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Human monoclonal antibody fragments targeting matrilin-3 in growth plate cartilage

Crystal Sao-Fong Cheung¹, Zhongyu Zhu², Julian Chun-Kin Lui¹, Dimiter Dimitrov², and Jeffrey Baron¹

¹Section on Growth and Development, National Institute of Child Health and Development, National Institutes of Health, Bethesda, Maryland, USA

²Cancer and Inflammation Program, National Cancer Institute, National Institutes of Health, Frederick, Maryland, USA

Abstract

Purpose—Many genetic disorders, including chondrodysplasias, and acquired disorders impair growth plate function, resulting in short and sometimes malformed bones. There are multiple endocrine and paracrine factors that promote chondrogenesis at the growth plate, which could potentially be used to treat these disorders. Targeting these growth factors specifically to the growth plate might augment the therapeutic skeletal effect while diminishing undesirable effects on non-target tissues.

Methods—Using yeast display technology, we selected singlechain variable antibody fragments that bound to human and mouse matrilin-3, an extracellular matrix protein specifically expressed in cartilage tissue. The ability of the selected antibody fragments to bind matrilin-3 and to bind cartilage tissue in vitro and in vivo was assessed by ELISA and immunohistochemistry.

Results—We identified antibody fragments that bound matrilin-3 with high affinity and also bound with high tissue specificity to cartilage homogenates and to cartilage structures in mouse embryo sections. When injected intravenously in mice, the antibody fragments specifically homed to cartilage.

Conclusions—Yeast display successfully selected antibody fragments that are able to target cartilage tissue in vivo. Coupling these antibodies to chondrogenic endocrine and paracrine signaling molecules has the potential to open up new pharmacological approaches to treat childhood skeletal growth disorders.

Keywords

scFv; yeast display; skeletal diseases; childhood growth; drug targeting

Introduction

The growth plate is a specialized cartilage structure present near the ends of tubular bones and vertebrae. The primary function of the growth plate is to generate new cartilage, which is then remodeled into bone tissue, leading to bone elongation. Longitudinal bone growth is a complex process which requires multiple intracellular, endocrine, and paracrine pathways to function normally. Consequently, mutations in hundreds of genes that are required for growth plate function give rise to disorders of skeletal growth, including the skeletal dysplasias, in which the bones are short and malformed, causing major disability. In addition to genetic disorders, acquired endocrine, nutritional, or inflammatory disorders can also impair bone growth at the growth plate, resulting in severe short stature.

Current treatment options for growth disorders are limited. Recombinant human growth hormone (GH) is used for both GH-deficiency and certain non-GH-deficient causes of short stature. However, the efficacy of GH treatment is often suboptimal. Even for growth hormone deficiency, the reported adult heights achieved in the majority of patients after GH supplementation remain below the normal range (1). In non-GH deficient conditions, including skeletal dysplasias, the efficacy is typically even more partial (2). Moreover, GH treatment carries a risk of increased intracranial pressure (3), slipped capital femoral epiphysis (4), insulin resistance (5,6), and possibly type II diabetes mellitus (7,8). Endogenous GH excess increases the risk of colon cancer; whether or not childhood GH treatment raises cancer risk in adulthood is not known (9). Because systemic administration of GH has limited efficacy and significant known and potential adverse effects, better treatments for growth plate disorders are needed.

Recent studies have identified many paracrine factors that positively regulate growth plate chondrogenesis and therefore might be used therapeutically, including Indian Hedgehog (IHH) (10, 11, 12, 13, 14), bone morphogenetic proteins (BMPs) (15, 16, 17, 18, 19), C-type natriuretic peptide (CNP) (20, 21, 22, 23, 24), and WNTs (25, 26, 27, 28). However, the development of these molecules into effective treatment has been hampered by their mechanism of action; these growth factors are produced locally and act locally in the growth plate, and thus do not lend themselves to systemic therapeutic approaches. We envision that these locally-acting molecules could be targeted to the growth plate by linking them to cartilage-binding proteins, such as antibody fragments. When administered systemically, these hybrid molecules would be preferentially taken up by growth plate cartilage, and thus might greatly augment the therapeutic effect on the target organ while diminishing adverse effects due to action on other tissues.

Similarly, growth-promoting endocrine factors, such as GH and insulin-like growth factor-I (IGF-I) might be linked to cartilage-binding polypeptides and thereby targeted to the growth plate. Targeted endocrine therapy could potentially enhance the therapeutic effects on chondrogenesis and reduce effects on non-target tissues, thereby decreasing risks such as malignancy and diabetes mellitus.

To develop cartilage-targeting therapy, we sought to identify polypeptides that home to cartilage tissue. We employed a yeast display human antibody library and selected high-

affinity binders to matrilin-3, an extracellular matrix protein expressed with high tissue specificity in cartilage (29, 30, 31). We identified three antibody fragments that bind with high affinity both to human and mouse matrilin-3, as well as to cartilage tissue in vitro. When these antibody fragments were administered to mice by tail-vein injection, they homed specifically to cartilage tissue. Coupling these antibody fragments to endocrine and paracrine factors that stimulate chondrogenesis could be used to direct these potent molecules specifically to cartilage tissue, providing important new therapeutic approaches to the treatment of growth plate disorders.

Material and methods

Selection of Extracellular Matrix Protein-Binding Antibody Fragments

Recombinant human and mouse matrilin-3 proteins that were biotinylated using an Avi-tag Specific Biotinylation kit (Aurora, CO) were used as the target for selection. In the first round of selection, approximately 5×10^{10} cells from the naïve antibody library were incubated with 10 μ g of biotinylated human matrilin-3 in 50 ml 0.1% bovine serum albumin (BSA)-phosphate-buffered saline (PBS), called PBSA, at room temperature for 2 h with gentle rotation. Then, the mixture was washed three times with 0.1% PBSA to remove unbound antibody fragments. Biotinylated matrilin-3 together with bound antibody fragments were subsequently incubated with 100 μ l of streptavidin-conjugated microbeads (Milenvi Biotec, Auburn, CA) and loaded onto the AutoMACS system for sorting. Cells which displayed antibody fragments with high affinity to matrilin-3 were collected and later amplified in SDCAA Medium (20 g Dextrose, 6.7 g Difco yeast nitrogen base without amino acids, 5 g Bacto casamino acids, 5.4 g Na₂HPO₄ and 8.56 g NaH₂PO₄. H₂O dissolved in 1 L of distilled water) at 250 rpm at 30°C for 24 h. After that, the culture was induced in SGCAA Medium (20 g Galactose, 20 g Raffinose, 1 g Dextrose, 6.7 g Difco yeast nitrogen base without amino acids, 5 g Bacto casamino acids, 5.4 g Na₂HPO₄ and 8.56 g NaH₂PO₄. H₂O dissolved in 1 L of distilled water) at 250 rpm at 20°C for 18 h. The pool obtained was subjected to another round of selection for binding to human matrilin-3. To ensure sufficient diversity of antibody fragments for second and third rounds of screening, the input cell number was increased by 100 fold compared to the prior round of sorting.

For the third round of selection, His-Tagged recombinant mouse matrilin-3 was employed. The screening was carried out in a similar way to the previous two rounds of selection toward human matrilin-3. Finally, antibody fragments that bound to mouse matrilin-3 were pulled down by anti-His-tag antibody-conjugated microbeads. The yeast cells expressing antibody fragments that possess high binding affinity to human and mouse matrilin-3 proteins were collected.

Cloning of Extracellular Matrix Protein-Binding Antibody Fragments in Mammalian Vectors

After the final round of cell sorting, DNA plasmids were extracted from the yeast cells using Yeast Plasmid Extraction Kit (Zymo Research, Irvine, CA) and then transformed into 10G chemical competent *E. coli* (Lucigen, Middleton, WI) for further amplification. The scFv-encoding DNA inserts were double-digested with restriction enzyme SfiI and ligated to a

modified pSecTagB vector, which bears the same set of SfiI sites and a downstream Fc-Avi tag.

Cell Culture

HEK293T cells were obtained from ATCC (Manassas, VA), and maintained in Dulbecco's modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (v/v) (Gibco) and 1% penicillin-streptomycin (PS) (v/v) (Gibco) at 37°C in a humidified atmosphere of 5% CO₂.

FreeStyle 293-F cells (Invitrogen, Grand Island, NY) were cultured in suspension in FreeStyle 293 Expression Medium (Gibco) shaking at 125 rpm at 37°C in a humidified atmosphere of 8% CO₂.

Small-Scale Expression of Antibody Fragments in HEK293T Cells

HEK293T cells were seeded in 24-well plates at a density of 5×10^4 cells/ well, and cultured at 37°C in 5% CO₂ overnight. On the next day, 0.5 µg of DNA and 5 µl of PolyFect reagent (Qiagen, Valencia, CA) were suspended in serum-free, antibiotic-free DMEM to give a final volume of 30 µl, which was allowed to stand at room temperature for 10 min. Then, the mixture was introduced to the wells containing cultured cells and 375 µl of fresh medium. At 48 h post-transfection, supernatant containing the secreted antibody fragments was collected for subsequent experiments.

Assessment of the Binding Ability and Specificity of Antibody Fragments

To assess the ability of the 36 selected clones of antibody fragments to bind to purified matrilin-3, 100 µl of human or mouse matrilin-3 protein (2 µg/ml) was coated onto 96-well plates at 4°C overnight. To assess binding to cartilage and non-cartilaginous tissues, heart, liver, lung, kidney, spleen, small intestine, muscle, and distal femoral and proximal tibial growth plate were dissected from 4-day-old C57BL/6 mice and homogenized in protein lysis buffer (150 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA (pH 8.0), 1% Triton X-100, 0.1% SDS) at 4°C. Tissue debris was removed by centrifugation. 100 µl (30 µg) of tissue lysate was coated onto 96-well plates at 4°C overnight. Each antibody clone was tested in triplicate wells. After blocking with 3% non-fat milk (200 µl/ well), 50 µl of the culture supernatant was introduced to each well and incubated at room temperature for 2 h. Supernatant from cells transfected with a non-specific antibody fragment (selected for binding to a protein of Dengue virus, an irrelevant protein target) was included as a negative control, while a commercial anti-matrilin-3 polyclonal antibody recognizing a 13-amino acid peptide from near the center of human matrilin-3 (Thermo Scientific, Rockford, IL) served as a positive control. The wells were then washed with 0.05% Tween-phosphate-buffered saline (PBST) four times and incubated with 50 µl of horseradish peroxidase (HRP)-conjugated anti-Fc antibody (Millipore, Temecula, CA) (diluted 1:5000 in 3% non-fat milk) at room temperature for 1 h. Finally, tetramethylbenzidine (TMB) substrate reagent (eBioscience, San Diego, CA) was added and the reaction was stopped by adding H₂SO₄ before absorbance was read at 450 nm.

Examination of Binding of Selected Antibody Fragments in Mouse Embryo Sections

Ten micrometers cryosections of frozen E15 mouse embryos were equilibrated to room temperature for 30 min, fixed in acetone for 15 min, air-dried for 30 min, and then blocked with 1% FCS in PBS at room temperature for 1 h. The sections were then incubated for 1 h with 100 μ l of the supernatant from transfected HEK293T cells expressing antibody fragments, washed with PBS for 5 min three times, incubated for 1 h with 100 μ l of HRP-conjugated anti-Fc antibody (1:2000 dilution in PBS containing 1% FCS), and washed with PBS for 5 min three times, all at room temperature. Binding of the selected antibody fragments to sections was detected using DAB substrate kit (Abcam, Cambridge, MA). An antibody fragment selected for binding to a protein of Dengue virus served as a negative control.

Large-Scale Expression of Selected Antibody Fragments in 293 FreeStyle-F Cells

For production and purification, antibody fragments were expressed in 293 FreeStyle-F, a suspension cell line which is adapted to serum-free medium and thus avoids serum IgG which may interfere with antibody fragment purification.

Twenty four hours before transfection, 3×10^7 293 FreeStyle-F cells were resuspended in 28 ml of FreeStyle 293 Expression Medium in a 250 ml Erlenmeyer flask on an orbital shaker rotating at 125 rpm, at 37°C in 8% CO₂. On the day of transfection, 30 μ g of DNA and 60 μ l of 293fectin reagent (Qiagen) were diluted in 2 ml of Opti-MEM, and the mixture was incubated at room temperature for 20 min. Afterward, the mixture was introduced to a flask containing the 293 FreeStyle-F cells in 28 ml medium and shaken for 3 days to express soluble protein fragments. The supernatant was subsequently collected for antibody purification using protein A columns.

Purification of Antibody Fragments by Protein A Column

Protein A resin (GenScript, Piscataway, NJ) slurry (2 ml) was packed into a glass column, and equilibrated with 50 ml of binding/ washing buffer (0.15 M NaCl, 20 mM Na₂HPO₄, pH 8.0). Culture supernatant was loaded onto the column. Unbound proteins were washed away with 100 ml of binding/ washing buffer. Bound antibodies were then eluted with 8 ml of elution buffer (100 mM acetic acid, pH 3.0). The eluate was neutralized by 1/10 volume of neutralization buffer (1 M Tris-HCl, pH 9.0) and dialyzed against 100 volumes of PBS at 4°C overnight. The purity of the antibodies was checked by SDS-PAGE (Figure S1).

Measurement of the Binding Affinity of Antibody Fragments

To assess the binding affinity of the purified antibody fragments, 100 μ l of human or mouse matrilin-3 protein (2 μ g/ml) was coated onto 96-well plates at 4°C overnight. Then, the wells were blocked with 200 μ l of 3% non-fat milk in PBS at room temperature for 2 h. 50 μ l of different concentrations of antibody fragments 13, 22, and 26 (230 nM, 57.5 nM, 14.375 nM, 3.594 nM, 0.899 nM, 0.225 nM, 0.056 nM, 0.014 nM) was added to incubate with the target at room temperature for 2 h. Duplicates were performed for each concentration of the selected antibody fragments. After binding, the wells were washed with 0.05% PBST four times and incubated with 50 μ l of HRP-conjugated anti-Fc antibody (diluted 1:5000 in 3%

non-fat milk) at room temperature for 1 h. Lastly, TMB substrate reagent was introduced and reaction was stopped by H₂SO₄ before absorbance was read at 450 nm.

Investigation of the In Vivo Homing Ability of Antibody Fragments

In order to evaluate whether the selected antibodies were able to home to growth plate cartilage, 50 µg of purified antibody fragments, diluted in 100 µl of sterile saline, was injected in 3-week-old C57BL/6 male mice intravenously. As control experiments, two groups of mice were injected with 100 µl of sterile saline or 50 µg (in 100 µl of sterile saline) of purified non-specific antibody fragment (selected for binding to a protein of Dengue virus). After 24 h, tissues were harvested and homogenized in protein lysis buffer. Tissue debris was removed by centrifugation, and supernatant was collected for ELISA to check for the localization of the antibody fragments. Briefly, 100 µl (30 µg) of tissue lysate was coated in triplicate wells in 96-well plates at 4°C overnight. Subsequent to blocking with 200 µl of 3% non-fat milk per well, 50 µl of HRP-conjugated anti-Fc antibody (diluted 1:5000 in 3% non-fat milk) was added to each well and incubated at room temperature for 1 h. TMB substrate reagent was then introduced and the reaction was stopped by adding H₂SO₄ before the absorbance was read at 450 nm. The detection limit of the ELISA (mean + 3 SD of growth plate lysate without antibody fragments) was 0.13, 0.22, 0.11 ng for antibody fragment 13, 22, and 26 respectively, which corresponded to an OD₄₀₅ of 0.057. For each organ or tissue in a particular group, statistical significance was assessed by ANOVA, followed by pairwise comparison of the growth plate signal to other tissues, with Holm-Sidak correction for multiple comparisons. Comparison of the growth plate signal between different groups were done by Student t-test.

Quantitative Histology of 6-week Old Mouse Growth Plate

Quantative histology was performed as previously described (32) with minor modifications. Formalin-fixed proximal tibiae were decalcified in 0.5 M EDTA, and embedded in paraffin. For each animal, 6 longitudinal sections (10-µm thickness, separated by at least 50 µm) were stained with Masson Trichrome and scanned with an Aperio ScanScope CS digital scanner. In each of the 6 sections, we measured proliferative and hypertrophic zone height (parallel to the chondrocyte columns) at 5 locations within the central two-thirds of the growth plate and averaged the 30 measurements.

Results

Selection of extracellular matrix protein-specific antibody fragments

Antibody fragments were selected from a large yeast display single-chain variable fragment (scFv) library for binding to matrilin-3, an extracellular matrix protein specifically expressed in cartilage (29, 30, 31). The sorting was carried out against human recombinant matrilin-3 protein in the first two rounds and mouse recombinant matrilin-3 protein in the third round of panning to increase the likelihood that the resulting antibody fragments would bind both to mouse cartilage matrix for preclinical safety and efficacy evaluations and human cartilage matrix, for clinical applications. After three rounds of selection, the enriched library exhibited a striking increase in binding to matrilin-3 of both species by flow cytometric analysis (Fig. 1a).

Binding of antibody fragments to cartilage matrix in vitro

Following the final round of selection, 36 yeast clones were randomly selected and the expression plasmids were isolated. The DNA sequences encoding the scFvs were then excised and subcloned into a mammalian expression vector pSecTagB, which was previously engineered to include a human Fc fragment-coding DNA sequence. The resulting construct consisted of a scFv antibody fragment fused with a human IgG1 Fc fragment. This construct was subsequently transfected into HEK293T cells for 48 h to express scFv-Fc proteins, and the ability of these 36 proteins to bind both human and mouse recombinant matrilin-3 was assessed by ELISA. Of these, 21 bound to human recombinant matrilin-3 (Fig. 1b, upper panel), and 17 bound to mouse recombinant matrilin-3 (Fig. 1b, lower panel), compared to bovine serum albumin and to a non-specific antibody fragment.

Binding of antibody fragments to cartilage extracts in vitro

To examine the tissue specificity of the antibody fragments, we used homogenized tissue from the growth plate cartilage, brain, heart, liver, lung, kidney, spleen, small intestine, and muscle from 4-day old mice to coat plastic wells and then assessed binding of the selected antibody fragments by ELISA. Fifteen antibody fragments that previously showed increased binding to both human and mouse matrilin-3 were examined for their specificity toward cartilage tissue. While most antibody fragments demonstrated poor tissue binding specificity, antibody fragments 13, 22, and 26 were found to preferentially bind to growth plate cartilage over other tissues (Fig. 1c), and thus were chosen for further studies. A non-specific antibody fragment showed little binding to cartilage or other tissues.

Immunohistochemical localization of matrilin-3-binding antibody fragments

To further characterize the ability and specificity of the antibody fragments to bind to cartilage tissue, we used the three antibody fragments (13,22,26) for immunohistochemical staining on frozen sections of C57BL/6 mouse embryos at embryonic day (E) 15. All three antibody fragments stained cartilage structures, such as the cartilaginous anlagen of the bones of the forelimb, rib cages, and vertebra, but there was no significant staining in non-cartilaginous tissues (Fig. 2). In contrast, a non-specific antibody fragment failed to show any staining in the cartilage tissues (data not shown).

Assessment of the binding affinity of purified matrilin-3-binding antibody fragments

To quantitatively assess binding affinity, we incubated either immobilized human or mouse matrilin-3 protein with different concentrations of purified antibody fragments 13, 22, and 26 and measured binding by ELISA. All three antibody fragments possessed high affinity towards both human and mouse matrilin-3 with EC50 values less than 1 nM (Fig. 3a, b).

Homing of matrilin-3-binding antibody fragments to cartilage in vivo

Purified antibody fragments were injected into mice via a tail vein. After 24 h, distal femoral and proximal tibial growth plate cartilage and various non-cartilaginous organs were isolated, homogenized and used to coat plastic wells. ELISA was performed using anti-human Fc antibody to detect the presence of antibody fragments delivered in vivo. For both the saline-injected and the non-specific antibody fragment-injected controls, the signals in

the growth plate and other non-cartilaginous organs were similar and below the ELISA detection limit. In contrast, for the three antibody fragments tested (13,22,26), the signals obtained in growth plate cartilage were significantly greater than the signals for saline or for non-specific antibody fragments (Fig. 4). Furthermore, for these three antibody fragments, the signals were significantly greater in cartilage than in non-cartilaginous organs (Fig. 4), suggesting that the selected antibody fragments homed to cartilage in vivo with high specificity.

Matrilin-3-binding antibody fragment administration in vivo showed no evidence of adverse effects on growth plate

To assess possible toxicity of the cartilage-binding antibody fragments to growth plate function in vivo, we injected antibody fragment 13, which showed the strongest binding, or non-targeting antibody fragment in 4-week old mice (n = 3 per group) every other day for 2 weeks (total of 6 injections, 30 ug each). Body weight, tibia length, proliferative zone height, and hypertrophic zone height at the end of the treatment period did not differ between mice that received antibody fragment 13 and those that received non-targeting antibody fragments (Fig. 5). However, preclinical assessment of the long-term safety of the antibody fragments would require extensive additional testing, including both growth plate-specific and systemic endpoints before clinical applications could be considered.

Discussion

We sought to identify antibody fragments that bind to cartilage with high affinity and specificity which could be used to target therapeutic molecules to growth plate cartilage. A naïve human scFv yeast display library was employed for selection of binders to matrilin-3, an extracellular matrix protein primarily expressed in growth plate cartilage. A sequential antigen panning approach was used (33), with the first two rounds of panning against human matrilin-3 and the last round against mouse matrilin-3. The resulting pool of clones was significantly enriched for binders to both human and mouse matrilin-3 proteins, compared to the naïve library. We identified individual clones that expressed antibody fragments that bound to both human and mouse matrilin-3. Of these, three antibody fragments showed specific binding in vitro to homogenates of cartilage tissue, but not homogenates of brain, heart, liver, lung, kidney, spleen, small intestine or muscle. These three fragments also showed tissue-specific binding to cartilage structures in sections of mouse embryos. Binding affinities of the selected antibody fragments 13, 22, and 26 were then assessed, demonstrating that all three purified antibody fragments exhibited high affinity for both human and mouse matrilin-3. Most importantly, after these antibody fragments were injected intravenously in mice, we found that they were specifically localized in cartilage and were not detectable in other tissues, including brain, heart, liver, lung, kidney, spleen, small intestine or muscle, indicating that the antibody fragments were capable of specifically targeting cartilage tissue in vivo.

The development of cartilage-targeting proteins opens up new potential approaches to treat growth plate disorders, including skeletal dysplasias, severe short stature due to systemic disease, and severe idiopathic short stature, by targeting growth-regulating endocrine factors

specifically to the growth plate. Current growth plate therapy generally involves the manipulation of systemic hormone levels, such as GH, IGF-I, estrogens, and androgens. However, these approaches have limited therapeutic efficacy for the more severe growth plate disorders, including many skeletal dysplasias, and exhibit undesirable effects due to actions on sites other than the growth plate. For instance, in achondroplasia, the most common type of skeletal dysplasia, growth hormone increases bone length, and therefore height, only modestly, and the dose is limited by adverse effects on other tissues (34). Coupling growth-regulating endocrine factors to cartilage-binding antibody fragments has the potential to direct endocrine therapeutic agents to cartilage. Our findings suggest that when administered systemically, the targeting antibody fragment-endocrine factor conjugates would be preferentially taken up by the growth plate cartilage, thereby creating a local depot which might allow sustained high local concentrations of molecules to improve efficacy at the growth plate and decrease adverse effects on other tissues.

In addition to endocrine factors, paracrine factors that stimulate growth plate chondrogenesis, including Indian Hedgehog (IHH) (10, 11, 12, 13, 14), bone morphogenetic proteins (BMPs) (15, 16, 17, 18, 19), and C-type natriuretic peptide (CNP) (20, 21, 22, 23, 24), could also be coupled to these antibody fragments. Because these paracrine factors are normally expressed and exert their action in the growth plate, targeted therapy would serve to localize these factors to their physiological site of action. For example, CNP is an important positive regulator of growth plate chondrogenesis (20, 21, 22, 23, 24). In mice, overexpression of CNP in growth plate compensates for mutations that cause achondroplasia (35). However, systemic administration of CNP in humans leads to natriuresis (36). CNP linked to a cartilage-targeting antibody fragments might increase the skeletal growth-promoting effect and reduce the effect on renal sodium handling, and so might emerge as an effective therapeutic approach to treat human achondroplasia. Similarly, targeting IHH or BMPs to growth plate cartilage might provide novel treatments for skeletal dysplasias or other causes of growth plate failure, such as systemic inflammatory diseases, renal failure, glucocorticoid therapy or radiation damage. Because matrilin-3 is expressed not only in growth plate cartilage but also in articular cartilage, this approach might also be used for targeted treatment of osteoarthritis and other articular disorders.

Targeting other tissues with peptides or antibody fragments have demonstrated promising results. Yokogawa and colleagues reported a short peptide of six aspartate residues that, upon conjugation to estradiol, was capable of localizing to bone in mice when administered systemically (37). Moreover, the peptide-estradiol complex could be retained in bone for an extended period of time, producing its biological effects primarily on bone tissue and minimizing effects on non-skeletal tissues (37). In addition, a ten-aspartate peptide was successfully utilized to direct alkaline phosphatase enzyme to bone matrix to treat hypophosphatasia in mice (38). This strategy was then applied successfully to treat children with severe hypophosphatasia in a recent clinical trial (39). Similarly, conjugation to a bisphosphonate has been used to target calcitonin analogues and osteoprotegerin successfully to bone matrix (40, 41, 42, 43).

There have also been some prior attempts to develop cartilage-binding peptides or polypeptides with the specific goal of treating articular cartilage disorders. A linear peptide

of six amino acids was reported to bind to collagen IIaI and direct nanoparticles to articular cartilage (44), when given locally by intraarticular injection. A scFv antibody fragment was identified that recognized reactive oxygen species-modified type II collagen, which is specifically present in inflamed joints (45). This scFv was then coupled to soluble tumor necrosis factor receptor II and administered systemically to reduce inflammation of articular cartilage in a murine arthritis model. We previously used 12-amino acid peptides to target growth plate cartilage (46) but were unable to demonstrate a high affinity and specificity for cartilage comparable to that achieved in the current study using antibody fragments.

Conclusion

In conclusion, we have identified antibody fragments that bind to cartilage matrix with high affinity and specificity and, when administered systemically in vivo, home specifically to cartilage structures. To our knowledge, ours is the first study to propose the concept of targeting growth plate for the treatment of linear growth disorders and the first study to produce an antibody capable of targeting normal cartilage after systemic administration. Coupling these cartilage-binding polypeptides to endocrine and paracrine signaling molecules that promote chondrogenesis could allow therapy targeted specifically to growth plate and articular cartilage, and thus open up broad new pharmacological approaches to treat skeletal dysplasias and other severe forms of linear growth failure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Ranke MB, Lindberg A, Mullis PE, Geffner ME, Tanaka T, Cutfield WS, Tauber M, and Dunger D. Towards optimal treatment with growth hormone in short children and adolescents: evidence and theses. *Horm Res Paediatr.* 79:51–67 (2013). [PubMed: 23446062]
2. Ranke MB. Human growth hormone therapy of non-growth hormone deficient children. *Pediatrician.* 14:178–182 (1987). [PubMed: 3331012]
3. Wilson TA, Rose SR, Cohen P, Rogol AD, Backeljauw P, Brown R, Hardin DS, Kemp SF, Lawson M, Radovick S, Rosenthal SM, Silverman L, and Speiser P. Update of guidelines for the use of growth hormone in children: the Lawson Wilkins Pediatric Endocrinology Society Drug and Therapeutics Committee. *J Pediatr.* 143:415–421 (2003). [PubMed: 14571209]
4. Wang SY, Tung YC, Tsai WY, Chien YH, Lee JS, and Hwu WL. Slipped capital femoral epiphysis as a complication of growth hormone therapy. *J Formos Med Assoc.* 106:S46–S50 (2007). [PubMed: 17493896]
5. Canete R, Valle M, Martos R, Sanchez-Carrion A, Canete MD, and van Donkelaar EL. Short-term effects of GH treatment on coagulation, fibrinolysis, inflammation biomarkers, and insulin resistance status in prepubertal children with GH deficiency. *Eur J Endocrinol.* 167:255–260 (2012). [PubMed: 22645201]

6. Darendeliler F, Karagiannis G, and Wilton P. Headache, idiopathic intracranial hypertension and slipped capital femoral epiphysis during growth hormone treatment: a safety update from the KIGS database. *Horm Res.* 68 Suppl 5:41–47 (2007). [PubMed: 18174706]
7. Cutfield WS, Wilton P, Bennmarker H, Albertsson-Wikland K, Chatelain P, Ranke MB, and Price DA. Incidence of diabetes mellitus and impaired glucose tolerance in children and adolescents receiving growth-hormone treatment. *Lancet.* 355:610–613 (2000). [PubMed: 10696981]
8. Yuen KC, Chong LE, and Riddle MC. Influence of glucocorticoids and growth hormone on insulin sensitivity in humans. *Diabet Med.* 30:651–663 (2013). [PubMed: 23510125]
9. Swerdlow AJ, Higgins CD, Adlard P, and Preece MA. Risk of cancer in patients treated with human pituitary growth hormone in the UK, 1959–85: a cohort study. *Lancet.* 360:273–277 (2002). [PubMed: 12147369]
10. Chau M, Forcinito P, Andrade AC, Hegde A, Ahn S, Lui JC, Baron J, and Nilsson O. Organization of the Indian hedgehog--parathyroid hormone-related protein system in the postnatal growth plate. *J Mol Endocrinol.* 47:99–107 (2011). [PubMed: 21642420]
11. Kronenberg HM. PTHrP and skeletal development. *Ann N Y Acad Sci.* 1068:1–13 (2006). [PubMed: 16831900]
12. Maeda Y, Nakamura E, Nguyen MT, Suva LJ, Swain FL, Razzaque MS, Mackem S, and Lanske B. Indian Hedgehog produced by postnatal chondrocytes is essential for maintaining a growth plate and trabecular bone. *Proc Natl Acad Sci U S A.* 104:6382–6387 (2007). [PubMed: 17409191]
13. Long F, Joeng KS, Xuan S, Efstratiadis A, and McMahon AP. Independent regulation of skeletal growth by Ihh and IGF signaling. *Dev Biol.* 298:327–333 (2006). [PubMed: 16905129]
14. Amizuka N, Warshawsky H, Henderson JE, Goltzman D, and Karaplis AC. Parathyroid hormone-related peptide-depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation. *J Cell Biol.* 126:1611–1623 (1994). [PubMed: 8089190]
15. Nilsson O, Parker EA, Hegde A, Chau M, Barnes KM, and Baron J. Gradients in bone morphogenetic protein-related gene expression across the growth plate. *J Endocrinol.* 193:75–84 (2007). [PubMed: 17400805]
16. De LF, Barnes KM, Uyeda JA, De-Levi S, Abad V, Palese T, Mericq V, and Baron J. Regulation of growth plate chondrogenesis by bone morphogenetic protein-2. *Endocrinology.* 142:430–436 (2001). [PubMed: 11145607]
17. Yoon BS, Pogue R, Ovchinnikov DA, Yoshii I, Mishina Y, Behringer RR, and Lyons KM. BMPs regulate multiple aspects of growth-plate chondrogenesis through opposing actions on FGF pathways. *Development.* 133:4667–4678 (2006). [PubMed: 17065231]
18. Yoon BS, Ovchinnikov DA, Yoshii I, Mishina Y, Behringer RR, and Lyons KM. *Bmpr1a* and *Bmpr1b* have overlapping functions and are essential for chondrogenesis in vivo. *Proc Natl Acad Sci U S A.* 102:5062–5067 (2005). [PubMed: 15781876]
19. Kobayashi T, Lyons KM, McMahon AP, and Kronenberg HM. BMP signaling stimulates cellular differentiation at multiple steps during cartilage development. *Proc Natl Acad Sci U S A.* 102:18023–18027 (2005). [PubMed: 16322106]
20. Olney RC. C-type natriuretic peptide in growth: a new paradigm. *Growth Horm IGF Res.* 16 Suppl A:S6–14 (2006). [PubMed: 16716628]
21. Olney RC, Bukulmez H, Bartels CF, Prickett TC, Espiner EA, Potter LR, and Warman ML. Heterozygous mutations in natriuretic peptide receptor-B (NPR2) are associated with short stature. *J Clin Endocrinol Metab.* 91:1229–1232 (2006). [PubMed: 16384845]
22. Agoston H, Khan S, James CG, Gillespie JR, Serra R, Stanton LA, and Beier F. C-type natriuretic peptide regulates endochondral bone growth through p38 MAP kinase-dependent and -independent pathways. *BMC Dev Biol.* 7:18 (2007). [PubMed: 17374144]
23. Mericq V, Uyeda JA, Barnes KM, De LF, and Baron J. Regulation of fetal rat bone growth by C-type natriuretic peptide and cGMP. *Pediatr Res.* 47:189–193 (2000). [PubMed: 10674345]
24. Teixeira CC, Agoston H, and Beier F. Nitric oxide, C-type natriuretic peptide and cGMP as regulators of endochondral ossification. *Dev Biol.* 319:171–178 (2008). [PubMed: 18514181]
25. Yates KE, Shortkroff S, and Reish RG. Wnt influence on chondrocyte differentiation and cartilage function. *DNA Cell Biol.* 24:446–457 (2005). [PubMed: 16008513]

26. Andrade AC, Nilsson O, Barnes KM, and Baron J. Wnt gene expression in the post-natal growth plate: regulation with chondrocyte differentiation. *Bone*. 40:1361–1369 (2007). [PubMed: 17337262]
27. Yang Y, Topol L, Lee H, and Wu J. Wnt5a and Wnt5b exhibit distinct activities in coordinating chondrocyte proliferation and differentiation. *Development*. 130:1003–1015 (2003). [PubMed: 12538525]
28. Akiyama H, Lyons JP, Mori-Akiyama Y, Yang X, Zhang R, Zhang Z, Deng JM, Taketo MM, Nakamura T, Behringer RR, McCrea PD, and de CB. Interactions between Sox9 and beta-catenin control chondrocyte differentiation. *Genes Dev*. 18:1072–1087 (2004). [PubMed: 15132997]
29. Lui JC, Nilsson O, Chan Y, Palmer CD, Andrade AC, Hirschhorn JN, and Baron J. Synthesizing genome-wide association studies and expression microarray reveals novel genes that act in the human growth plate to modulate height. *Hum Mol Genet*. 21:5193–5201 (2012). [PubMed: 22914739]
30. Lui JC, Andrade AC, Forcinito P, Hegde A, Chen W, Baron J, and Nilsson O. Spatial and temporal regulation of gene expression in the mammalian growth plate. *Bone*. 46:1380–1390 (2010). [PubMed: 20096814]
31. Klatt AR, Nitsche DP, Kobbe B, Morgelin M, Paulsson M, and Wagener R. Molecular structure and tissue distribution of matrilin-3, a filament-forming extracellular matrix protein expressed during skeletal development. *J Biol Chem*. 275:3999–4006 (2000). [PubMed: 10660556]
32. Forcinito P, Andrade AC, Finkielstain GP, Baron J, Nilsson O, Lui JC. Growth-inhibiting conditions slow growth plate senescence. *J Endocrinol*. 2011;208:59–67. [PubMed: 20974641]
33. Zhang MY, Shu Y, Phogat S, Xiao X, Cham F, Bouma P, Choudhary A, Feng YR, Sanz I, Rybak S, Broder CC, Quinnan GV, Evans T, and Dimitrov DS. Broadly cross-reactive HIV neutralizing human monoclonal antibody Fab selected by sequential antigen panning of a phage display library. *J Immunol Methods*. 283:17–25 (2003). [PubMed: 14659896]
34. Horton WA, Hall JG, and Hecht JT. Achondroplasia. *Lancet*. 370:162–172 (2007). [PubMed: 17630040]
35. Yasoda A, Komatsu Y, Chusho H, Miyazawa T, Ozasa A, Miura M, Kurihara T, Rogi T, Tanaka S, Suda M, Tamura N, Ogawa Y, and Nakao K. Overexpression of CNP in chondrocytes rescues achondroplasia through a MAPK-dependent pathway. *Nat Med*. 10:80–86 (2004). [PubMed: 14702637]
36. Igaki T, Itoh H, Suga SI, Hama N, Ogawa Y, Komatsu Y, Yamashita J, Doi K, Chun TH, and Nakao K. Effects of intravenously administered C-type natriuretic peptide in humans: comparison with atrial natriuretic peptide. *Hypertens Res*. 21:7–13 (1998). [PubMed: 9582102]
37. Yokogawa K, Miya K, Sekido T, Higashi Y, Nomura M, Fujisawa R, Morito K, Masamune Y, Waki Y, Kasugai S, and Miyamoto K. Selective delivery of estradiol to bone by aspartic acid oligopeptide and its effects on ovariectomized mice. *Endocrinology*. 142:1228–1233 (2001). [PubMed: 11181539]
38. Millan JL, Narisawa S, Lemire I, Loisel TP, Boileau G, Leonard P, Gramatikova S, Terkeltaub R, Camacho NP, McKee MD, Crine P, and Whyte MP. Enzyme replacement therapy for murine hypophosphatasia. *J Bone Miner Res*. 23:777–787 (2008). [PubMed: 18086009]
39. Yadav MC, Lemire I, Leonard P, Boileau G, Blond L, Beliveau M, Cory E, Sah RL, Whyte MP, Crine P, and Millan JL. Dose response of bone-targeted enzyme replacement for murine hypophosphatasia. *Bone*. 49:250–256 (2011). [PubMed: 21458605]
40. Bhandari KH, Newa M, Uludag H, and Doschak MR. Synthesis, characterization and in vitro evaluation of a bone targeting delivery system for salmon calcitonin. *Int J Pharm*. 394:26–34 (2010). [PubMed: 20412845]
41. Bhandari KH, Newa M, Chapman J, and Doschak MR. Synthesis, characterization and evaluation of bone targeting salmon calcitonin analogs in normal and osteoporotic rats. *J Control Release*. 158:44–52 (2012). [PubMed: 22001608]
42. Yang Y, Bhandari KH, Panahifar A, and Doschak MR. Synthesis, characterization and biodistribution studies of (125)I-radioiodinated di-PEGylated bone targeting salmon calcitonin analogue in healthy rats. *Pharm Res*. 31:1146–1157 (2014). [PubMed: 24357414]

43. Doschak MR, Kucharski CM, Wright JE, Zernicke RF, and Uludag H. Improved bone delivery of osteoprotegerin by bisphosphonate conjugation in a rat model of osteoarthritis. *Mol Pharm.* 6:634–640 (2009). [PubMed: 19718808]
44. Rothenfluh DA, Bermudez H, O'Neil CP, and Hubbell JA. Biofunctional polymer nanoparticles for intra-articular targeting and retention in cartilage. *Nat Mater.* 7:248–254 (2008). [PubMed: 18246072]
45. Hughes C, Faurholm B, Dell'Accio F, Manzo A, Seed M, Eltawil N, Marrelli A, Gould D, Subang C, Al-Kashi A, De BC, Winyard P, Chernajovsky Y, and Nissim A. Human single-chain variable fragment that specifically targets arthritic cartilage. *Arthritis Rheum.* 62:1007–1016 (2010). [PubMed: 20131274]
46. Cheung CS, Lui JC, and Baron J. Identification of chondrocyte-binding peptides by phage display. *J Orthop Res.* 31:1053–1058 (2013). [PubMed: 23440926]

Figure 1. Selection of matriline-3-binding antibody fragments and assessment of their binding characteristics.

(a) A yeast display antibody library was panned against human (first and second rounds of panning) and mouse (third round) matriline-3 protein. Fluorescence-activated cell sorting showed that the binding affinity of the enriched pool of yeast display antibody fragments toward matriline-3 dramatically increased after three rounds of selection, when compared to the naïve library. (b) The binding abilities of 36 clones of antibody fragments to human or mouse recombinant matriline-3 protein were evaluated by ELISA. Plastic wells were coated with matriline-3 protein or bovine serum albumin (BSA) and then incubated with antibody fragments, washed and detected using a HRP-conjugated anti-Fc antibody. Absorbance was normalized to background signal of 3% non-fat milk. (c) The tissue binding specificity of 15 selected clones of antibody fragments were assessed by ELISA. Tissue lysates from a panel of organs from 4-day old mice were used to coat plastic wells. Individual antibody fragments

were incubated in the wells, then washed and detected using HRP-conjugated anti-Fc antibody. A non-specific antibody fragment directed against an irrelevant protein served as a negative control and a commercial antibody against matrilin-3 served as a positive control. For panels b-d, data represent mean $\pm$ SEM from three independent experiments (n=9).

Figure 2. Binding of antibody fragments to tissues in frozen mouse embryo sections. Antibody fragments 13, 22, and 26 were incubated with frozen E15 mouse embryo sections, then detected with HRP-conjugated anti-Fc antibody and stained with DAB substrate to produce a brown color. Immunostaining was observed in cartilage tissues, including digits of forepaw (first row) and rib cages (second row), but not in non-cartilaginous structures.

Figure 3. Assessment of the binding affinity of purified martilin-3-binding antibody fragments. The three selected antibody fragments, 13, 22 and 26, were expressed and purified using protein A columns. Various concentrations of each antibody fragment were incubated with recombinant human (a) and mouse (b) martilin-3 proteins, and binding was measured by ELISA. The negative control (background) lacked an antibody fragment.

Figure 4. Homing of selected matrilin-3-binding antibody fragments to cartilage *in vivo*. Saline, non-specific antibody fragment, or purified antibody fragments 13, 22, and 26 was injected intravenously in 3-week old mice. After 24 hours, various organs were collected and homogenized, and tissue lysates were used to coat plastic wells. The presence of antibody fragments was detected by ELISA with an anti-Fc antibody. All three antibody fragments were detected in cartilage, but not in non-cartilaginous organs. Saline and non-specific antibody served as negative controls. Data represent mean $\pm$ SEM from five independent experiments (n=5).

Figure 5. No evident toxicity after injection of antibody fragments.

Representative sections showing the histology of 6-week old mouse tibial growth plate after injection of non-targeting antibody fragment (A) or antibody fragment 13 (B) every other day for 2 weeks starting at 4 weeks of age. At the end of the treatment period, injection of antibody fragment 13 did not affect the overall histology of the growth plate (A,B), body weight (C), tibia length (D), height of the proliferative zone (E) or height of the hypertrophic zone (F). Statistical significance were defined as $P < 0.05$ by t-test. RZ, resting zone; PZ, proliferative zone; HZ, hypertrophic zone. The superimposed dotted curves represent the boundaries of the proliferative and hypertrophic zones.