growth, such as during pregnancy and in cancer, ADAM12-S is present in high amounts in body fluids, including serum<sup>(25–27)</sup> and urine.<sup>(6)</sup> Thus, to study in vivo functions of ADAM12 in bone, we examined the skeletal phenotype in transgenic mice expressing full-length ADAM12-S or a truncated metalloprotease-deficient form of ADAM12-S in the circulation.

## MATERIALS AND METHODS

### ADAM12-transgenic and -deficient mice

Transgene constructs encoding the full-length human ADAM12-S or a truncated form of ADAM12-S, lacking the pro- and metalloprotease domains (ADAM12- $\Delta$ PM), under control of the muscle creatine kinase (MCK) promoter, were used to generate transgenic mice.<sup>(15)</sup> Two different transgenic founder lines expressing high (A12-high, 32 transgene copies) and low (A12-low, 4 transgene copies) levels of secreted ADAM12-S (68 kDa) in serum, as well as a single founder line (A12- $\Delta$ PM) expressing the truncated protein ADAM12- $\Delta$ PM (45 kDa) in serum, were used in this study (Fig. 1A).<sup>(15)</sup> The A12-high founder line was maintained on a B6CBAF1 background, whereas the A12-low and A12- $\Delta$ PM founder lines were backcrossed to C57BL/6. All mice were bred as heterozygotes to obtain littermate controls. Genotyping of the mice was done by PCR using primers specific for human ADAM12 on DNA derived from mouse tails as described.<sup>(15)</sup> Transgenic mice and control littermates were weighed once a week until killed at 5, 7.5, 10, or 26 weeks of age to isolate bone samples. For bromodeoxyuridine (BrdU) incorporation, 2-, 4-, or 7.5-week-old mice were injected intraperitoneally



**FIG. 1.** ADAM12 proteins in serum and bone marrow from transgenic mice. (A) Western blot analysis using polyclonal rabbit anti-serum (rb122) to detect ADAM12 protein in serum from transgenic mice (TG) or littermate control mice (LC), from founder lines expressing high (A12-high) or low (A12-low) levels of full-length ADAM12-S (68 kDa), or a truncated form of ADAM12-S, lacking the pro- and metalloprotease domains (A12- $\Delta$ PM; 45 kDa). (B) Immunoprecipitation using a mix of monoclonal antibodies (6E6, 8F8, 6C10) and subsequent Western blotting using polyclonal rabbit antiserum (rb122) was used to detect ADAM12-S (68 kDa) in the bone marrow from transgenic mice expressing high levels of ADAM12-S (TG) or littermate control mice (LC).

with BrdU (50  $\mu$ g/g body weight) 1.5 h before death. ADAM12 (meltrin  $\alpha$ )-deficient mice were kindly provided by Dr A Sehara-Fujisawa, Kyoto University, Kyoto, Japan, and were generated and genotyped as described.<sup>(8)</sup> In addition, lack of ADAM12 transcripts in the homozygous knockout mice was confirmed by RT-PCR, as previously described.<sup>(17)</sup> All animal experiments were done in accordance with the guidelines of the Animal Inspectorate, Denmark.

### Antibodies and recombinant protein

Mouse monoclonal antibodies recognizing the ADAM12 disintegrin domain (6E6, 8F8, 6C10) and polyclonal rabbit antiserum (rb122) that binds the cysteine-rich domain of ADAM12 were generated and characterized as previously described.<sup>(3,28)</sup> The polyclonal antibody against mouse  $\beta$ 1 integrin was kindly provided by Dr S Johansson, Uppsala University, Uppsala, Sweden.<sup>(29)</sup> Mouse monoclonal antibody to BrdU was obtained from Roche (Indianapolis, IN, USA), mouse monoclonal antibody to collagen X was obtained from Laboratory Vision (Fremont, CA, USA), and secondary antibodies were obtained from Dako A/S (Glostrup, Denmark). Recombinant full-length ADAM12-S and ADAM12-S with a E351Q catalytic site mutation were produced in EBNA-293 cells, purified, and shown to be biologically active in protease and cell adhesion assays as previously described.<sup>(5,12)</sup>

### Bone length measurements, histology, and immunohistochemistry

Whole body X-ray analysis of ADAM12-S transgenic mice and control littermates was performed using a Siemens Mammomat 3000 with exposure parameters of 25 mA and 27 kV. The X-ray images were documented by an

ADC system (Analog to Digital Converting System-AGFA 70 8 × 10'). The images were stored in the PACS system (Picture Archiving and Communicating System AGFA), and the length of the extremity bones were measured digitally on the screen. Alternatively, the femur and tibia were removed, fixed in 4% formaldehyde, and measured manually using a Callimax. Bone samples were decalcified in 10% EDTA, processed, and embedded in paraffin. Deparaffinized sections (5 µm thick) were stained with hematoxylin/eosin, Alcian blue/Van Gieson, or used for immunohistochemistry. Apoptotic cells were detected using ApoTag reagents according to the manufacturer's instructions (Invitrogen A/S, Taastrup, Denmark). For collagen type X immunohistochemistry, deparaffinized sections were blocked for endogenous peroxidase activity with 1% hydrogen peroxide in methanol, treated with 0.4% pepsin in 0.01 M HCl and 1% bovine testicular hyaluronidase (Sigma-Aldrich A/S, Brøndby, Denmark) to unmask antigens, blocked in 2% BSA, and incubated with primary antibodies diluted in 2% BSA overnight at 4°C. To detect BrdU<sup>+</sup> chondrocytes, deparaffinized sections were incubated for 20 minutes in 4N HCl and then treated with 0.1% trypsin and 0.1% CaCl<sub>2</sub> in 0.05 M Tris, pH 7.6. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide in methanol before incubating with primary anti-BrdU antibody overnight at 4°C. For all immunohistochemistry, primary antibody detection was performed using the ChemMate kit (Dako A/S), and stained sections were examined using a Zeiss Axioplan microscope equipped with an AxioCam camera. The AxioVision 4.4 software was used to measure the width of the total growth plate in the proximal tibia and distal femur on both hematoxylin/eosin and Alcian blue/Van Gieson-stained sections. Collagen type X is a marker of hypertrophic chondrocytes<sup>(30)</sup> and collagen type X-stained sections were used to determine the width of the hypertrophic zone relative to the proliferative zone of the growth plate. The mean width was calculated as the average of several digital measurements from at least five different animals in each group.

#### *Immunoprecipitation, Western blot, and immunocytochemistry*

Mouse serum and bone marrow samples, as well as total cell lysates, were examined by immunoprecipitation and subsequent Western blot analysis or by direct Western blotting as described previously.<sup>(15,28)</sup> A mix of mouse monoclonal antibodies (6E6, 8F8, 6C10) coupled to protein G Sepharose was used for ADAM12 immunoprecipitation, and rabbit polyclonal antiserum (rb122) was used for ADAM12 Western blotting together with horseradish peroxidase-conjugated secondary antibodies and the chemiluminescence ECL Plus detection system (Amersham Biosciences, Hilleroed, Denmark). Immunocytochemistry using polyclonal antibodies against β1 integrin and ADAM12 (rb122) was performed on either adherent cells or cells in suspension as previously described.<sup>(15,28)</sup>

#### *Quantitative RT-PCR*

Total RNA was isolated using the TRIzol Reagent (Invitrogen), according to the manufacturer's protocol. One

microgram of RNA was treated with DNase I (Invitrogen) and reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche). The quantitative PCR (QPCR) was performed using the LightCycler FastStart DNA Master SYBR Green I and the LightCycler QPCR machine (Roche). Primers targeting the reference gene 18S rRNA (forward: 5'-CGCCGCTAGAGGTGAAATTC-3' and reverse: 5'-TTGGCAAATGCTTTTCGCTC-3') produced a 62-bp band.<sup>(31)</sup> Primers targeting mouse ADAM12 (forward: 5'-TGTGGAAATGGCTATGTGGA-3' and reverse: 5'-CAGGTGGTAGCGTTACAGCA-3') produced a 89-bp band. QPCR products were sequenced and checked for the expected sizes on a 2% agarose gel. The data were analyzed using the 2<sup>-ΔΔC<sub>t</sub></sup> method.<sup>(32)</sup>

#### *In vitro assays*

Murine β1 integrin-deficient chondrocytes (β1<sup>-/-</sup>) and control chondrocytes (β1<sup>+/+</sup>), kindly provided by Dr R Fassler, Max Planck Institute of Biochemistry, Martinsried, Germany, were generated and cultured as described.<sup>(10,33)</sup> To examine the effects of ADAM12-S on chondrocyte proliferation, cells were treated with 2 µg/ml recombinant ADAM12-S or vehicle in serum-free media for 24 h, followed by 60-minute incubation at 37°C with 10 µM BrdU. BrdU<sup>+</sup> cells were subsequently detected using a mouse monoclonal antibody to BrdU as described by the manufacturer (Roche). In addition, DNA content in ADAM12-S- and control-treated cells was examined by flow cytometry. In short, cells were trypsinized, digested with RNase A for 30 minutes at 37°C, stained with propidium iodide for 30 minutes at room temperature, and analyzed with a FACScalibur machine (BD Biosciences, Palo Alto, CA, USA). For cell attachment assays, 96-well plates were first coated with 10 µg/ml fibronectin or collagen type II. Cells that had been treated with 2 µg/ml recombinant ADAM12-S, ADAM12-S with a catalytic site mutation, or vehicle for 1 h in serum-free media were allowed to attach for 1 h at 37°C in the presence of recombinant protein. Adherent cells were fixed, stained, and examined at OD 590 nm, as previously described.<sup>(34)</sup> Each assay was carried out in six separate wells and was repeated in three independent experiments.

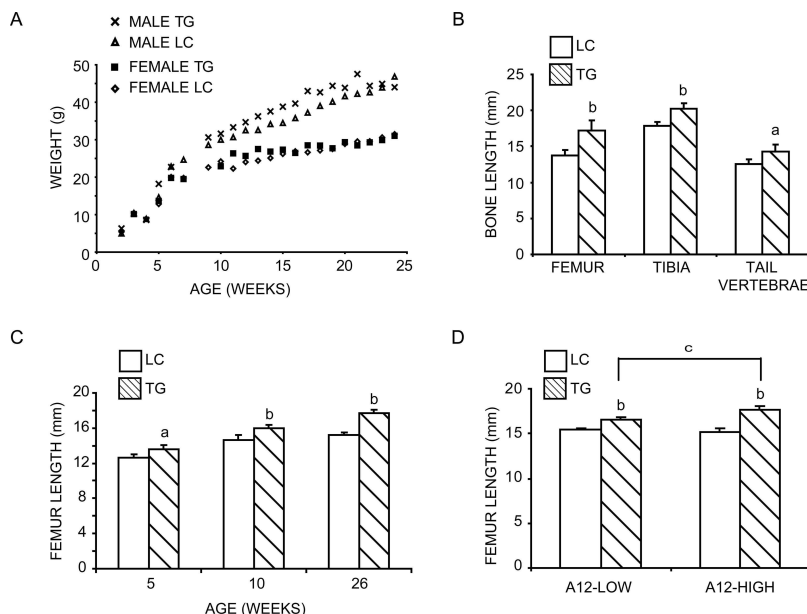
#### *Statistical analysis*

All data are expressed as means ± SD. The Student's *t*-test or Mann-Whitney *U*-Wilcoxon rank sum *W*-test was used to compare two groups of data, with *p* < 0.05 considered statistically significant.

## RESULTS

### *Longitudinal bone growth is increased in ADAM12-S transgenic mice*

To study the functional role of ADAM12 in bone tissue, we examined transgenic mice expressing human ADAM12-S under control of the MCK promoter<sup>(15)</sup> that directs transgene expression to striated muscle.<sup>(35)</sup> Because ADAM12-S is a secreted protein, it is released into the circulation and can be detected in serum from transgenic mice (Fig. 1A). Moreover, ADAM12-S protein can access bone tissues as shown by immunoprecipitation and



**FIG. 2.** Increased bone length in ADAM12-S transgenic mice. (A) Total body weight of female and male transgenic mice expressing high levels of ADAM12-S (TG) versus female and male littermate controls (LC), up to 6 months of age ( $n > 5$  in each group). (B) Digital measurements of the length of femur, tibia, and three consecutive tail vertebrae in 6-month-old transgenic mice expressing high levels of ADAM12-S (TG) or littermate controls (LC). (C) Femoral length at 5, 10, or 26 weeks of age in transgenic mice expressing high levels of ADAM12-S (TG) or littermate controls (LC). (D) Femoral length in 6-month-old mice from two independent ADAM12-S transgenic founder lines expressing high (A12-high) or low (A12-low) levels of ADAM12-S. For B-D,  $n > 7$  in each group. <sup>a</sup> $p < 0.005$ , <sup>b</sup> $p < 0.001$  when comparing transgenic vs. control mice; <sup>c</sup> $p < 0.001$  when comparing the two transgenic founder lines.

subsequent Western blot analysis of bone marrow flushed from long bones of transgenic mice and littermate controls (Fig. 1B). Up to 25 weeks of age, no overall difference in the size or total body weight of transgenic mice expressing high levels of ADAM12-S was observed compared with control littermates (Fig. 2A). However, older mice have previously been reported to exhibit increased total body fat mass<sup>(15)</sup> and some transgenic mice (especially females) developed kyphosis, a characteristic bending of the vertebral column. Both digital X-ray analysis and manual measurements of the length of tail vertebrae as well as the femur and tibia revealed a marked increase in longitudinal bone growth in ADAM12-S transgenic mice compared with littermate controls at 6 months of age (Fig. 2B). The increase in bone length is progressive and age dependent, rising from an 8% increase in femur length at 5 weeks of age to 9% at 10 weeks and 17% at 6 months of age (Fig. 2C). Moreover, comparing femoral length in independent ADAM12-S transgenic founder lines expressing high (A12-high) and lower (A12-low) levels of ADAM12-S in the circulation showed that the effect is gene dose dependent; that is, longitudinal bone growth was increased in both transgenic lines compared with littermate controls, but more so in mice expressing the higher ADAM12-S levels (Fig. 2D).

#### *ADAM12-S-induced bone growth requires the pro- and metalloprotease domains*

The findings of increased bone length in ADAM12-S transgenic mice raised the question of whether ADAM12 metalloprotease activity is involved. To address this issue, we analyzed transgenic mice expressing a truncated form of ADAM12-S that lacked the entire pro- and metalloprotease domains (ADAM12- $\Delta$ PM). No change in longitudinal bone growth was observed in 6-month-old ADAM12- $\Delta$ PM transgenic mice compared with littermate controls (Fig. 3A), indicating that the effect of ADAM12-S on bone growth is metalloprotease dependent. To determine whether ADAM12 is essential for proper growth of long bones,

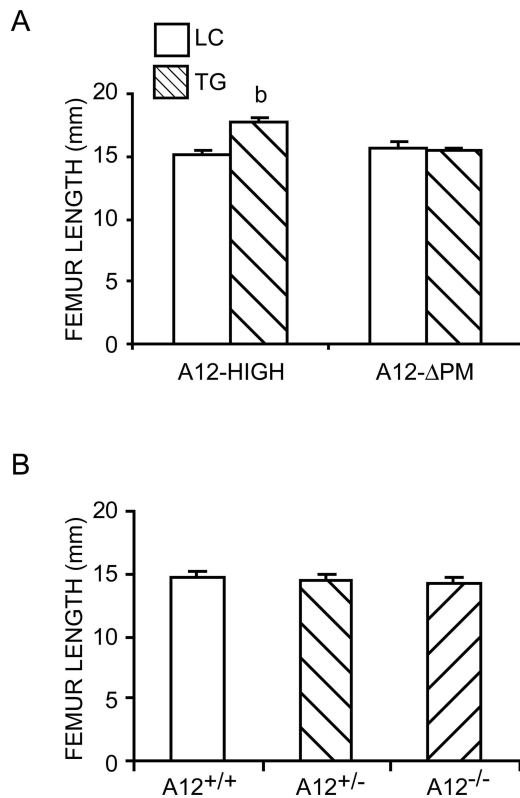
we examined femoral length in 10-week-old mice with a targeted deletion of the *ADAM12* gene. No difference was observed when comparing ADAM12-deficient mice with mice heterozygous for the targeted allele or wildtype mice (Fig. 3B).

#### *ADAM12-S modulates proliferation and maturation of growth plate chondrocytes*

Longitudinal bone growth is regulated at the growth plate formed between the diaphysis and epiphysis by the segregation of chondrocytes at different stages of differentiation.<sup>(36,37)</sup> To examine the increased bone length associated with ADAM12-S expression in more detail, we performed a histological analysis of the growth plate in the proximal tibia from 5-, 7.5-, or 10-week-old ADAM12-S transgenic mice and littermate controls. ADAM12-S transgenic mice were found to have an apparently normal organization of the growth plate; however, the overall width of the growth plate was statistically significantly increased in ADAM12-S transgenic mice compared with littermate control mice (Figs. 4A and 4B). Furthermore, immunohistochemical staining of the growth plate in transgenic and littermate control mice for collagen type X showed no obvious change in either staining pattern or intensity (Figs. 4C and 4D). However, digital measurements of collagen type X-stained sections revealed an increased width of the collagen type X-positive region, representing the prehypertrophic and hypertrophic zones, in transgenic mice, whereas no change in the collagen type X-negative region (including the proliferative and resting zones) was observed (Figs. 4C, 4D, and 4G).

Because hypertrophic chondrocytes are terminally differentiated cells, derived from the maturation of proliferating chondrocytes, expansion of the hypertrophic zone may be caused by enhanced proliferation and maturation of chondrocytes. To test this possibility, we examined chondrocyte proliferation by the incorporation of BrdU. The number of BrdU<sup>+</sup> cells in the proliferative zone of the growth plate





**FIG. 3.** The increase in bone length requires the pro- and metalloprotease domains. (A) Femoral length in 6-month-old transgenic mice (TG) expressing high levels of full-length ADAM12-S (A12-high) or a truncated form of ADAM12-S, lacking the pro- and metalloprotease domains (A12-ΔPM) vs. the respective littermate controls (LC). (B) Femoral length in 10-week-old ADAM12-deficient mice (A12<sup>-/-</sup>), mice heterozygous for the targeted allele (A12<sup>+/-</sup>), or littermate wildtype controls (A12<sup>+/+</sup>). In both panels,  $n > 7$  in each group. <sup>b</sup> $p < 0.001$ .

was increased in 7.5-week-old ADAM12-S transgenic mice compared with control littermates (Figs. 4E, 4F, and 4H). However, counting the number of cells per column revealed no significant difference (data not shown). In addition, detection of apoptotic cells in the growth plate by ApoTag-staining showed only a few apoptotic cells, and no difference between transgenic mice and littermate controls was detected (data not shown).

To examine the effect of ADAM12-S on chondrocyte proliferation *in vitro*, we treated murine chondrocytes with purified recombinant ADAM12-S or vehicle control. However, no effect of ADAM12-S treatment on cell proliferation was observed by either BrdU incorporation or by flow cytometry of propidium iodide-stained cells (data not shown), indicating that the effect of ADAM12-S on chondrocyte proliferation involves additional factors present only *in vivo*.

#### *Endogenous ADAM12 interacts with integrins in chondrocytes*

Chondrocytes express several different members of the integrin family,<sup>(38)</sup> and chondrocyte proliferation and dif-

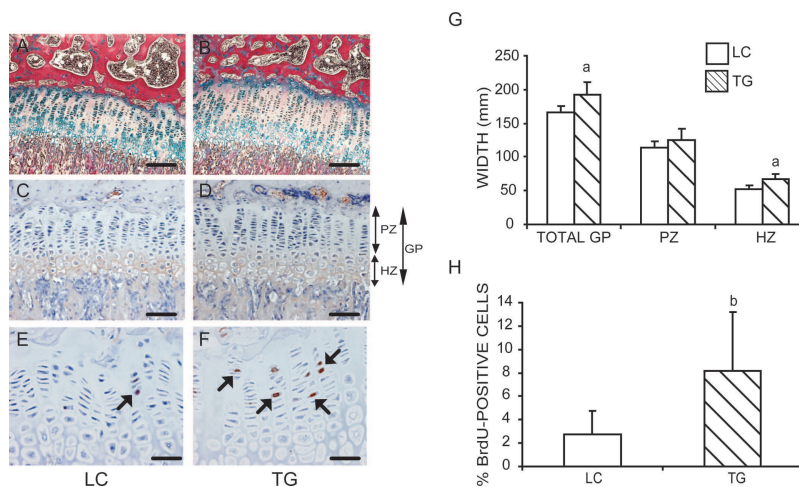
ferentiation is highly dependent on the contact between these cell surface receptors and the surrounding ECM.<sup>(39)</sup> ADAM12 is known to mediate some of its biological functions through a direct interaction with integrins, including  $\beta 1$  and  $\beta 3$  heterodimers.<sup>(11,12,40)</sup> To study whether the described effects of ADAM12 on chondrocytes may also be mediated through integrin-dependent pathways, we examined the expression of ADAM12 in chondrocytes derived from mice with a targeted deletion of  $\beta 1$  integrin in cartilage and control chondrocytes.<sup>(33)</sup> Western blot analysis showed that ADAM12 is expressed in apparently equal amounts in  $\beta 1$  integrin-expressing chondrocytes and  $\beta 1$  integrin-deficient chondrocytes; however, quantitative RT-PCR analysis showed an ~4-fold higher level of ADAM12 mRNA in control chondrocytes compared with chondrocytes lacking  $\beta 1$  integrin (data not shown). As expected,  $\beta 1$  integrin expression was detected at the surface of control chondrocytes (Fig. 5A), whereas no  $\beta 1$  integrin was observed in  $\beta 1$  integrin-deficient chondrocytes (Fig. 5B). Intriguingly, ADAM12 immunocytochemistry of both permeabilized adherent chondrocytes (Figs. 5C and 5D) and unpermeabilized cells in suspension (Figs. 5E and 5F) showed a clear staining at the surface of  $\beta 1$  integrin-deficient chondrocytes (Figs. 5D and 5F), whereas ADAM12 was only weakly detected in cells with intact  $\beta 1$  integrin expression (Figs. 5C and 5E). Because ADAM12 mRNA and protein were clearly detected in both control and  $\beta 1$  integrin-deficient chondrocytes, the difference in ADAM12 immunocytochemical detection is most likely caused by an endogenous interaction between  $\beta 1$  integrin and ADAM12 changing the ADAM12 immunoreactivity *in situ*.

#### *ADAM12-S reduces chondrocyte attachment on extracellular matrix components *in vitro**

The perceived interaction between ADAM12 and integrins in chondrocytes led us to test whether ADAM12-S affects integrin-mediated chondrocyte attachment on ECM components. For this purpose, we treated murine chondrocytes with recombinant ADAM12-S and studied the effect on cellular attachment to collagen type II and fibronectin *in vitro*. Interestingly, ADAM12 acts as a disintegrin, reducing cellular attachment on collagen type II and fibronectin by 46% and 36%, respectively (Fig. 6A). On the contrary, ADAM12-S with the catalytic site mutated did not show any significant effect on chondrocyte attachment (data not shown). Previous findings have shown that, although ADAM12 interacts primarily with  $\beta 1$  integrin, in its absence,  $\beta 3$  integrin can act as its binding partner.<sup>(41)</sup> This mechanism is supported by our findings that, similar to the effect in  $\beta 1$  integrin-expressing control chondrocytes, ADAM12-S inhibited the cellular interaction with collagen type II by 17% and with fibronectin by 38% in cells lacking  $\beta 1$  integrin (Fig. 6B).

## DISCUSSION

Several studies have reported a regulated expression of ADAM12 in bone cells *in vitro*.<sup>(18–23,42)</sup> Here, we describe



**FIG. 4.** ADAM12-S modulates proliferation and maturation of growth plate chondrocytes. (A and B) Alcian blue/Van Gieson-stained growth plates in the proximal tibia of 5-week-old transgenic mice expressing high levels of ADAM12-S (TG) or littermate control mice (LC). (C and D) Collagen type X immunohistochemistry in the growth plate of 7.5-week-old transgenic mice expressing high levels of ADAM12-S (TG) or littermate control mice (LC). (E and F) BrdU incorporation in proliferating chondrocytes in the tibial growth plate from 7.5-week-old transgenic mice expressing high levels of ADAM12-S (TG) or littermate control mice (LC). Bars in A–D are 80  $\mu$ m and bars in E and F are 40  $\mu$ m. (G) Digital measurements of the width of the total growth plate (total GP), the collagen type X-negative region of the growth plate, including resting and proliferative zones (PZ), or the collagen type X-positive region of the growth plate, including the prehypertrophic and hypertrophic zones (HZ) in the proximal tibia from 7.5-week-old transgenic mice expressing high levels of ADAM12-S (TG) or littermate controls (LC). (H) Percentage BrdU<sup>+</sup> cells in the growth plate of 7.5-week-old transgenic mice expressing high levels of ADAM12-S (TG) or littermate control mice (LC). For G and H,  $n = 5$  in each group. <sup>a</sup> $p < 0.05$ , <sup>b</sup> $p < 0.01$ .

for the first time an *in vivo* analysis of the role of ADAM12 in the skeleton using a transgenic mouse model. Overexpression of human ADAM12-S under control of the MCK promoter leads to high levels of secreted ADAM12-S in serum, which can therefore access bone tissue through the blood stream. Interestingly, longitudinal bone growth is markedly increased in ADAM12-S transgenic mice compared with littermate controls. The effect is gene dose dependent and occurs in a progressive and age-related manner.

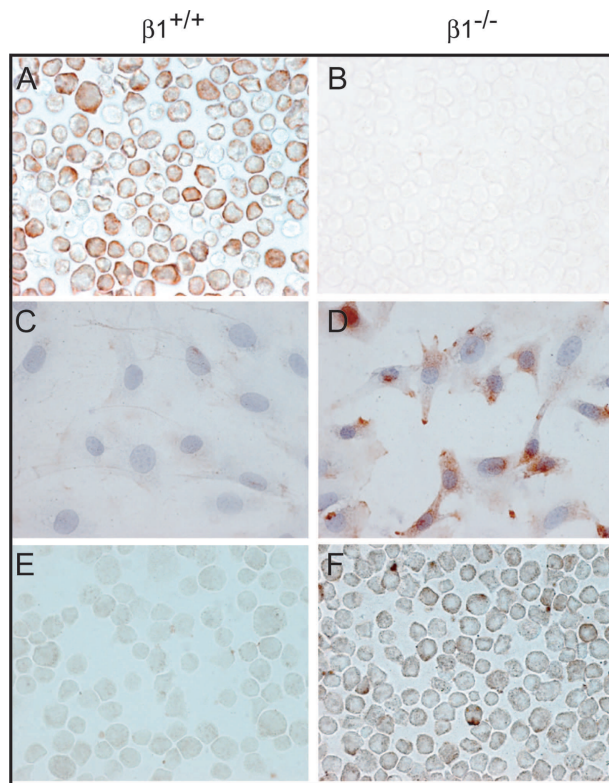
The regulation of postnatal bone growth is a complex process,<sup>(30)</sup> involving a multitude of hormones and growth factors.<sup>(43,44)</sup> Thus, the observed increase in bone growth in ADAM12-S transgenic mice may likely implicate changes in some of these regulatory factors, either systemically or locally in bone tissue. For instance, ADAM12-S acts as an IGF-BP protease *in vitro*,<sup>(5)</sup> and although no difference in the level of total IGF-I in serum from ADAM12-S transgenic mice and littermate controls was observed (unpublished results), it is possible that the amount of IGF-I bound to IGF-BPs, and thereby free to act on bone cells, is altered. In addition, sex steroids, such as estrogens, may play a role in the effect of ADAM12 on longitudinal bone growth. Estrogen receptor  $\beta$ -deficient female mice have been reported to exhibit increased femoral length.<sup>(45,46)</sup> However, this effect is somewhat smaller than in ADAM12-S transgenic mice, and in contrast to the increased bone growth in ADAM12-S transgenic mice, which is progressive, the increase in bone length in young estrogen receptor  $\beta$  null mice is reversed in older animals.

In many ways, the observed bone phenotype in ADAM12-S transgenic mice resembles that of mice with a

loss-of-function mutation in the fibroblast growth factor receptor 3 (Fgfr3). Like mice overexpressing ADAM12-S, mice lacking Fgfr3 function exhibit a marked increase in the length of the vertebral column and long bones, characterizing Fgfr3 as a negative regulator of bone growth.<sup>(47)</sup> As in the Fgfr3 null mice, the kyphosis in ADAM12-S transgenic mice is most likely caused by overgrowth rather than a collapse of the vertebral body, yet detailed BMD studies are under preparation to clarify this. Also, the enhanced growth in both these mouse models is accompanied by expansion of the epiphyseal growth plates.

The growth plate is formed between the diaphysis and epiphysis by the segregation of chondrocytes at different stages of differentiation.<sup>(37)</sup> Subsequent longitudinal bone growth is strictly regulated by the balanced proliferation and differentiation of growth plate chondrocytes. The width of the hypertrophic zone is maintained by the rate at which chondrocytes enter the hypertrophic phase, as well as by the rate of invasion at the chondro-osseous junction. Neither TRACP staining of bone sections or preliminary bone histomorphometry revealed any difference in the number of osteoclasts in the proximal tibia of ADAM12-S transgenic mice compared with littermate controls (unpublished results). However, an increase in the number of chondrocytes incorporating BrdU, an established marker of cell proliferation, was observed and may well explain the expansion of the hypertrophic zone in ADAM12-S transgenic mice.

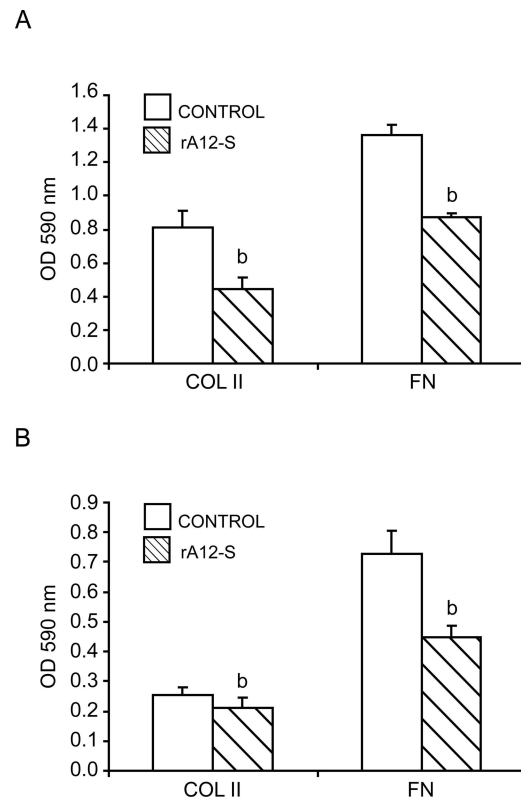
To study the effect of ADAM12 on chondrocyte proliferation in more detail, we tested the effect of treating murine chondrocytes with purified recombinant ADAM12-S protein *in vitro*. However, no difference in proliferation was



**FIG. 5.** Endogenous ADAM12 interacts with integrins in chondrocytes. (A and B) Immunocytochemical staining of  $\beta 1$  integrin in nonpermeabilized  $\beta 1$  integrin-expressing chondrocytes ( $\beta 1^{+/+}$ ) or  $\beta 1$  integrin-deficient ( $\beta 1^{-/-}$ ) chondrocytes in suspension. (C and D) Immunocytochemical staining of ADAM12 in permeabilized adherent  $\beta 1$  integrin-expressing chondrocytes ( $\beta 1^{+/+}$ ) or  $\beta 1$  integrin-deficient ( $\beta 1^{-/-}$ ) chondrocytes. (E and F) Immunocytochemical staining of ADAM12 in nonpermeabilized  $\beta 1$  integrin-expressing ( $\beta 1^{+/+}$ ) chondrocytes or  $\beta 1$  integrin-deficient ( $\beta 1^{-/-}$ ) chondrocytes in suspension.

detected when comparing ADAM12-S- and vehicle-treated cells. This discrepancy between *in vivo* and *in vitro* findings may indicate that the effect of ADAM12-S on chondrocyte proliferation seen in the growth plate of ADAM12-S transgenic mice requires additional factors present only *in vivo*. ADAM12-S is an active metalloprotease<sup>(5)</sup>; thus, it is possible that in the ADAM12-S transgenic mice, ADAM12-S stimulates chondrocyte proliferation indirectly by cleaving IGFBPs or other unidentified substrates, thereby stimulating growth factors.

The indication that ADAM12 may mediate its effect on the skeleton, at least in part, through its metalloprotease activity is strongly supported by the observation that expression of a truncated form of ADAM12-S, lacking the entire pro- and metalloprotease domains, caused no difference in bone length. Moreover, ADAM12 does not seem to be essential for proper regulation of bone length, because no abnormal femur length was seen in ADAM12-deficient mice. However, a certain redundancy between different ADAM proteins has previously been shown<sup>(24)</sup> and may account for the apparently normal bone growth in ADAM12-deficient mice.



**FIG. 6.** Recombinant ADAM12-S inhibits chondrocyte attachment *in vitro*. Attachment of (A)  $\beta 1$  integrin-expressing chondrocytes ( $\beta 1^{+/+}$ ) or (B)  $\beta 1$  integrin-deficient chondrocytes ( $\beta 1^{-/-}$ ) after 1-h treatment with 2  $\mu$ g/ml purified recombinant ADAM12-S (rA12-S) or vehicle control, followed by 1-h attachment on plates coated with 10  $\mu$ g/ml fibronectin (FN) or collagen type II (Col II). The quantitations represent the average measurement at OD 590 nm of six different wells after crystal violet staining and a representative of three independent experiments is shown. <sup>b</sup>*p* < 0.01.

ADAM12-S exhibits other functions beyond its proteolytic activity. For example, it is known to interact with cell surface adhesion molecules, including syndecans and integrins.<sup>(10–12,41)</sup> Integrins are highly expressed on chondrocytes, where they mediate adhesion to cartilage matrix proteins.<sup>(38)</sup> Cell-ECM interactions in the growth plate are believed to regulate, in concert with growth factors and cytokines, chondrocyte proliferation and maturation.<sup>(30,39)</sup> Accordingly, specific inactivation of the  $\beta 1$  integrin gene in chondrocytes causes a severe chondrodysplasia and shortening of the long bones, due in part to loss of adhesion to collagen type II and fibronectin, as well as decreased chondrocyte proliferation.<sup>(33)</sup> Interestingly, the decreased chondrocyte proliferation in these mice is partially caused by overexpression of Fgfr3. With this in mind, we tested whether ADAM12 may mediate its effect on chondrocytes by modulating integrin functions. Both RT-PCR and Western blot analysis revealed the expression of ADAM12 in murine chondrocytes; however, little ADAM12 was detected by immunocytochemistry. In contrast, clear immunostaining was observed in chondrocytes derived from mice with a targeted deletion of  $\beta 1$  integrin, indicating that an



endogenous interaction between ADAM12 and  $\beta 1$  integrin somehow regulates the bioavailability of ADAM12 in chondrocytes and thereby possibly its functions.

The hypothesis that ADAM12 may exert integrin-dependent functions in the growth plate is supported by in vitro studies showing that ADAM12-S inhibits cell adhesion on both fibronectin and collagen type II. No significant effect was seen when cells were treated with a catalytically dead ADAM12-S mutant, suggesting that ADAM12 protease activity is somehow involved.  $\beta 1$  integrin heterodimers are thought to serve as receptors for fibronectin and collagen type II in chondrocytes.<sup>(38,48)</sup> However, ADAM12 still inhibited adhesion to both fibronectin and collagen type II in  $\beta 1$  integrin-deficient chondrocytes, indicating that other cell adhesion receptors are involved. This confirms our recent findings showing that loss of  $\beta 1$  integrin-ADAM12 interactions in  $\beta 1$  integrin-deficient chondrocytes can be compensated for by other integrins, including  $\beta 3$  integrin.<sup>(41)</sup>

In conclusion, the naturally occurring soluble form of human ADAM12 stimulates longitudinal bone growth in mice by mechanisms requiring an intact metalloprotease domain. In addition, molecular interactions between ADAM12 and  $\beta 1$  integrins may likely change cell-matrix contacts in the growth plate, which can modulate both chondrocyte proliferation and differentiation, and consequently, longitudinal bone growth.

## ACKNOWLEDGMENTS

The authors thank Dr Reinhard Fassler for providing the chondrocytes and Dr Atsuko Sehara-Fujisawa for providing the ADAM12-deficient mice. We also thank Signe Breum, Brit Valentin, and Jacqueline Tybjerg for technical assistance and Linda Raab and Dana Beitner-Johnson for editing the manuscript. UW was supported by the Danish Cancer Society, the Danish Medical Research Council, Dansk Kræftforskningsfond, and the Neye, Velux, Novo Nordisk, Lundbeck, Munksholm, Friis, and Thaysen Foundations. MK was supported by a postdoctoral fellowship from the Danish Medical Research Council and by the Novo Nordisk and Haensch Foundations.

## REFERENCES

1. Wewer UM, Engvall E, Albrechtsen R 2005 ADAM12: The long and the short of it. In: Hooper NM, Lendeckel U (eds.) *The ADAM Family of Proteases, Proteases in Biology and Disease*, vol. 4. Springer, Dordrecht, The Netherlands, pp. 123–146.
2. Blobel CP 2005 ADAMs: Key components in EGFR signalling and development. *Nat Rev Mol Cell Biol* **6**:32–43.
3. Gilpin BJ, Loechel F, Mattei MG, Engvall E, Albrechtsen R, Wewer UM 1998 A novel, secreted form of human ADAM 12 (meltrin alpha) provokes myogenesis in vivo. *J Biol Chem* **273**:157–166.
4. Loechel F, Gilpin BJ, Engvall E, Albrechtsen R, Wewer UM 1998 Human ADAM 12 (meltrin alpha) is an active metalloprotease. *J Biol Chem* **273**:16993–16997.
5. Loechel F, Fox JW, Murphy G, Albrechtsen R, Wewer UM 2000 ADAM 12-S cleaves IGFBP-3 and IGFBP-5 and is inhibited by TIMP-3. *Biochem Biophys Res Commun* **278**:511–515.
6. Roy R, Wewer UM, Zurakowski D, Pories SE, Moses MA 2004 ADAM 12 cleaves extracellular matrix proteins and correlates with cancer status and stage. *J Biol Chem* **279**:51323–51330.
7. Asakura M, Kitakaze M, Takashima S, Liao Y, Ishikura F, Yoshinaka T, Ohmoto H, Node K, Yoshino K, Ishiguro H, Asanuma H, Sanada S, Matsumura Y, Takeda H, Beppu S, Tada M, Hori M, Higashiyama S 2002 Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: Metalloproteinase inhibitors as a new therapy. *Nat Med* **8**:35–40.
8. Kurisaki T, Masuda A, Sudo K, Sakagami J, Higashiyama S, Matsuda Y, Nagabukuro A, Tsuji A, Nabeshima Y, Asano M, Iwakura Y, Sehara-Fujisawa A 2003 Phenotypic analysis of Meltrin alpha (ADAM12)-deficient mice: Involvement of Meltrin alpha in adipogenesis and myogenesis. *Mol Cell Biol* **23**:55–61.
9. Ito N, Nomura S, Iwase A, Ito T, Kikkawa F, Tsujimoto M, Ishiura S, Mizutani S 2004 ADAMs, a disintegrin and metalloproteinases, mediate shedding of oxytocinase. *Biochem Biophys Res Commun* **314**:1008–1013.
10. Iba K, Albrechtsen R, Gilpin B, Frohlich C, Loechel F, Zolkiewska A, Ishiguro K, Kojima T, Liu W, Langford JK, Sanderson RD, Brakebusch C, Fassler R, Wewer UM 2000 The cysteine-rich domain of human ADAM 12 supports cell adhesion through syndecans and triggers signaling events that lead to beta1 integrin-dependent cell spreading. *J Cell Biol* **149**:1143–1156.
11. Thodeti CK, Albrechtsen R, Grauslund M, Asmar M, Larsson C, Takada Y, Mercurio AM, Couchman JR, Wewer UM 2003 ADAM12/syndecan-4 signaling promotes beta 1 integrin-dependent cell spreading through protein kinase Calpha and RhoA. *J Biol Chem* **278**:9576–9584.
12. Thodeti CK, Frohlich C, Nielsen CK, Holck P, Sundberg C, Kveiborg M, Mahalingam Y, Albrechtsen R, Couchman JR, Wewer UM 2005 Hierarchy of ADAM12 binding to integrins in tumor cells. *Exp Cell Res* **309**:438–450.
13. Kurisaki T, Masuda A, Osumi N, Nabeshima Y, Fujisawa-Sehara A 1998 Spatially- and temporally-restricted expression of meltrin alpha (ADAM12) and beta (ADAM19) in mouse embryo. *Mech Dev* **73**:211–215.
14. Yagami-Hiromasa T, Sato T, Kurisaki T, Kamijo K, Nabeshima Y, Fujisawa-Sehara A 1995 A metalloprotease-disintegrin participating in myoblast fusion. *Nature* **377**:652–656.
15. Kawaguchi N, Xu X, Tajima R, Kronqvist P, Sundberg C, Loechel F, Albrechtsen R, Wewer UM 2002 ADAM 12 protease induces adipogenesis in transgenic mice. *Am J Pathol* **160**:1895–1903.
16. Masaki M, Kurisaki T, Shirakawa K, Sehara-Fujisawa A 2005 Role of meltrin {alpha} (ADAM12) in obesity induced by high-fat diet. *Endocrinology* **146**:1752–1763.
17. Kronqvist P, Kawaguchi N, Albrechtsen R, Xu X, Schroder HD, Moghadaszadeh B, Nielsen FC, Frohlich C, Engvall E, Wewer UM 2002 ADAM12 alleviates the skeletal muscle pathology in mdx dystrophic mice. *Am J Pathol* **161**:1535–1540.
18. Abe E, Mocharla H, Yamate T, Taguchi Y, Manolagas SC 1999 Meltrin-alpha, a fusion protein involved in multinucleated giant cell and osteoclast formation. *Calcif Tissue Int* **64**:508–515.
19. Bartholin L, Destaing O, Forissier S, Martel S, Maguer-Satta V, Jurdic P, Rimokh R 2005 FLRG, a new ADAM12-associated protein, modulates osteoclast differentiation. *Biol Cell* **97**:577–588.
20. Boissy P, Lenhard TR, Kirkegaard T, Peschon JJ, Black RA, Delaisse JM, del Carmen OM 2003 An assessment of ADAMs in bone cells: Absence of TACE activity prevents osteoclast recruitment and the formation of the marrow cavity in developing long bones. *FEBS Lett* **553**:257–261.
21. Inoue D, Reid M, Lum L, Kratzschmar J, Weskamp G, Myung YM, Baron R, Blobel CP 1998 Cloning and initial character-

- ization of mouse meltrin beta and analysis of the expression of four metalloprotease-disintegrins in bone cells. *J Biol Chem* **273**:4180–4187.
22. Harris HA, Murrills RJ, Komm BS 1997 Expression of meltrin-alpha mRNA is not restricted to fusagenic cells. *J Cell Biochem* **67**:136–142.
  23. Verrier S, Hogan A, McKie N, Horton M 2004 ADAM gene expression and regulation during human osteoclast formation. *Bone* **35**:34–46.
  24. Horiuchi K, Zhou HM, Kelly K, Manova K, Blobel CP 2005 Evaluation of the contributions of ADAMs 9, 12, 15, 17, and 19 to heart development and ectodomain shedding of neuregulins beta1 and beta2. *Dev Biol* **283**:459–471.
  25. Laigaard J, Sorensen T, Frohlich C, Pedersen BN, Christiansen M, Schiott K, Uldbjerg N, Albrechtsen R, Clausen HV, Ottesen B, Wewer UM 2003 ADAM12: A novel first-trimester maternal serum marker for Down syndrome. *Prenat Diagn* **23**:1086–1091.
  26. Shi Z, Xu W, Loechel F, Wewer UM, Murphy LJ 2000 ADAM 12, a disintegrin metalloprotease, interacts with insulin-like growth factor-binding protein-3. *J Biol Chem* **275**:18574–18580.
  27. Wewer UM, Morgelin M, Holck P, Jacobsen J, Lydolph MC, Johnsen AH, Kveiborg M, Albrechtsen R 2006 ADAM12 is a four-leafed clover -the excised prodomain remains bound to the mature enzyme. *J Biol Chem* **281**:9418–9422.
  28. Sundberg C, Thodeti CK, Kveiborg M, Larsson C, Parker P, Albrechtsen R, Wewer UM 2004 Regulation of ADAM12 cell-surface expression by protein kinase C epsilon. *J Biol Chem* **279**:51601–51611.
  29. Wennerberg K, Lohikangas L, Gullberg D, Pfaff M, Johansson S, Fassler R 1996 Beta 1 integrin-dependent and -independent polymerization of fibronectin. *J Cell Biol* **132**:227–238.
  30. Karsenty G, Wagner EF 2002 Reaching a genetic and molecular understanding of skeletal development. *Dev Cell* **2**:389–406.
  31. Le Pabic H, Bonnier D, Wewer UM, Coutand A, Musso O, Baffet G, Clement B, Theret N 2003 ADAM12 in human liver cancers: TGF-beta-regulated expression in stellate cells is associated with matrix remodeling. *Hepatology* **37**:1056–1066.
  32. Livak KJ, Schmittgen TD 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). *Method Methods* **25**:402–408.
  33. Aszodi A, Hunziker EB, Brakebusch C, Fassler R 2003 Beta1 integrins regulate chondrocyte rotation, G1 progression, and cytokinesis. *Genes Dev* **17**:2465–2479.
  34. Kawaguchi N, Sundberg C, Kveiborg M, Moghadaszadeh B, Asmar M, Dietrich N, Thodeti CK, Nielsen FC, Moller P, Mercurio AM, Albrechtsen R, Wewer UM 2003 ADAM12 induces actin cytoskeleton and extracellular matrix reorganization during early adipocyte differentiation by regulating beta1 integrin function. *J Cell Sci* **116**:3893–3904.
  35. Lee CC, Pons F, Jones PG, Bies RD, Schlang AM, Leger JJ, Caskey CT 1993 Mdx transgenic mouse: Restoration of recombinant dystrophin to the dystrophic muscle. *Hum Gene Ther* **4**:273–281.
  36. Kronenberg HM 2003 Developmental regulation of the growth plate. *Nature* **423**:332–336.
  37. Ortega N, Behonick DJ, Werb Z 2004 Matrix remodeling during endochondral ossification. *Trends Cell Biol* **14**:86–93.
  38. Loeser RF 2000 Chondrocyte integrin expression and function. *Biorheology* **37**:109–116.
  39. Behonick DJ, Werb Z 2003 A bit of give and take: The relationship between the extracellular matrix and the developing chondrocyte. *Mech Dev* **120**:1327–1336.
  40. Huang J, Bridges LC, White JM 2005 Selective modulation of integrin-mediated cell migration by distinct ADAM family members. *Mol Biol Cell* **16**:4982–4991.
  41. Thodeti CK, Frohlich C, Nielsen CK, Takada Y, Fassler R, Albrechtsen R, Wewer UM 2005 ADAM12-mediated focal adhesion formation is differently regulated by beta1 and beta3 integrins. *FEBS Lett* **579**:5589–5595.
  42. Tian BL, Wen JM, Zhang M, Xie D, Xu RB, Luo CJ 2002 The expression of ADAM12 (meltrin alpha) in human giant cell tumours of bone. *Mol Pathol* **55**:394–397.
  43. Nilsson A, Ohlsson C, Isaksson OG, Lindahl A, Isgaard J 1994 Hormonal regulation of longitudinal bone growth. *Eur J Clin Nutr* **48**(Suppl 1):S150–S158.
  44. Le Roith D, Bondy C, Yakar S, Liu JL, Butler A 2001 The somatomedin hypothesis: 2001. *Endocr Rev* **22**:53–74.
  45. Chagin AS, Lindberg MK, Andersson N, Moverare S, Gustafsson JA, Savendahl L, Ohlsson C 2004 Estrogen receptor-beta inhibits skeletal growth and has the capacity to mediate growth plate fusion in female mice. *J Bone Miner Res* **19**:72–77.
  46. Lindberg MK, Alatalo SL, Halleen JM, Mohan S, Gustafsson JA, Ohlsson C 2001 Estrogen receptor specificity in the regulation of the skeleton in female mice. *J Endocrinol* **171**:229–236.
  47. Deng C, Wynshaw-Boris A, Zhou F, Kuo A, Leder P 1996 Fibroblast growth factor receptor 3 is a negative regulator of bone growth. *Cell* **84**:911–921.
  48. Scully SP, Lee JW, Ghert PMA, Qi W 2001 The role of the extracellular matrix in articular chondrocyte regulation. *Clin Orthop Relat Res* **S72**–S89.

Address reprint requests to:  
 Marie Kveiborg, PhD  
 Institute of Molecular Pathology  
 University of Copenhagen  
 Frederik V's vej 11  
 2100 Copenhagen, Denmark  
 E-mail: kveiborg@pai.ku.dk

Received in original form January 11, 2006; revised form March 21, 2006; accepted May 11, 2006.