とである。

B. 研究方法

1) ラット脊椎硬膜外癒着モデルの確立

生後 7−8 週令（体重 200−220 g）の Sprague-Dawley(SD)ラットを使用した。

a) 麻酔・体位：ジエチルエーテルによる麻酔導入後、腹腔内麻酔（4%抱水クロラール 2 ml）および 1%キシロカイン 5 ml で局所麻酔を実施し、ラットを腹臥位とした。背部を電動式バリカンにて剃毛し、四肢を軽く固定し体位をとった（図 1）。

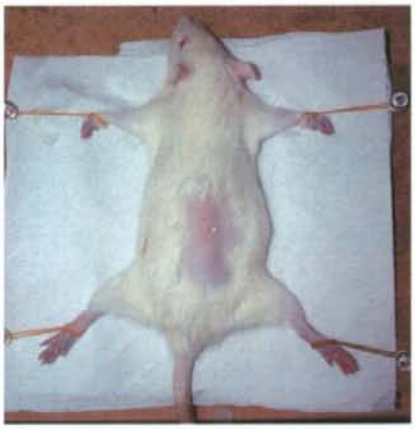

図 1. ラットを腹臥位とし剃毛

b) 腰椎椎弓切除：背側正中切開をおき、第 1 腰椎（L1）から第 4 腰椎（L4）までの棘突起および椎弓を展開した。次に手術用顕微鏡下において電動式ドリル、リュエル、ケリソン鉗子を用いて L1〜L4 の椎弓切除を行い、硬膜背側を展開した（図 2）。筋層からの出血に対してはバイポーラメスで焼灼し止血した。

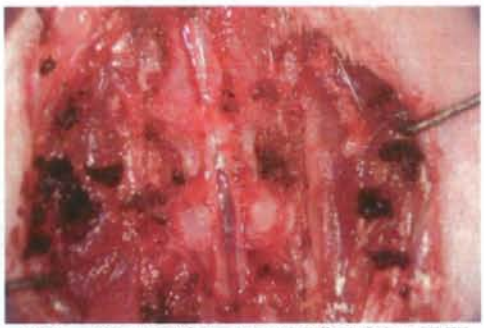

図 2. 椎弓切除および硬膜背側の展開

c) 硬膜周囲の処置：剥離子を用いて硬膜側面および前面の剥離操作を実施し、硬膜表面の血管をバイポーラメスで焼灼した。

d) 閉創：止血を確認した後、創内を生理食塩水にて洗浄、4-0 ナイロン糸にて皮膚を縫合し、閉創した。縫合部分にゲンタマイシン軟膏を塗布した。

e) 術後処置：手術後はケージ内で自由に運動させた。

f) 硬膜周囲癒着の評価：術後 4 週、6 週、8 週の時点で、術後の硬膜周囲組織の癒着の状態を以下の 2)~5)で評価した。

2) 硬膜周囲癒着の肉眼的評価

硬膜周囲の癒着の程度を肉眼所見で分類した。Grade0（癒着なし）、1（弱い引っ張り力ではがれる）、2（中等度から強度の引っ張り力で剥離できる）、3（鋭的な切除でのみ剥離できる）の 4 段階評価を行った。

3) 硬膜周囲の組織学的評価

摘出した脊椎および硬膜から組織切片を作製し、ヘマトキシリン−エオジン（HE）染色によって組織学的に観察した。硬膜表面から瘢痕組織までの距離・硬膜の厚み・瘢痕中の炎症細胞数・クモ膜下腔の面積について測定

した。同時に癒着防止材料の残存の有無・量についても評価した。

4) 神経学的評価

癒着防止材が脊髄・硬膜の治癒を妨げず、神経機能に障害を与えないことを確認するため、神経学的評価を行った。評価基準には、ラット後肢の運動機能評価を行う Basso, Bresnahan, and Beatle (BBB) open field locomotor rating score (*Experimental Neurology* 139, 244, 1996)を使用した。

5) MPC ポリマーゲルの被覆による硬膜周囲の癒着防止効果についての検討

1) で確立したラット脊椎硬膜外癒着モデルを使用して、実験動物を 2 群に分け、1 群は MPC ゲル 1 ml を硬膜周囲に注入し、他群は生理食塩水を硬膜周囲に注入した。液を注入後数分でゲル化し、体位を移動させても流出しないことを確認後閉創した（図 3, 4）。

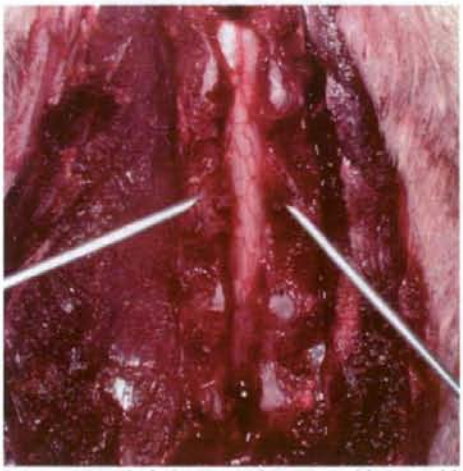

図 3. MPC ポリマーゲルは A 液と B 液を混和して注入

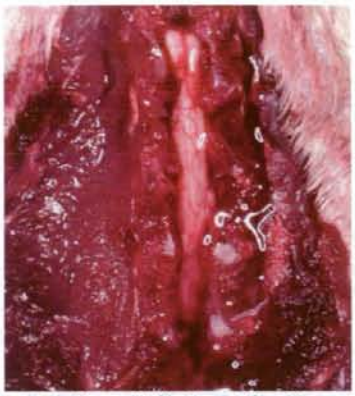

図 4. 注入後数分でゲル化

（倫理面への配慮）

すべての動物実験は「動物の保護及び管理に関する法律」、「実験動物の飼育及び保管等に関する基準総理府告示」、「東京大学医学部動物実験指針」に従って、東京大学医学部倫理委員会の承諾の下で行った。

C. 研究結果

1) ラット脊椎硬膜外癒着モデルの確立

麻酔による術中・術後死例はなく、麻酔からの覚醒も安定していた。また手術中の中途覚醒はなく十分な麻酔深度が得られていた。手術用顕微鏡を用いた慎重な操作により、出血のコントロールも良好であり、手術行程を妨げたり、生命の危険が心配されたりするような出血量はなかった。また椎弓切除や硬膜の剥離操作において硬膜および脊髄神経の肉眼的損傷はなかった。

2) 硬膜周囲癒着の肉眼的評価

肉眼での観察では、術後 6 週および 8 週の時点において、硬膜周囲の癒着がみられ、鈍的剥離のみでは硬膜を周囲組織から遊離させることは困難で、剪刀による鋭的切離を必要とした（図

5）。癒着の程度が比較的軽度であったものもあったが（図 6）全例で何らかの癒着が確認できた。

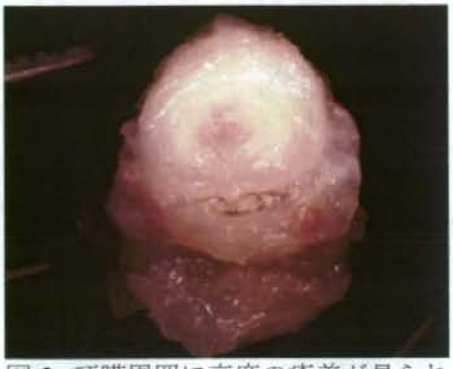

図 5. 硬膜周囲に高度の癒着が見られる：Grade 3（術後 6 週時点）

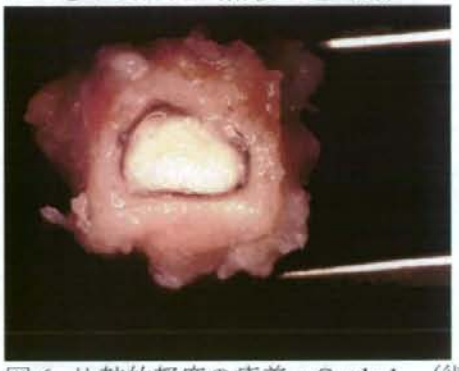

図 6. 比較的軽度の癒着：Grade 1 （術後 8 週時点）

術後 4 週の時点では硬膜周囲に癒着の見られたものもあったが、癒着がほとんどないものもあり癒着モデルとしては適当でないと判断した（図 7）。

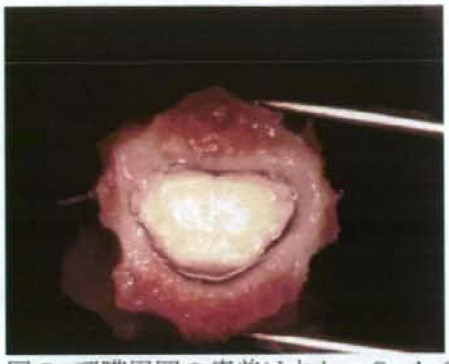

図 7. 硬膜周囲の癒着はなし：Grade 0（術後 4 週時点）

3) 硬膜周囲の組織学的評価

術後 6 週および 8 週での組織学的評価を行ったところ、硬膜周囲の癒着および脊柱管の圧迫所見が見られた。椎弓切除を行った硬膜背側に肉芽組織が増生し脊柱管は圧排され扁平化していた。硬膜と肉芽組織との境界は不明瞭で高度に癒着している所見であった（図 8）。強拡大では硬膜周囲に炎症細胞の浸潤および線維性組織の増生が見られた（図 9）。

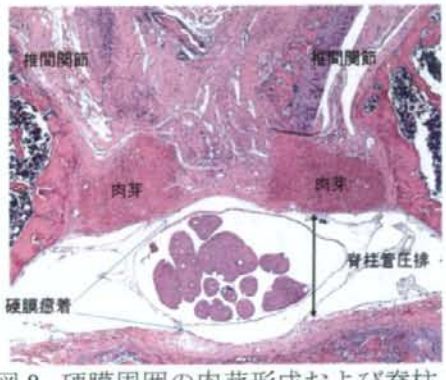

図 8. 硬膜周囲の肉芽形成および脊柱管の圧排あり（術後 6 週）

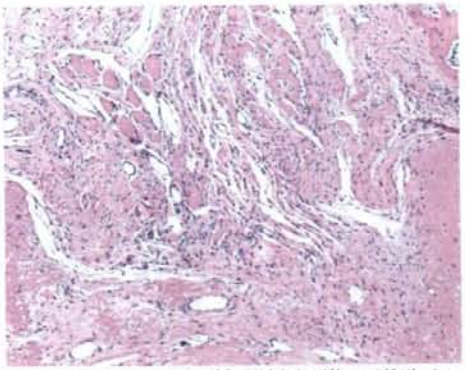

図 9. 硬膜周囲に線維性組織の増生および炎症細胞の浸潤あり（術後 6 週：強拡大）

術後 4 週の時点では椎弓切除部位の硬膜に癒着が見られたもの（図 10）と癒着が見られなかったもの（図 11）が混在した。

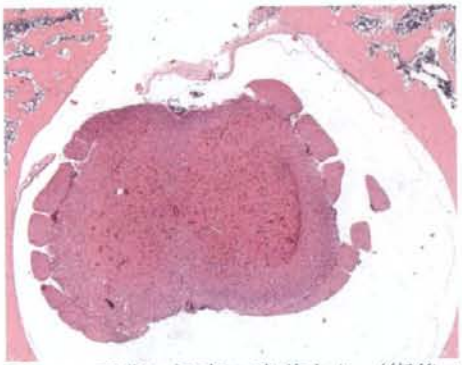

図 10. 硬膜に軽度の癒着あり（術後 4 週）

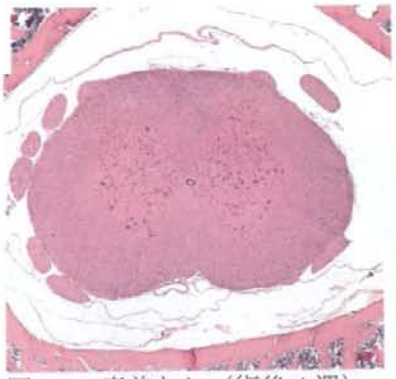

図 11. 癒着なし（術後 4 週）

硬膜の損傷・硬膜周囲組織の異常所見・創部感染等は特にみられなかった。また、術後 4 週、6 週、8 週いずれの時点においても MPC ポリマーゲルの残存はなかった。

4) 神経学的評価

術翌日および術後 4 週、6 週、8 週の時点において BBB score はいずれも 21 点であり神経障害は見られなかった。

以上より、このラットの硬膜周囲癒着モデルは今回の研究において適当であると考えられたため、次年度以降の実験でも使用することとした。

D. 考察

MPC ポリマーゲルの組織癒着防止効果を検討する今年度の研究では、脊椎外科手術後の硬膜周囲癒着防止材として臨床応用するための基礎的検討を開始した。

脊椎硬膜周囲の癒着は重篤な合併症であるにもかかわらず、これを防止する材料・手法は未だ確立されておらず、現時点ではいくつかの材料の単発的な報告が散見するにとどまっている。いずれの報告においても、癒着の防止効果を客観的かつ明確に検討したものではなく、また、副作用の問題もあり、実用化には至っていない。本研究は、MPC ポリマーゲルを脊椎硬膜周囲の癒着防止材と

して用いるという、従来行われてこなかった視点からの研究開発である。評価方法においては従来の肉眼的・組織学的評価のみにとどまらず、癒着の力学的評価、画像評価を行うことにより、癒着防止効果を客観的に明らかにする最初の研究となることが予想される。また運動機能評価、残存物性評価も実施し、この癒着防止材が脊椎硬膜周囲の治癒を妨げないことを確認する予定である。

今年度の研究では、これまでに硬膜周囲癒着の動物モデルが確立されていないため、まずラット脊椎硬膜外癒着モデルを確立し、癒着の評価方法の検討を行った。本研究の結果から、確立した癒着モデルおよび硬膜周囲癒着の評価方法が適切であることを確認した。

次年度以降は、今年度確立した硬膜周囲癒着モデルを用い、MPC ポリマーゲルの被覆による硬膜周囲の癒着防止効果についての検討を実施する予定である。今年度実施した評価項目に加え、X 線および microCT 撮影による評価を予定している。方法として硬膜外腔に造影剤（オムニパーク）を注入し、X 線および microCT 撮影を実施し硬膜外腔の面積および形状を測定する。

さらに硬膜周囲癒着の力学的検討を予定している。方法としては、硬膜および脊椎を周囲組織から切離後に、手術施行部位の全長にわたって採取する。図 12 に示すレオメーターシステムを用い、椎体および硬膜管をそれぞれエアチャックで把持し、遠位方向へ 10 mm/min の速度で硬膜と椎体とが破断するまで牽引する（図 13）。この際の最大破断張力を癒着程度の指標として測定し、統計学的解析を実施する予定である。

図 12. レオメーターシステム

図 13. レオメーターでの硬膜―椎体破断張力の測定

次年度は本年度実施した評価に加えてこれらの評価を行い、組織癒着防止材としての有効性を検討する予定である。

E. 結論

今年度の研究により、ラット硬膜外癒着モデルを確立し、癒着程度の生体工学的な評価を可能にした。またこのモデルを使用して MPC ポリマーゲルの被覆による硬膜周囲の癒着防止効果についての実験を開始した。

F. 健康危険情報

特になし。

G. 研究発表

1.論文発表

1) Kyomoto M, Moro T, Miyaji F, Konno T, Hashimoto M, Kawaguchi H, Takatori Y, Nakamura K, and Ishihara K: Enhanced wear resistance of orthopaedic bearing due to the cross-linking of poly (MPC) graft chains induced by gamma-ray irradiation. *J Biomed Mater Res B* 84: 320-327, 2008.

2) Kawasaki Y, Kugimiya F, Chikuda H, Kamekura S, Ikeda T, Kawamura N, Saito T, Shinoda Y, Higashikawa A, Yano F, Ogasawara T, Ogata N, Hoshi K, Hofmann F, Woodgett JR, Nakamura K, Chung UI, and Kawaguchi H: Phosphorylation of GSK-3β by cGMP-dependent protein kinase II promotes hypertrophic differentiation of murine chondrocytes. *J Clin Invest* 118: 2506-2515, 2008.

3) Kyomoto M, Moro T, Miyaji F, Hashimoto M, Kawaguchi H, Takatori Y, Nakamura K, and Ishihara K: Effect of 2-methacryloyloxyethyl phosphorylcholine concentration on photo-induced graft polymerization of polyethylene in reducing the wear of orthopaedic bearing surface. *J Biomed Mater Res A* 86: 439-47, 2008.

4) Sato S, Kimura A, Ozdemir J, Asou Y, Miyazaki M, Jinno T, Ae K, Liu X, Osaki M, Takeuchi Y, Fukumoto S, Kawaguchi H, Haro H, Shinomiya K, Karsenty G, and Takeda S: The Distinct role of the Runx proteins in chondrocyte differentiation and intervertebral disc degeneration: Findings in murine models and in human disease. *Arthritis Rheum* 58: 2764-2775, 2008.

5) Shinoda Y, Ogata N, Higashikawa A, Manabe I, Shindo T, Yamada T, Kugimiya F, Ikeda T, Kawamura N, Kawasaki Y, Tsushima K, Takeda N, Nagai R, Hoshi K, Nakamura K, Chung UI, and Kawaguchi H: Krüppel-like factor 5 causes cartilage degradation through transactivation of matrix metalloproteinase 9. *J Biol Chem* 283: 24682-24689, 2008.

6) Oka H, Muraki S, Akune T, Mabuchi A, Suzuki T, Yoshida H, Yamamoto S, Nakamura K, Yoshimura N, and Kawaguchi H: Fully automatic quantification of knee osteoarthritis severity on plain radiographs. *Osteoarthritis Cartilage* 16: 1300-1306, 2008.

7) Kawaguchi H: Endochondral ossification signals in cartilage degradation during osteoarthritis progression in experimental mouse models. *Mol Cells* 25: 1-6, 2008.

8) Kawaguchi H: Regulation of osteoarthritis development by Wnt-β-catenin signaling through the endochondral ossification process. *J Bone Miner Res* 24: 8-11, 2009.

9) Higashikawa A, Saito T, Ikeda T, Kamekura S, Kawamura N, Kan A, Oshima Y, Ohba S, Ogata N, Takeshita K, Nakamura K, Chung UI, and Kawaguchi H: Identification of the core element responsive to runt-related transcription factor 2 in the promoter of human type x collagen gene. *Arthritis Rheum* 60: 166-178, 2009.

10) Chikuda H, Seichi A, Takeshita K, Shoda N, Ono T, Matsudaira K, Kawaguchi H, and Nakamura K: Radiographic analysis of the cervical spine in patients with retro-odontoid pseudotumors. *Spine* 34: E110-114, 2009.
11) Kyomoto M, Moro T, Miyaji F, Hashimoto M, Kawaguchi H, Takatori Y, Nakamura K, and Ishihara K. Effects of mobility/immobility of surface modification by 2-methacryloyloxyethyl phosphorylcholine polymer on the durability of polyethylene for artificial joints. *J Biomed Mater Res A* (in press).
12) Liu G, Iwata K, Ogasawara T, Watanabe J, Fukazawa K, Ishihara K, Asawa Y, Fujihara Y, Chung UI, Moro T, Takatori Y, Takato T, Nakamura K, Kawaguchi H, and Hoshi K: Selection of highly osteogenic and chondrogenic cells from bone marrow stromal cells in biocompatible polymer-coated plates. *J Biomed Mater Res A* (in press).
13) Kyomoto M, Moro T, Iwasaki Y, Miyaji F, Kawaguchi H, Takatori Y, Nakamura K, Ishihara K. Superlubricious surface mimicking articular cartilage by grafting poly(2-methacryloyloxyethyl phosphorylcholine) on orthopaedic metal bearings. *J Biomed Mater Res A* (in press).
14) Hirata M, Kugimiya F, Fukai A, Ohba S, Kawamura N, Ogasawara T, Kawasaki Y, Saito T, Yano F, Ikeda T, Nakamura K, Chung UI, and Kawaguchi H: C/EBPβ promotes transition from proliferation to hypertrophic differentiation of chondrocytes through transactivation of $p57^{Kip2}$. *PLoS ONE* (in press).
15) Muraki S, Oka H, Akune T, Mabuchi A, En-yo Y, Yoshida M, Saika A, Suzuki T, Yoshida H, Ishibashi H, Yamamoto S, Nakamura K, Kawaguchi H, and Yoshimura N: Prevalence of radiographic knee osteoarthritis and its association with knee pain in the elderly of Japanese population-based cohorts: The ROAD study. *Osteoarthritis Cartilage* (in press).
16) Muraki S, Akune T, Oka H, Mabuchi A, En-yo Y, Yoshida M, Saika A, Nakamura K, Kawaguchi H, and Yoshimura N: Association of occupational activity with radiographic knee osteoarthritis and lumbar spondylosis in the elderly of population-based cohorts: The ROAD study. *Arthritis Rheum* (in press).
17) Ushita M, Saito T, Ikeda T, Yano F, Higashikawa A, Ogata N, Chung UI, Nakamura K, and Kawaguchi H: Transcriptional induction of SOX9 by NF-κB family member RelA in chondrogenic cells. *Osteoarthritis Cartilage* (in press).
18) Moro T, Kawaguchi H, Ishihara K, Kyomoto M, Karita T, Ito H, Nakamura K, and Takatori Y: Wear resistance of artificial hip joints with poly(2-methacryloyloxyethyl phosphorylcholine) grafted polyethylene: Comparisons with the effect of polyethylene cross-linking and ceramic femoral heads. *Biomaterials* (in press).
19) 高本康史, 三浦俊樹, 大数加光治, 田

中栄，中村耕三：上腕骨滑車中央部に生じた無症候性離断性骨軟骨炎の1例．*関東整災誌* 39(4)：143-147，2008.

20) 大数加光治，三浦俊樹，石山典幸，大江隆史，中村耕三：橈骨遠位端骨折手術における術中Cアーム CT の有用性について．*日本手の外科学会雑誌* 24(5)：593-596，2008.

2．学会発表

① 国内学会

1) 石山典幸、茂呂徹、三浦俊樹、大江隆史、川口浩：生体適合性ポリマーハイドロゲルによる癒着防止効果の検討．第51回日本手の外科学会学術集会．2008.4.17-18（つくば）

2) 三浦俊樹、石山典幸、大数加光治、伊藤祥三、中村耕三：母指CM関節障害に対するLRTI法術後の早期回復過程．第51回日本手の外科学会学術集会．2008.4.17-18.（つくば）

3) 伊藤祥三、三浦俊樹、岡敬之、中村耕三：母指外転測定法の信頼性．第51回日本手の外科学会学術集会．2008.4.17-18.（つくば）

4) 大数加光治、三浦俊樹：橈骨遠位端骨折手術における遠位スクリュー位置と術後矯正損失．第51回日本手の外科学会学術集会．2008.4.17-18.（つくば）

5) 茂呂徹、高取吉雄、石原一彦、山本基、苅田達郎、伊藤英也、金野智浩、京本政之、山脇昇、中村耕三、川口浩：人工股関節のポリエチレンライナーにMPC処理を加える範囲が摩耗抑制効果に与える影響．第81回日本整形外科学会学術総会．2008．5．22-25（札幌）

6) 川口浩、村木重之、岡敬之、阿久根徹、馬淵昭彦、中村耕三、吉村典子：変形性関節症の大規模臨床統合データベースの構築と、これを用いた観察疫学・ゲノム疫学研究．第81回日本整形外科学会学術総会．2008．5．22-25（札幌）．

7) 延與良夫、吉田宗人、吉村典子、村木重之、岡敬之、馬淵昭彦、中村耕三、川口浩：頸椎症における高位別有病率と頚部痛との関係 - Research on Osteoarthritis against Disabilit（ROAD）プロジェクト -．第81回日本整形外科学会学術総会．2008．5．22-25（札幌）．

8) 川口浩：変形性関節症：その病態解明・診断・治療の最前線．福岡県臨床整形外科医会．2008．6．7（博多）．

9) 川口浩：変形性関節症：研究・診療の現状と問題点（シンポジウム：骨粗鬆症と変形性関節症：研究と診療の最前線）．第50回日本老年医学会学術集会・総会．2008．6.19-21（千葉）．

10) 川口浩：変形性関節症研究の現状と問題点（シンポジウム：プロテオーム解析技術の新展開：創薬ターゲット/臨床マーカー発見を目指して）．日本ヒトプロテオーム機構第6回大会．2008.7.29-30（大阪）．

11) 川口浩：変形性関節症 up-to-date：診断と治療の最前線．第1回TCOA（東京都臨床整形外科医会）up-to-dateセミナー．2008．8.30（東京）．

12) 川口浩：変形性関節症 - その病態解明・診断・治療の最前線（ランチョンセミナー）．第36回九州リウマチ学会．2008．9.6-7（佐賀）．

13) 京本政之、茂呂徹、金野智浩、川口浩、高取吉雄、中村耕三、石原一彦：MPCポリマーによる高潤滑インターフェイスが長寿命型人工関節を実現する．東京大学生命科学研究ネットワークシンポジウム．2008．9.23（東京）

14) 雑賀健一、京本政之、茂呂徹、金野智浩、川口浩、高取吉雄、中村耕三、石原一彦：高潤滑性ポリマーを用いた光開始グラフト重合法による長寿命

型人工関節の開発．東京大学生命科学研究ネットワークシンポジウム．2008．9.23（東京）

15) 石山典幸、茂呂徹、三浦俊樹、大江隆史、伊藤祥三、森崎裕、金野智浩、吉河美都奈、大山但、石原一彦、中村耕三、川口浩：組織癒着防止効果を有する生体適合性MPCゲルの開発．東京大学生命科学研究ネットワークシンポジウム．2008．9.23（東京）

16) 川口浩：変形性関節症：その病態解明・診断・治療の最前線．平成20年度 堺市医師会整形外科医会講演会．2008．10.4（大阪）．

17) 村木重之、岡敬之、阿久根徹、馬渕昭彦、中村耕三、川口浩、吉村典子：高齢者では腰椎椎間狭小化と腰痛との関連は女性の方が男性よりも強い－ROADプロジェクト－．第23回日本整形外科学会基礎学術集会．2008.10.23-24（京都）．

18) 村木重之、岡敬之、阿久根徹、馬渕昭彦、中村耕三、川口浩、吉村典子：変形性膝関節症における骨棘形成および関節裂隙狭小化の膝痛への影響－ROADプロジェクト－．第23回日本整形外科学会基礎学術集会．2008.10.23-24（京都）．

19) 石山典幸、茂呂徹、三浦俊樹、大江隆史、中村耕三、川口浩：腱癒着防止効果を有する生体内解離性MPCゲルの開発．第23回日本整形外科学会基礎学術集会．2008.10.23-24（京都）．

20) 茂呂徹、川口浩、石原一彦、京本政之、山本基、苅田達郎、伊藤英也、齊藤貴志、中村耕三、高取吉雄：人工股関節ライナー表面のMPCグラフト処理による摩耗抑制効果：ライナーの架橋の有無および骨頭の材質による比較．第23回日本整形外科学会基礎学術集会．2008.10.23-24（京都）．

21) 深井厚、河村直洋、東川晶朗、斎藤琢、菅哲徳、平田真、川崎洋介、大島寧、鄭雄一、中村耕三、川口浩：Akt1は軟骨内骨化における軟骨細胞の石灰化を維持して骨格成長を制御する．第23回日本整形外科学会基礎学術集会．2008.10.23-24（京都）．

22) 岡敬之、阿久根徹、村木重之、吉村典子、中村耕三、川口浩：変形性膝関節症におけるレントゲン上の外側関節裂隙狭小は膝痛増悪予測因子である－重症度自動計測システムKOACADを用いた縦断研究－．第26回日本骨代謝学会．2008．10.29-31（大阪）．

23) 阿久根徹、村木重之、岡敬之、中村耕三、川口浩、吉村典子：変形性膝関節症のX線学的重症度と膝症状および下肢機能との関連 － ROAD（Research on Osteoarthritis against Disabilit）プロジェクト－．2008．10.29-31（大阪）．

24) 吉村典子、村木重之、岡敬之、中村耕三、川口浩、阿久根徹：変形性膝関節症および変形性脊椎症とメタボリック症候群との関連；ROAD（Research on Osteoarthritis against Disabilit）プロジェクト．第26回日本骨代謝学会．2008．10.29-31（大阪）．

25) 吉村典子、阿久根徹、村木重之、岡敬之、川口浩、中村耕三、Lau Edith、Kwok Anthony、Leung Ping-chung：アジア諸国における脊椎椎体骨折有病率の比較：The Asian Vartebral Osteoporosis Study（AVOS）．第10回日本骨粗鬆症学会．2008．10.31-11.2（大阪）．

26) 石山典幸、茂呂徹、三浦俊樹、大江隆史、伊藤祥三、森崎裕、大山但、吉河美都奈、金野智浩、中村耕三、川口浩、石原一彦：生体内解離性ポリマーハイドロゲルの癒着防止効果の組織学的・分子生物学的検討．日本バイオマテリアル学会シンポジウム2008．2008.11.17-18．（東京）

27) 京本政之、茂呂徹、岩崎泰彦、宮路史

明、金野智浩、川口浩、高取吉雄、中村耕三、石原一彦：リン脂質グラフトポリマーによる超潤滑性 Co-Cr-Mo 合金表面の創製．日本バイオマテリアル学会シンポジウム 2008.
2008.11.17-18.（東京）

② 国際学会

1) Moro T, Takatori Y, Ishihara K, Konno T, Kyomoto M, Yamamoto M, Karita T, Ito H, Nakamura K, Kawaguchi H: Grafting of biocompatible polymer on the liner surface for extending longevity of artificial hip joints. 8th World Biomaterials Congress (WBC) 2008. 5. 28-6. 1 (Amsterdam, The Netherlands)
2) Kyomoto M, Moro T, Kawaguchi H, Takatori Y, Nakamura K, Ishihara K: Nanometer-scale high lubricious surface modification extends the durability of artificial joint. 8th World Biomaterials Congress (WBC) 2008. 5. 28-6. 1 (Amsterdam, The Netherlands)
3) Ishiyama N, Moro T, Miura T, Ohe T, Ito S, Konno T, Yoshikawa M, Ohyama T, Ishihara K, Nakamura K, Kawaguchi H: Investigation of biodissociated phospholipid polymer hydrogel that prevents tissue adhesion without impairing healing. 8th World Biomaterials Congress (WBC) 2008. 5. 28-6. 1 (Amsterdam, The Netherlands)
4) Moro T, Takatori Y, Kyomoto M, Ishihara K, Nakamura K, Kawaguchi H: Biocompatible phospholipid polymer grafting on liner surface of artificial hip joints enhances the wear resistance independently of liner cross-linking of femoral head material. 2008 World Congress on Osteoarthritis (OARSI). 2008. 9.18-21 (Rome, Italy).
5) Akune T, Muraki S, Oka H, Nakamura K, Kawaguchi H, Yoshimura N: Association of radiographic severity of knee osteoarthritis with symptomatic parameters: The ROAD study. 2008 World Congress on Osteoarthritis (OARSI). 2008. 9.18-21 (Rome, Italy).
6) Oka H, Akune T, Muraki S, Nakamura K, Yoshimura N, Kawaguchi H: Lateral joint space narrowing on radiographs predicts pain progression in knee osteoarthritis patients: Application of fully automatic KOACAD system to OAI public data. 2008 World Congress on Osteoarthritis (OARSI). 2008. 9.18-21 (Rome, Italy).
7) Morita M, Yamada H, Yoshimura N, Date H, Oka H, Muraki S, Akune T, Ichinose H, Kawaguchi H, Nakamura K: Evaluation of the usefulness of biomarkers in knee osteoarthritis or lumbar spondylosis in mass cohort study of Japan. 2008 World Congress on Osteoarthritis (OARSI). 2008. 9.18-21 (Rome, Italy).
8) Fukai A, Kawamura N, Nakamura K, Chung UI, Kawaguchi H: Akt1 contributes to chondrocyte calcification during endochondralossification. . 2008 World Congress on Osteoarthritis (OARSI). 2008. 9.18-21 (Rome, Italy).
9) Kawaguchi H: Transcriptional regulation of osteoarthritis progression (Symposium: Update on Osteoarthritis: from Bench to Bedside -APLAR-OARSI Co-Sponsored

Symposium). The 13th Congress of the Asia Pacific League of Associations for Rheumatology (APLAR 2008). 2008. 9.23-27 (Yokohama, Japan).

10) Akune T, Muraki S, Oka H, Nakamura K, Kawaguchi H, Yoshimura N: Association of radiographic severity of knee osteoarthritis with symptomatic parameters: The ROAD study. The 13th Congress of the Asia Pacific League of Associations for Rheumatology (APLAR 2008). 2008. 9.23-27 (Yokohama, Japan).

11) Oka H, Akune T, Muraki S, Nakamura K, Yoshimura N, Kawaguchi H: Lateral joint space narrowing on radiographs predicts pain progression in knee osteoarthritis patients: Application of fully automatic KOACAD system to OAI public data. The 13th Congress of the Asia Pacific League of Associations for Rheumatology (APLAR 2008). 2008. 9.23-27 (Yokohama, Japan).

12) Moro T, Takatori Y, Ishihara K, Kyomoto M, Karita T, Ito H, Nakamura K, Kawaguchi H: The effect of biocompatible polymer grafting onto polyethylene liner surface: Improvement of lubricity regardless of the characteristics of bearing materials. The 55th Annual Meeting of the Orthopaedic Research Society (ORS). 2009.2.22-25 (Las Vegas, USA)

13) Ishiyama N, Moro T, Miura T, Ohe T, Ito S, Konno T, Yoshikawa M, Ohyama T, Ishihara K, Nakamura K, Kawaguchi H: Anti-adhesion effect without impairing healing of biocompatible phospholipid polymer hydrogel. The 55th Annual Meeting of the Orthopaedic Research Society (ORS). 2009.2.22-25 (Las Vegas, USA)

14) Kyomoto M, Moro T, Miyaji F, Kawaguchi H, Takatori Y, Nakamura K, Ishihara K: Brush-like structure only gives high durability to cross-linked polyethylene among various surface-modified layers with MPC polymer. The 55th Annual Meeting of the Orthopaedic Research Society (ORS). 2009.2.22-25 (Las Vegas, USA)

15) Higashikawa A, Saito T, Kamekura S, Ikeda T, Kan A, Moro T, Ohba S, Ogata N, Nakamura K, Chung UI, Kawaguchi H: Transcriptional Regulation of Type X Collagen Expression and Hypertrophic Differentiation of Chondrocytes by Runx2 during Osteoarthritis Progression. The 55th Annual Meeting of the Orthopaedic Research Society (ORS). 2009.2.22-25 (Las Vegas, USA)

③ シンポジウム

1) 茂呂徹、川口浩、石原一彦、金野智浩、京本政之、山脇昇、橋本雅美、苅田達郎、伊藤英也、齋藤貴志、中村耕三、高取吉雄：MPC ポリマーの表面処理による低摩耗型人工関節. 日本バイオマテリアル学会シンポジウム 2008. 2008.11.17-18. (東京)

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雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kawasaki Y, Kugimiya F, Chikuda H, Kamekura S, Ikeda T, Kawamura N, Saito T, Shinoda Y, Higashikawa A, Yano F, Ogasawara T, Ogata N, Hoshi K, Hofmann F, Woodgett JR, Nakamura K, Chung UI, Kawaguchi H	Phosphorylation of GSK-3β by cGMP-dependent protein kinase II promotes hypertrophic differentiation of murine chondrocytes.	***J Clin Invest***	118	2506 -2515	2008
Sato S, Kimura A, Ozdemir J, Asou Y, Miyazaki M, Jinno T, Ae K, Liu X, Osaki M, Takeuchi Y, Fukumoto S, Kawaguchi H, Haro H, Shinomiya K, Karsenty G, Takeda S	The Distinct role of the Runx proteins in chondrocyte differentiation and intervertebral disc degeneration: Findings in murine models and in human disease.	***Arthritis Rheum***	58	2764 -2775	2008
Shinoda Y, Ogata N, Higashikawa A, Manabe I, Shindo T, Yamada T, Kugimiya F, Ikeda T, Kawamura N, Kawasaki Y, Tsushima K, Takeda N, Nagai R, Hoshi K, Nakamura K, Chung UI, Kawaguchi H	Krüppel-like factor 5 causes cartilage degradation through transactivation of matrix metalloproteinase 9.	***J Biol Chem***	283	24682 -24689	2008
Oka H, Muraki S, Akune T, Mabuchi A,	Fully automatic quantification of knee osteoarthritis severity on plain radiographs.	***Osteoarthritis Cartilage***	16	1300 -1306	2008

Suzuki T, Yoshida H, Yamamoto S, Nakamura K, Yoshimura N, Kawaguchi H					
Kitano K, Matsuno R, Konno T, Takai M, Ishihara K	Nanoscale Structured Phospholipid Polymer Brush for Biointerface.	***Tans Mater Res Soc Jpn***	33(3)	771-774	2008
Koyama Y, Miyashita M, Irie S, Yamamoto M, Karita T, Moro T, Takatori Y, Kazuma K	A study of disease management activities of hip osteoarthritis patients under conservative treatment.	***J Orthop Nurs***	12	75-83	2008
Kyomoto M, Moro T, Miyaji F, Konno T, Hashimoto M, Kawaguchi H, Takatori Y, Nakamura K, Ishihara K	Enhanced wear resistance of orthopaedic bearing due to the cross-linking of poly(MPC) graft chains induced by gamma-ray irradiation.	***J Biomed Mater Res B Appl Biomater***	84	320-327	2008
Kyomoto M, Moro T, Miyaji F, Hashimoto M, Kawaguchi H, Takatori Y, Nakamura K, Ishihara K	Effect of 2-methacryloyloxyethyl phosphorylcholine concentration on photo-induced graft polymerization of polyethylene in reducing the wear of orthopaedic bearing surface.	***J Biomed Mater Res A***	86	439-447	2008
Seo JH, Matsuno R, Konno T, Takai M, Ishihara K	Surface Tethering of Phosphorylcholine Groups onto Poly(dimethylsiloxane) through Swelling-deswelling Methods with Phospholipids Moiety Containing ABA-type Block Copolymers.	***Biomaterials***	29	1367 -1376	2008
Futamura K, Matsuno R, Konno T, Takai M, Ishihara K	Rapid Development of Hydrophilicity and Protein Adsorption Resistance by Polymer Surfaces Bearing Phosphorylcholine and Naphthalene Groups.	***Langmuir***	24	10340 -10344	2008
Hoshi T, Matsuno R, Sawaguchi T, Konno T, Takai M, Ishihara K	Protein adsorption resistant surface on polymer composite based on 2D/3D controlled grafting of phospholipid polymers.	***Appl Surf Sci***	255	379 -383	2008
Choi J, Konno T, Matsuno R, Takai M, Ishihara K	Surface Immobilization of Biocompatible Phospholipid Polymer Multilayered Hydrogel on Titanium Alloy. ,	***Colloid and Surfaces B : Biointerfaces***	67	216 -223	2008
Morisaku T, Watanabe J, Konno T, Takai M,	Hydration of Phosphorylcholine Groups Attached to Highly Swollen Polymer Hydrogels Studied by Thermal Analysis.	***Polymer***	49(21)	4652 -4657	2008

Ishihara K					
Fujii K, Matsumoto H, Koyama Y, Iwasaki Y, Ishihara K, Takakuda K	Prevention of Biofilm Formation with a Coating of 2-Methacryloyloxyethyl Phosphorylcholine Polymer.	***J Vet Med Sci***	70(2)	167-173	2008
Higashikawa A, Saito T, Ikeda T, Kamekura S, Kawamura N, Kan A, Oshima Y, Ohba S, Ogata N, Takeshita K, Nakamura K, Chung UI, Kawaguchi H	Identification of the core element responsive to runt-related transcription factor 2 in the promoter of human type x collagen gene.	***Arthritis Rheum***	60	166-178	2009
Chikuda H, Seichi A, Takeshita K, Shoda N, Ono T, Matsudaira K, Kawaguchi H, Nakamura K	Radiographic analysis of the cervical spine in patients with retro-odontoid pseudotumors.	***Spine***	34	E110-114	2009
Kyomoto M, Moro T, Miyaji F, Hashimoto M, Kawaguchi H, Takatori Y, Nakamura K, Ishihara K	Effects of mobility/immobility of surface modification by 2-methacryloyloxyethyl phosphorylcholine polymer on the durability of polyethylene for artificial joints.	***J Biomed Mater Res A***			in press.
Liu G, Ogasawara T, Watanabe J, Ishihara K, Asawa Y, Chung UI, Moro T, Takatori Y, Takato T, Nakamura K, Kawaguchi H, Hoshi K	Selection of highly osteogenic and chondrogenic cells from bone marrow stromal cells in biocompatible polymer-coated plates.	***J Biomed Mater Res A***			in press.
Kyomoto M, Moro T, Iwasaki Y, Miyaji F, Kawaguchi H, Takatori Y, Nakamura K, Ishihara K	Super-lubricious surface mimicking articular cartilage by grafting poly(2-methacryloyloxyethyl phosphorylcholine) on orthopaedic metal bearings.	***J Biomed Mater Res A***			in press.
Kyomoto M, Ishihara K	Self-initiated surface graft polymerization of 2-methacryloyloxyethyl phosphorylcholine on poly(ether-ether-ketone) by photo-irradiation.	***ACS Applied Materials & Interfaces***			in press
Hirata M, Kugimiya F,	C/EBPβ promotes transition from proliferation to hypertrophic	***PLoS ONE***			in press

Fukai A, Ohba S, Kawamura N, Ogasawara T, Kawasaki Y, Saito T, Yano F, Ikeda T, Nakamura K, Chung UI, Kawaguchi H	differentiation of chondrocytes through transactivation of $p57^{Kip2}$.				
Ushita M, Saito T, Ikeda T, Yano F, Higashikawa A, Ogata N, Chung UI, Nakamura K, Kawaguchi H	Transcriptional induction of SOX9 by NF-κB family member RelA in chondrogenic cells.	***Osteoarthritis Cartilage***			in press

Phosphorylation of GSK-3β by cGMP-dependent protein kinase II promotes hypertrophic differentiation of murine chondrocytes

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cGMP-dependent protein kinase II (cGKII; encoded by *PRKG2*) is a serine/threonine kinase that is critical for skeletal growth in mammals; in mice, cGKII deficiency results in dwarfism. Using radiographic analysis, we determined that this growth defect was a consequence of an elongated growth plate and impaired chondrocyte hypertrophy. To investigate the mechanism of cGKII-mediated chondrocyte hypertrophy, we performed a kinase substrate array and identified glycogen synthase kinase–3β (GSK-3β; encoded by *Gsk3b*) as a principal phosphorylation target of cGKII. In cultured mouse chondrocytes, phosphorylation-mediated inhibition of GSK-3β was associated with enhanced hypertrophic differentiation. Furthermore, cGKII induction of chondrocyte hypertrophy was suppressed by cotransfection with a phosphorylation-deficient mutant of GSK-3β. Analyses of mice with compound deficiencies in both protein kinases (*Prkg2*$^{-/-}$*Gsk3b*$^{+/-}$) demonstrated that the growth retardation and elongated growth plate associated with cGKII deficiency were partially rescued by haploinsufficiency of *Gsk3b*. We found that β-catenin levels decreased in *Prkg2*$^{-/-}$ mice, while overexpression of cGKII increased the accumulation and transactivation function of β-catenin in mouse chondroprogenitor ATDC5 cells. This effect was blocked by coexpression of phosphorylation-deficient GSK-3β. These data indicate that hypertrophic differentiation of growth plate chondrocytes during skeletal growth is promoted by phosphorylation and inactivation of GSK-3β by cGKII.

Introduction

Skeletal growth is achieved by endochondral ossification in the growth plate cartilage, with orderly columnar arrays of resting, proliferative, and hypertrophic zones of chondrocytes. During the process, chondrocytes undergo proliferation, hypertrophic differentiation, and apoptosis, each of which is regulated by distinct molecular signaling systems (1). Among them, C-type natriuretic peptide (CNP; encoded by *Nppc*), a humoral factor that can regulate a variety of homeostatic processes by binding the membrane-bound guanylyl cyclase–coupled receptor B (GC-B; encoded by *Npr2*), has been shown to play important roles in skeletal growth, because mice deficient in either gene exhibit impaired skeletal growth (2, 3). Loss-of-function mutations in *Npr2* also show dwarfism in patients known as acromesomelic dysplasia, type Maroteaux (4), demonstrating the importance of CNP/GC-B signaling in the skeletal growth of humans as well. This signaling causes the intracellular accumulation of cGMP, which then activates cGMP-dependent protein kinases (cGKs) (5). In mammalian cells, there are 2 cGK isoforms, cGKI and cGKII (encoded by *Prkg1* and *Prkg2*, respectively), which show distinct distributions and functions (6, 7). Although both are expressed in growth plate cartilage, *Prkg2*$^{-/-}$ mice show postnatal dwarfism with about 20%–30% reduction in the length of limbs and trunk (6), while *Prkg1*$^{-/-}$ mice show a normal skeleton (8), indicating that only cGKII is indispensable for skeletal growth.

cGKII is a membrane-bound serine/threonine kinase with a cGMP-binding domain and a catalytic domain in the C terminus (7). In addition to growth retardation resulting from cGKII deficiency in mice, our previous positional cloning analysis identified a deletion in *Prkg2*, the rat gene encoding cGKII, in the Komeda miniature rat Ishikawa (KMI), a naturally occurring mutant rat, which also exhibited dwarfism with 20%–30% shorter long bones and vertebrae (9). The deletion resulted in a frame shift and a premature stop codon, predicting a truncated cGKII protein that lacks the kinase domain (cGKII-Δkinase). KMI rats show an elongated growth plate, whose height is about 2.5-fold that of WT littermates. This is caused by the existence of an abnormal intermediate layer between the proliferative and hypertrophic zones with accumulation of few proliferative or hypertrophic chondrocytes, which indicates that the kinase activity of cGKII is necessary for

Nonstandard abbreviations used: ALP, alkaline phosphatase; Bad, BCL2-antagonist of cell death; cdc25, cell division cycle 25 homolog; cGK, cGMP-dependent protein kinase; cGKII-Δkinase, truncated cGKII protein that lacks the kinase domain; CNP, C-type natriuretic peptide; COL10, type X collagen; GC-B, guanylyl cyclase–coupled receptor B; GSK-3β, glycogen synthase kinase–3β; GSK-3β^{S9A}, phosphorylation-deficient mutant of GSK-3β with a serine-to-alanine substitution; PLK, polo-like kinase; p90RSK, 90-kDa ribosomal protein S6 kinase; PTH, parathyroid hormone; PTHrP, parathyroid hormone–related protein; TCF, T cell factor; VASP, vasodilator-stimulated phosphoprotein.

Conflict of interest: The authors have declared that no conflict of interest exists.

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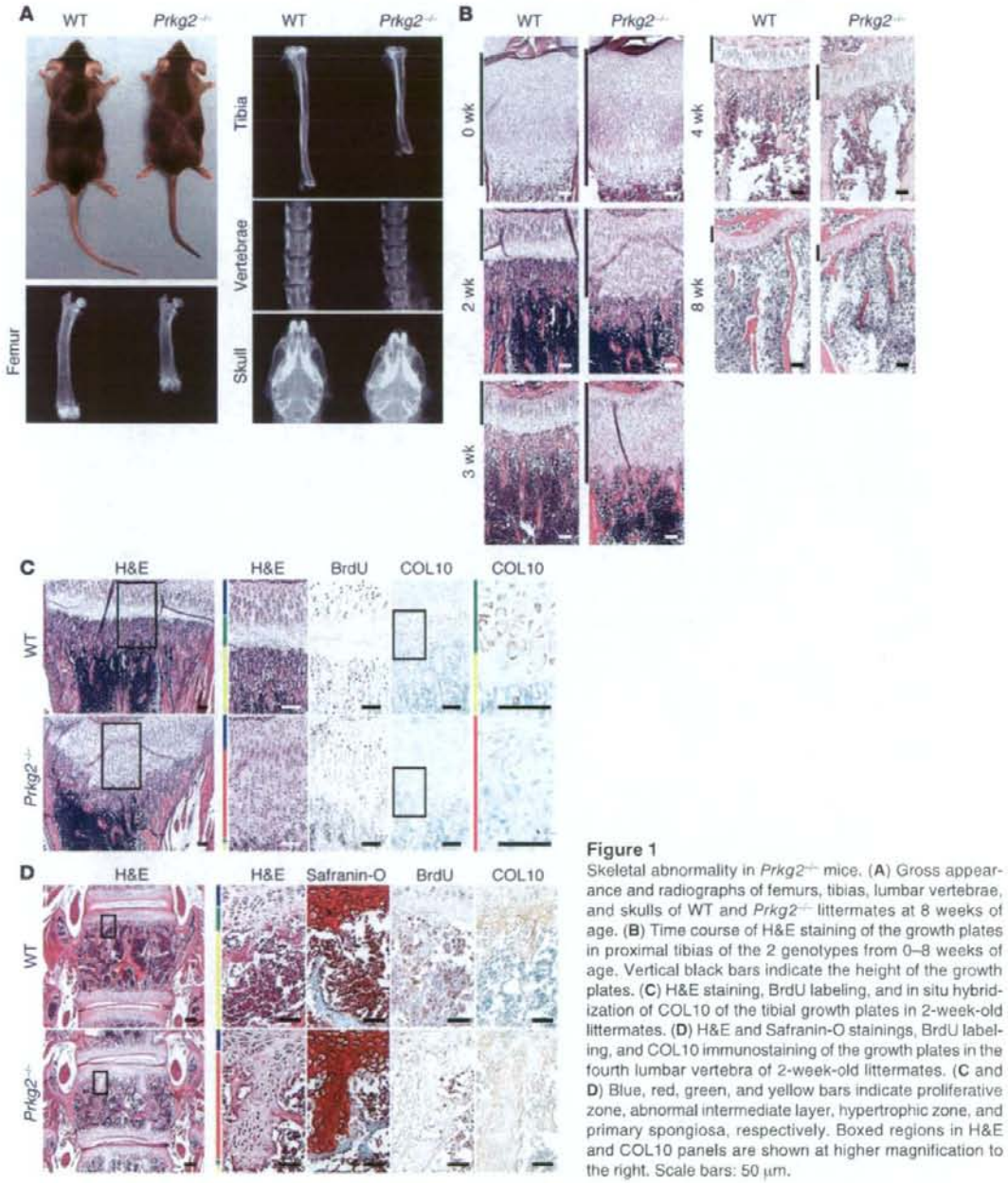

Figure 1

Skeletal abnormality in *Prkg2*$^{-/-}$ mice. (**A**) Gross appearance and radiographs of femurs, tibias, lumbar vertebrae, and skulls of WT and *Prkg2*$^{-/-}$ littermates at 8 weeks of age. (**B**) Time course of H&E staining of the growth plates in proximal tibias of the 2 genotypes from 0–8 weeks of age. Vertical black bars indicate the height of the growth plates. (**C**) H&E staining, BrdU labeling, and in situ hybridization of COL10 of the tibial growth plates in 2-week-old littermates. (**D**) H&E and Safranin-O stainings, BrdU labeling, and COL10 immunostaining of the growth plates in the fourth lumbar vertebra of 2-week-old littermates. (**C** and **D**) Blue, red, green, and yellow bars indicate proliferative zone, abnormal intermediate layer, hypertrophic zone, and primary spongiosa, respectively. Boxed regions in H&E and COL10 panels are shown at higher magnification to the right. Scale bars: 50 μm.

hypertrophic differentiation of growth plate chondrocytes (9). To investigate the mechanism underlying cGKII kinase activity in chondrocyte hypertrophy, in the present study we performed a screen of its potential phosphorylation targets and identified glycogen synthase kinase-3β (GSK-3β; encoded by *Gsk3b*) as a significant phosphorylation target of cGKII. Because the phosphorylation of GSK-3β at Ser9 is known to cause its inactivation (10), we further examined the functional involvement of GSK-3β in the cGKII-induced hypertrophic differentiation of chondrocytes and investigated the underlying mechanism. Our results demonstrated that cGKII promotes chondrocyte hypertrophy and skeletal growth through phosphorylation and inactivation of GSK-3β.

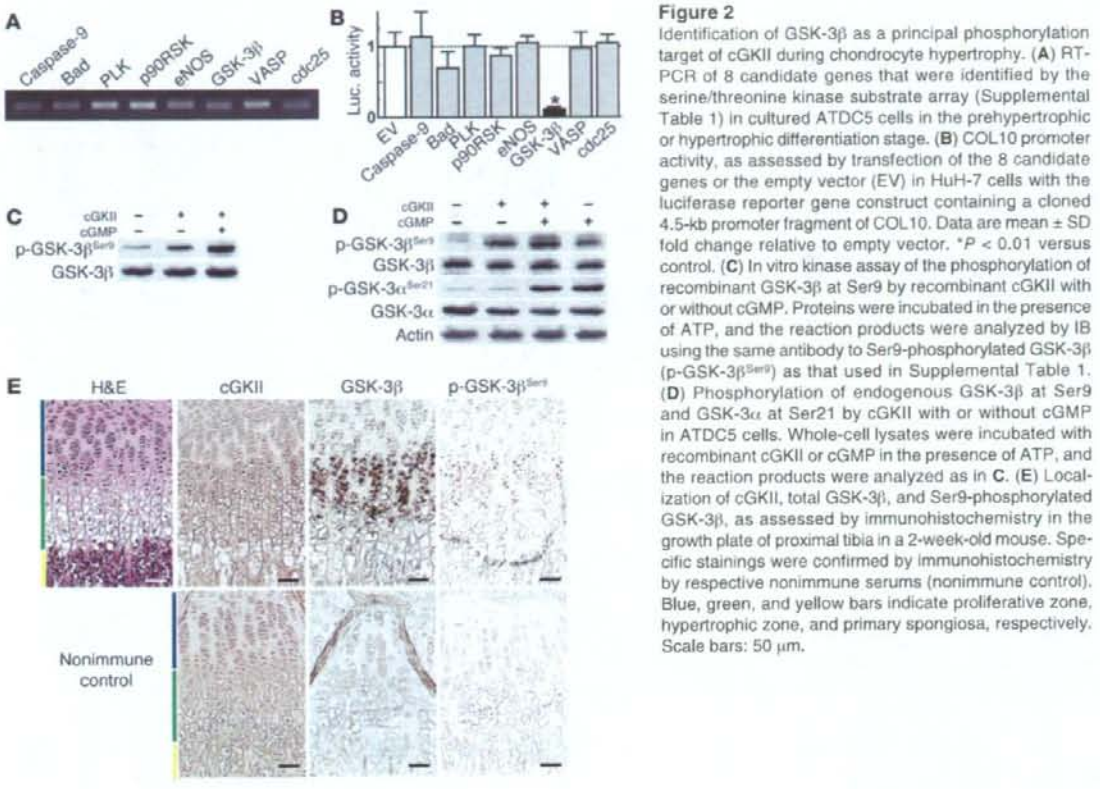

Figure 2

Identification of GSK-3β as a principal phosphorylation target of cGKII during chondrocyte hypertrophy. (**A**) RT-PCR of 8 candidate genes that were identified by the serine/threonine kinase substrate array (Supplemental Table 1) in cultured ATDC5 cells in the prehypertrophic or hypertrophic differentiation stage. (**B**) COL10 promoter activity, as assessed by transfection of the 8 candidate genes or the empty vector (EV) in HuH-7 cells with the luciferase reporter gene construct containing a cloned 4.5-kb promoter fragment of COL10. Data are mean ± SD fold change relative to empty vector. $^*P < 0.01$ versus control. (**C**) In vitro kinase assay of the phosphorylation of recombinant GSK-3β at Ser9 by recombinant cGKII with or without cGMP. Proteins were incubated in the presence of ATP, and the reaction products were analyzed by IB using the same antibody to Ser9-phosphorylated GSK-3β (p-GSK-3β^{Ser9}) as that used in Supplemental Table 1. (**D**) Phosphorylation of endogenous GSK-3β at Ser9 and GSK-3α at Ser21 by cGKII with or without cGMP in ATDC5 cells. Whole-cell lysates were incubated with recombinant cGKII or cGMP in the presence of ATP, and the reaction products were analyzed as in **C**. (**E**) Localization of cGKII, total GSK-3β, and Ser9-phosphorylated GSK-3β, as assessed by immunohistochemistry in the growth plate of proximal tibia in a 2-week-old mouse. Specific stainings were confirmed by immunohistochemistry by respective nonimmune serums (nonimmune control). Blue, green, and yellow bars indicate proliferative zone, hypertrophic zone, and primary spongiosa, respectively. Scale bars: 50 μm.

Results

Growth plate abnormality in Prkg2$^{-/-}$ mice. *Prkg2*$^{-/-}$ mice showed postnatal dwarfism with short limbs and trunk compared with WT littermates (Figure 1A), as previously reported (6). Radiographic analysis at 8 weeks of age revealed that the lengths of femur, tibia, and vertebra, which are known to be primarily formed through endochondral ossification, were shorter in *Prkg2*$^{-/-}$ mice. The longitudinal length of the *Prkg2*$^{-/-}$ skull was also shorter, while the width was comparable to WT. This finding is probably attributable to 2 types of the skull growth via endochondral ossification and intramembranous ossification (11), although this needs to be further investigated. The time course of histological observation of the tibial growth plate revealed that the height was greater in *Prkg2*$^{-/-}$ than WT mice from 2 to 4 weeks after birth but was restored to a level comparable to that of WT mice by 8 weeks of age (Figure 1B). As previously observed in KMI rats (9), growth plate elongation during these ages was caused by an abnormal intermediate layer between the proliferative and hypertrophic zones, with accumulation of few proliferative or hypertrophic chondrocytes, as determined by BrdU uptake and expression of type X collagen (COL10), respectively (Figure 1C). The growth plate of the *Prkg2*$^{-/-}$ vertebral bones also contained the abnormal intermediate layer, which was intermittently focal in the elongated growth plate (Figure 1D). These results indicate that cGKII is necessary for hypertrophic differentiation of growth plate chondrocytes during endochondral ossification for longitudinal growth of limbs and trunk not only in rats, but also in mice.

Phosphorylation targets of cGKII in chondrocyte hypertrophy. To investigate the mechanism underlying cGKII activity in hypertrophic differentiation of chondrocytes, we performed a screen of its phosphorylation targets by in vitro kinase assay using a serine/threonine kinase substrate array. From 87 candidate peptides containing serine/threonine phosphorylation sites, we identified 8 substrates that were most strongly phosphorylated by cGKII: caspase-9, BCL2-antagonist of cell death (Bad), polo-like kinase (PLK), 90-kDa ribosomal protein S6 kinase (p90RSK), eNOS, GSK-3β, vasodilator-stimulated phosphoprotein (VASP), and cell division cycle 25 homolog (cdc25) (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI35243DS1). All of these molecules were confirmed to be expressed in mouse chondrogenic ATDC5 cells in the prehypertrophic or hypertrophic differentiation stage (Figure 2A). However, a luciferase reporter assay revealed that GSK-3β markedly suppressed COL10 promoter activity, while none of the other candidates had a significant effect (Figure 2B). These data suggest that GSK-3β might be functionally involved in chondrocyte hypertrophy, although involvement of the other factors cannot be ruled out. Direct phosphorylation of recombinant GSK-3β at Ser9, the crucial site for inactivation of GSK-3β (10), by recombinant cGKII protein was confirmed by in vitro kinase assay using the same antibody as the screening array above, and the phos-

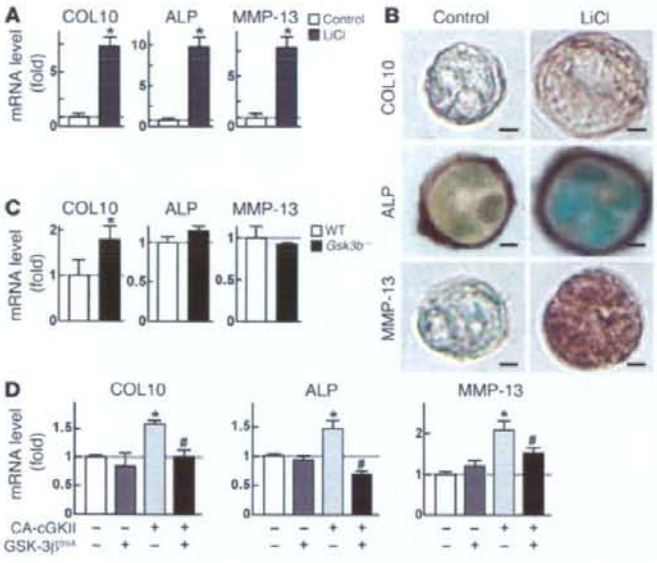

Figure 3
Regulation of chondrocyte hypertrophy by GSK-3β. (**A**) Effects of LiCl on mRNA levels of the hypertrophic markers COL10, ALP, and MMP-13, as assessed by real-time RT-PCR in ATDC5 cells cultured in 3-dimensional alginate beads. (**B**) Effects of LiCl on the hypertrophic markers, as assessed by immunocytochemistry in primary costal chondrocytes cultured in 3-dimensional alginate beads. For morphological comparison, sections of the representative colonies containing 4 cells were selected. Scale bars: 10 μm. (**C**) mRNA levels of the hypertrophic markers, as assessed by real-time RT-PCR in cultured costal chondrocytes from WT and *Gsk3b*$^{+/-}$ mice. (**D**) mRNA levels of the hypertrophic markers in stable lines of ATDC5 cells retrovirally transfected with the constitutively active form of cGKII (CA-cGKII), GSK-3β^{S9A}, or the control GFP (–). Data are mean ± SD of the relative amount compared with control or WT. *$P < 0.01$ versus control or WT. #$P < 0.01$ versus constitutively active cGKII alone.

phorylation was enhanced by the addition of cGMP (Figure 2C). Furthermore, endogenous GSK-3β in cell lysates of ATDC5 cells was phosphorylated at Ser9 by recombinant cGKII protein, which was further enhanced by the addition of cGMP. On the other hand, GSK-3α, the closely related isoform of GSK-3β, was not phosphorylated by cGKII, nor were protein levels of GSK-3β and GSK-3α altered by cGKII or cGMP (Figure 2D). Immunohistochemistry revealed that cGKII, total GSK-3β, and Ser9-phosphorylated GSK-3β were colocalized in prehypertrophic chondrocytes of the growth plate, implicating the interaction of these molecules in vivo as well (Figure 2E). Compared with the respective nonimmune controls, the localization of Ser9-phosphorylated GSK-3β appeared to be restricted to those cells that also produced cGKII, whereas total GSK-3β was more broadly distributed, which supports the notion that cGKII is an important regulator of GSK-3β phosphorylation.

Regulation of chondrocyte hypertrophy by GSK-3β. In the 3-dimensional cultures of ATDC5 cells and primary costal chondrocytes in alginate beads, LiCl, a selective inhibitor of GSK-3β, stimulated the expression of chondrocyte hypertrophic differentiation markers COL10, alkaline phosphatase (ALP), and MMP-13 and induced morphological hypertrophy of the cells (Figure 3, A and B). COL10 expression also increased in cultured primary costal chondrocytes from *Gsk3b*$^{+/-}$ mice compared with WT chondrocytes, while ALP and MMP-13 levels were comparable between the genotypes (Figure 3C). Introduction of a constitutively active allele of cGKII into ATDC5 cells stimulated expression of hypertrophic markers, and this was attenuated by the cointroduction of a phosphorylation-deficient mutant of GSK-3β with a serine-to-alanine substitution (GSK-3β^{S9A}), which is known to have constitutive activity (Figure 3D). These data demonstrated that Ser9 phosphorylation of GSK-3β is necessary for the induction of chondrocyte hypertrophy by cGKII. The GSK-3β^{S9A} introduction alone altered none of the 3 markers (Figure 3D), which indicates that an endogenous GSK-3β level was sufficient for the suppression of chondrocyte hypertrophy in this culture system.

Mechanism underlying cGKII/GSK-3β signaling in chondrocyte hypertrophy. We further examined the molecular mechanism whereby GSK-3β phosphorylation by cGKII regulates hypertrophic differentiation of chondrocytes. Because GSK-3β is known to be a negative regulator of β-catenin through its phosphorylation and degradation (10), we compared the localization of β-catenin, Ser9-phosphorylated GSK-3β, and total GSK-3β in the growth plates of WT and *Prkg2*$^{-/-}$ littermates. In the WT growth plate, β-catenin as well as both GSK-3β proteins were localized mainly in the cytoplasm of prehypertrophic chondrocytes (Figure 4A). cGKII deficiency caused similar decreases in β-catenin and Ser9-phosphorylated GSK-3β levels with little effect on the total GSK-3β level in the abnormal intermediate layer. In cultured ATDC5 cells, cGKII induced cytosolic accumulation of β-catenin after stimulation by 8-bromo-cGMP, while cGKII-Δkinase had a minimal effect (Figure 4B). Overexpression of constitutively active cGKII enhanced the promoter activity of the β-catenin target T cell factor (TCF), which was markedly suppressed by cotransfection of GSK-3β^{S9A} (Figure 4C). Again, GSK-3β^{S9A} alone did not have an effect, which indicates that an endogenous GSK-3β level is sufficient for β-catenin suppression. We next examined the involvement of a scaffolding peptide, Axin, which is known to associate with GSK-3β and promotes effective phosphorylation and degradation of β-catenin under conditions of Wnt stimulation (10). IP/IB analysis using HEK293 cells transfected with Myc-tagged Axin and cGKII revealed that cGKII formed a complex with Axin and phosphorylated GSK-3β not only in the whole-cell lysates, but also in the IP with Axin, suggesting some interaction between Ser9 phosphorylation and coupling with Axin in regulation of GSK-3β by cGKII (Figure 4D).

In our previous study, we showed that cGKII caused attenuation of Sox9 transcriptional function through inhibition of nuclear entry (9). Because Sox9 is known not only to induce chondrogenic differentiation of mesenchymal cells, but also to prevent hypertrophic differentiation of chondrocytes (12), this may contribute

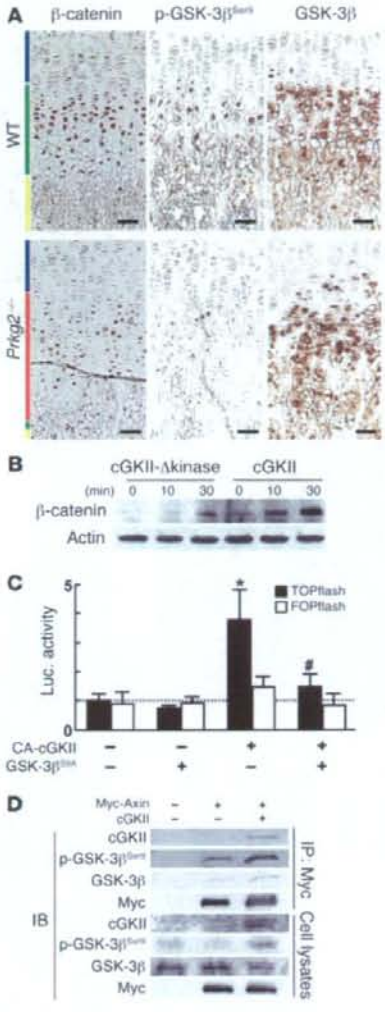

Figure 4

Mechanism underlying cGKII/GSK-3β signaling in chondrocyte hypertrophy. (**A**) Localization of β-catenin, Ser9-phosphorylated GSK-3β, and total GSK-3β, as assessed by immunohistochemistry in the growth plates of the proximal tibias of WT and *Prkg2*$^{-/-}$ mice at 2 weeks of age. Blue, red, green, and yellow bars indicate proliferative zone, abnormal intermediate layer, hypertrophic zone, and primary spongiosa, respectively. Scale bars: 50 μm. (**B**) Time course of β-catenin protein level after stimulation by 8-bromo-cGMP, as assessed by IB in the cytosolic fraction of ATDC5 cells with retroviral introduction of cGKII or cGKII-Δkinase. (**C**) Promoter activity of the β-catenin target TCF, as assessed by luciferase (Luc) assay using TOPflash and FOPflash reporter plasmids in HEK293 cells transfected with constitutively active cGKII, GSK-3β^{S9A}, or the control GFP (–). Data are mean ± SD fold change compared with control (–/–). $^{*}P < 0.01$ versus control. $^{\#}P < 0.01$ versus constitutively active cGKII alone. (**D**) Physical association of cGKII and GSK-3β with Axin by IP/IB analysis. HEK293 cells were transfected with Myc-tagged Axin (Myc-Axin) and/or cGKII, and an aliquot of the cell lysates underwent IP with the high-affinity anti–c-Myc antibody–coupled agarose as described in Methods. The IP (Myc) or the whole-cell lysates underwent IB with an antibody to cGKII, Ser9-phosphorylated GSK-3β, GSK-3β, or Myc.

to the mechanism whereby cGKII promotes chondrocyte hypertrophy. However, inhibition of Sox9 nuclear entry by cGKII was independent of phosphorylation of Sox9 itself, because cGKII inhibited not only the nuclear entry of the WT Sox9, but also that of the phosphorylation-deficient Sox9 mutants with serine-to-alanine substitutions at putative phosphorylation sites Ser64 and Ser181 (Figure 5A), which suggests that other phosphorylation targets of cGKII are important. We therefore examined the involvement of GSK-3β phosphorylation in the inhibition of Sox9 nuclear entry by cGKII. Neither addition of the GSK-3β inhibitor LiCl nor overexpression of GSK-3β^{S9A} altered cGKII-dependent inhibition of Sox9 nuclear entry, which indicates that the inhibitory effect of cGKII was independent of GSK-3β phosphorylation by cGKII (Figure 5B).

We next assessed involvement of other putative signaling systems in cGKII action on chondrocyte hypertrophy. Although Runx2 has previously been shown to be an important transcription factor that induces hypertrophic differentiation of chondrocytes (13), its expression was visible in the abnormal intermediate layer of the *Prkg2*$^{-/-}$ growth plate (Supplemental Figure 1A). In addition, neither the mRNA level nor the subcellular localization of Runx2 was altered by cGKII overexpression in cultured ATDC5 cells (Supplemental Figure 1, B and C).

FGF signaling has also been shown to be important for chondrocyte differentiation and endochondral ossification in mice and humans (14). Considering that targeted overexpression of CNP in chondrocytes counteracts dwarfism in a mouse model of achondroplasia with activated FGF receptor 3 (15) and that the mutant mice exhibit an elongated growth plate similar to that of *Prkg2*$^{-/-}$ mice (16), there might be cross-talk between cGKII and FGF signaling. Because FGF signaling stimulates MAPK or STAT-1 signaling pathways, we examined the interaction of phosphorylation of Erk1, Erk2, p38 MAPK, JNK1, JNK2/3, and STAT-1. Among these, FGF-2 most strongly phosphorylated Erk1 and Erk2; however, overexpression of cGKII affected none of these in the presence or absence of FGF-2, indicating no apparent interaction between cGKII and FGF, MAPK, or STAT-1 signaling (Supplemental Figure 2A).

Partial reversal of the skeletal abnormality in Prkg2$^{-/-}$ *mice by GSK-3β insufficiency.* To test whether GSK-3β plays a role in mediating the effect of cGKII-induced skeletal growth changes in vivo, we examined the effect of genetic insufficiency of GSK-3β on the skeletal abnormality apparent in *Prkg2*$^{-/-}$ mice. Although *Gsk3b*$^{-/-}$ mice were embryonically lethal (17), *Gsk3b*$^{+/-}$ mice developed and grew normally (Supplemental Figure 3). We therefore crossed *Prkg2*$^{-/-}$ mice with *Gsk3b*$^{+/-}$ mice to generate compound *Prkg2*$^{-/-}$ *Gsk3b*$^{+/-}$ mice. Radiographic analysis and total axial length measurement showed that *Prkg2*$^{-/-}$ *Gsk3b*$^{+/-}$ mice exhibited partial, but significant, restoration (about 30%–40%) of the impaired skeletal growth of *Prkg2*$^{-/-}$ mice at 8, 12, and 16 weeks after birth (Figure 6, A and B). Measurement of skeletal length confirmed that the endochondral ossification of femur, tibia, humerus, ulna, vertebra, and skull were decreased by cGKII deficiency, while skull width and clavicle length — which are known to develop by endochondral and intra-