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クロマチン転写制御を目的とした人工酵素の開発に関する研究

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（総合）研究報告書

クロマチン転写制御を目的とした人工酵素の開発に関する研究

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研究要旨

真核生物における転写制御を理解するためには、クロマチン構造を解除する機構を解明しない限り、転写調節の制御機構論を理解することはできない。クロマチンの制御には３種類の酵素（化学修飾酵素、ATP非依存ならびに依存のクロマチン構造変換因子）が重要な役割を果たすことが近年明らかになった。クロマチン構造変換酵素は、DNA結合型転写因子と相互作用することにより特定のプロモーターへ誘導されるという真核遺伝子転写の選択性、特異性を説明しうる新しいメカニズムを我々は提唱してきた。

本計画では、クロマチン構造変換酵素とDNA結合型転写因子の相互作用に着目し、協調性を生み出す酵素・基質両者の機能上重要な領域を特定し、その制御（増減）を可能にすることを目的としている。DNA結合蛋白とクロマチン構造変換因子の相互作用は細胞や個体分化において重要な働きがある事を示唆する知見が得られており、本研究は細胞分化制御の理解、さらに細胞分化誘導をはじめとした疾患（癌、臓器再生）の新しい治療法の開発につながると期待できる。最終的にはヒトに代表される真核細胞のクロマチンからの選択的な転写制御を可能にし、人工転写酵素（クロマチン構造変換酵素）を用いたピンポイントでのナノ治療（ナノミセル等の導入）の基盤となる情報及び技術を開発する。

A．研究目的

本研究の目的は、ヒトにおけるクロマチン状態からの遺伝子転写を制御することを可能にするための基盤情報の集積及び技術の開発であり、最終的にはナノ技術を応用したクロマチンからの転写の操作を可能にする新しい治療法を開発することである。

転写因子をターゲットとする治療戦略の開発は受容体や酵素に比し、遅れている状況にある。その理由は、従来の転写研究は裸のDNAの状態を想定した研究が中心に発展したためと考えられる。すなわち、ヒトでは遺伝子転写を解明するうえで、クロマチン状態での制御の理解が不可欠であるが、過去の研究はin vitroでの裸のDNAの状態を対象としてきため、クロマチン状態からの転写を十分に説明できなかった。そのため、真核転写を対象とした治療戦略が発展しなかったと考えられる。しかしながら、遺伝子転写制御は病態をはじめ、あらゆる生命現象において中心的な役割を担っている。遺伝子転写制御を解明することは極めて重要な分野であり、クロマチンからの転写制御の理解が真核転写を理解する鍵になると考えられる。本計画で真核転写の基盤を明らかにし、真核細胞のクロマチンをターゲットとする遺伝子発現転写調節の新しい治療戦略を可能にすることが目的である。クロマチン構造変換酵素とDNA結合型転写因子の相互作用の制御は、細胞分化や癌化と密接に関わっていることは既に知られており、幅広く生命現象の制御に関わっている。この相互作用の制御が可能になれば、細胞分化誘導をはじめとする疾患（癌、臓器再生）の新しい治療法の開発につながると期待できる。具体的な例としては、ホメオボックス蛋白を標的とした臓器再生、E2Fによる細胞周期制御あるいはオンコジーン転写因子の不活化を通した抗癌療法などが考えられる。特定組織、細胞への導入には人工的なナノ技術が理想と考えられる研究課題である。このように、クロマチンレベルでの遺伝子発現転写調節を通した普遍的な治療法は、ナノマシンによるピンポイント・デリバリーとの併用により、癌から特定臓器疾患（心血管疾患等）に幅広く応用できる新しい治療法の開発につながると期待できる。

B．研究方法

遺伝子発現転写制御におけるクロマチンの役割及びその制御は約５年前から内外において注目されている分野である。DNAとともにクロマチンの基本コンポーネントを構成するヒストンの役割が主に研究されてきた。しかしながら、ヒストンは普遍的に存在するため、遺伝子特異性・選択性は説明できないといち早く注目した我々は、DNA結合蛋白とクロマチン構造変換因子の相互作用が真核生物での転写活性化の機構論の解明の糸口になると考え、転写反応の特異性を決定する上でもっとも重要なDNA結合型転写因子との協調的な相互作用を通した制御機構に注目した。

具体的には、以下のような実験を行った。

１。クロマチン構造変換酵素とDNA結合方転写因子の相互作用の単離・同定

・相互作用因子単離同定法：Sp/KLF因子の6HIS (hexahistidine)エピトープを付加したリコンビナント蛋白質を調製し、細胞核抽出液からSp/KLF因子と相互作用する因子をHISエピトープに対するアフィニティ精製を用いて単離した。SDS-PAGEゲル電気泳動にて展開後、Coomassie Brilliant Blueで染色し、分離の良いバンドは切り出した。分離の悪いバンドについては、pH勾配で二次元電気泳動を行い、切り出した。切り出したバンドは、酵素消化後質量分析器(MALDI TOF-MS)にて質量パターンを解析し、さらにフィンガープリント法でアミノ酸配列を推定した。

２。クロマチン構造変換酵素とDNA結合方転写因子の相互作用の機能的意義の解析

・相互作用の検討：リコンビナント蛋白質を用いた直接相互作用の解析には、GST pull-down法を用いた。具体的には、リコンビナント蛋白質にはGSTを付加し、GSTに対するアフィニティ・レジンで固定した。相互作用因子側（エピトープに対するウエスタン、His等）で検出した。細胞免疫沈降にはProtein-A/G sepharoseに抗体を固定し、細胞抽出液を添加後、相互作用因子側に対する抗体でイムノブロットした。
・DNA結合能の検討：ゲルシフトアッセイをSuzuki et al. 2000 の方法にしたがって行った。^{32}Pでラベルした DNAプローブにリコンビナント蛋白質を反応後、アクリルアミドゲルにて電気泳動し、オートラジオグラフィーにて結合の有無と程度を検出した。Sp1はSV40のGC配列、KLF5はSE1配列をプローブに用いた。
・転写活性の検討：レポーター・トランスフェクション・アッセイを行った。4x10^{5}個の細胞を24穴プレートにまき、24時間後にリポフェクチン法を用いてプラスミドを細胞に導入し、さらに48時間後に細胞を回収し、ルシフェラーゼアッセイのプロトコール通り(Promega)にルシフェラーゼ・レポーター活性を測定した。Sp1はSV40のearly プロモーターを、KLF5にはSMembとPDGF-A鎖両遺伝子のプロモーターのレポーター・コンストラクトを用いた。
・アセチル化能の検討：アセチル化反応をSuzuki et al. 2000の方法にしたがって行った。具体的には、^{14}Cでラベルした acetyl CoAをアセチル化酵素反応に加え、SDS-PAGE電気泳動後、オートラジオグラフィにてアセチル化の有無と程度を評価した。
・細胞内遺伝子発現調節の検討：フォルボル・エステルをはじめとする増殖刺激剤を添加した培養細胞株を経時的にサンプリングし、RT-PCR解析(PDGF-A鎖遺伝子）用にRNAを、ウエスタンブロット解析用に蛋白質をそれぞれ調製した。
・血管病態モデルでの検討：ラットでのバルーン障害モデルは、頸動脈をバルーンで拡張後、２週後に病理切片を作製し、免疫組織評価を行った。
・安定発現細胞株の作製並びに細胞増殖能の検討：安定発現細胞株は、G418カセットを含む発現ベクターを構築後、ネオマイシンに対する耐性を基に安定発現細胞を株化した。細胞増殖の検討は、細胞の経時的な細胞数の計測の他、BrdUの導入効率を測定した。
・点変異ならびにアデノウイルスのコンストラクトの作製：アセチル化残基の点変異はPCR法を用いて導入した。アデノウイルスはDNA-TPC法を用いて作製した。

３。クロマチン構造変換酵素の結晶構造解

析

・結晶作製、結晶構造解析：リコンビナントを大腸菌内で発現後、アフィニティ精製した。濃縮とサイズ分画を行い、高純度の標品を得た。hanging drop法で結晶を作製し、X線ビームで回折像を得た。

（倫理面への配慮）
本計画は、本学の組み替え実験計画の承認を得ている。生化学、細胞生物学的実験は、当施設の実験ガイドラインを遵守する。また、動物実験については、動物愛護の観点から配慮する。

C．研究結果

クロマチン構造を解除する機構を解明しない限り、真核生物における転写調節の制御機構論を理解することはできない。クロマチンの制御には３種類の酵素（化学修飾酵素、ATP非依存ならびに依存のクロマチン構造変換因子）が重要な役割を果たすことが近年明らかになった。クロマチン構造変換因子は化学修飾酵素（アセチル化、メチル化、燐酸化、ユビキチン化）及びATP依存（SWI/SNF等）とATP非依存（ヒストンシャペロンASF/CIA/RCAF、TAF-1等）のクロマチン構造変換因子の３群に大別されるが、これらの因子の酵素活性の機能ならびに構造は十分に明らかにされていない。しかしながら、酵素活性がクロマチン構造変換にとって必須であるため、活性制御がクロマチン構造変換の制御の鍵になると考えられる。そのため、酵素活性領の制御（増減）を通したクロマチンへのアクセスの調節の視点からの研究を進めることは、真核転写を解明し、さらに調節を可能にするうえで重要と考えられる。実際に、世界でもっとも解析が進んでいるアセチル化酵素p300の酵素活性領域をマップするための点変異ミュータントは我々自身が世界に先駆けて作製した(Suzuki et al., 2000)。

クロマチン構造変換酵素は、DNA結合型転写因子と相互作用することにより特定のプロモーターへ誘導されるという真核遺伝子転写の選択性、特異性を説明しうる新しいメカニズムを我々は提唱してきた。今までに、DNA結合蛋白とクロマチン構造変換因子の相互作用及び制御の機能的な意義を明らかにしてきた。まず、アセチル化酵素とDNA結合蛋白の相互作用を解析した。過去に我々が単離同定したDNA結合蛋白Sp/KLFファミリー因子間(Suzuki et al., 1998) にアセチル化酵素との相互作用に特異性があり、また相互間の制御を明らかにした(Suzuki et al., 2000)。ファミリー因子間の特異的な制御におけるアセチル化酵素との相互作用の役割、またDNA結合蛋白によるアセチル化酵素活性の制御を明らかにした世界ではじめての例であった。

研究期間中に、同DNA結合蛋白Sp/KLFファミリー因子の相互作用因子を単離・同定し、ATP非依存のクロマチン構造変換因子との相互作用を明らかにした。その結果、世界ではじめてDNA結合蛋白ファミリー因子とATP非依存クロマチン構造変換因子間の相互作用とその特異性を示し、さらにその機能的な意義を示した(Suzuki et al., 2003; Miyamoto, Suzuki et al., 2003)。

具体的には、Sp/KLFのリコンビナント蛋白質を用いて細胞核抽出液から相互作用因子をアフィニティ精製後、バンドをTOF-MS法にて同定した。今回は、ATP非依存のクロマチン構造変換因子TAF-Iの単離に成功した。Sp/KLF因子とTAF-Iの相互作用を確認するために、リコンビナント蛋白質を用いたin vitroでのGST pull-downアッセイで直接結合を確認後、抗体を用いた免疫沈降を施行し、細胞内で実際に相互作用することを確認した。Sp1はHeLa細胞からアフィニティ精製の結果、TAF-Iのαとβの両アイソフォームと相互作用したが(Suzuki et al., 2003)、KLF5はTAF-Iβ/SETのみと相互作用した(Miyamoto, Suzuki et al., 2003)。また、TAF-Iはp53, MyoD, NFkappaB等他のDNA結合型転写因子と相互作用しなかった。さらに、TAF-Iと同様にATP非依存クロマチン構造変換因子（ヒストンシャペロン）であるASF/CIA/RCAFはSp/KLFと相互作用しなかったため、DNA結合型転写因子Sp/KLFとATP非依存クロマチン構造変換因子（ヒストンシャペロン)TAF-Iの相互作用に特異性を認めた。

次に、相互作用の機能的な意義を検討するために、Sp/KLF因子のDNA結合能ならびに転写活性化能への影響を検討した。ゲルシフトアッセイ、レポーター・コトランスフェクションアッセイを施行した結果、TAF-IはSp/KLF因子(Sp1, KLF5) のDNA結合

活性及び転写活性化能を抑制し、リプレッサーとして作用することを明らかにした。具体的には、TAF-Iαとβは両方ともSp1のDNA結合配列(SV40遺伝子配列) への結合を抑制し、Sp1によるSV40遺伝子プロモーターの転写活性化を阻害した。また、SETはKLF5のDNA結合配列(SMemb遺伝子SE配列) への結合を抑制し、KLF5によるSMemb、PDGF-A鎖両遺伝子のプロモーターの活性化を阻害した。よって、TAF-IはSp/KLF因子の複数の活性を抑制した。

TAF-Iの細胞ないし個体のレベルでの機能を解析するために、細胞機能が明らかなKLF5を中心に解析した。KLF5はprotooncogeneであり、心血管系を中心に細胞レベルでは細胞増殖を促進し、病態形成に関わる。組織レベルでは、心血管系臓器の病態刺激に対するリモデリング反応を制御する (Shindo et al., 2002)。一方、TAF-Iはtumor suppressor因子と考えられており、それに対してKLF5はprotooncogeneであることから、TAF-IはKLF5の細胞機能を負に制御する可能性があると考えた。KLF5を発現する細胞株を樹立した結果、細胞増殖が促進された。TAF-1を同細胞にトランスフェクトした結果、細胞増殖が抑制された。また、病態においてもKLF5とTAF-Iは興味深くカップリングした発現パターンを認めた。KLF5とTAF-Iはともに正常血管ではほとんど発現を認めないが、バルーン障害後の新生内膜で両者とも発現が誘導された。病態における発現を検討するために、モデルアゴニストとしてphorbol ester刺激を用いた細胞実験の結果、刺激後KLF5は発現が誘導されるのに対して、TAF-Iは発現が抑制された。これらの知見より、TAF-IはKLF5の抑制因子であり、刺激(phorbol ester)によりTAF-I自身の発現が抑制され、さらにKLF5の発現が亢進することにより、KLF5が活性化されるメカニズムが考えられた。TAF-Iのアデノウイルス等の発現コンストラクトを用いて、遺伝子導入による治療効果を検討した結果、KLF5導入により血管病態モデルでの新生内膜が増大するのに対し、TAF-Iの導入によりその増大の阻害が確認された。遺伝子治療の可能性が示された。

同時に、KLF5とアセチル化酵素p300が相互作用し、その結果、後者が前者をアセチル化し、さらに転写を活性化することを示した。アセチル化修飾を受ける残基を質量分析器で特定した。点変異を作成した結果、アセチル化されない点変異体は、KLF5 wild typeでみられる細胞増殖促進作用を有さず、また転写活性化を示さなかった。アデノウイルスコンストラクトを血管病態モデルに投与した結果、新生内膜の増大を認めず、KLF5の作用においてアセチル化が重要であることを示した。さらに、TAF-Iはp300によるKLF5のアセチル化を阻害し、KLF5に対するTAF-Iの作用は、アセチル化の阻害も介することを示した。DNA結合転写因子に対して、p300とTAF-Iがそれぞれ正負に作用し、そのメカニズムが相互作用並びに化学修飾のカップリングを介することをはじめて示した。

また、脱アセチル化酵素によるDNA結合転写因子への直接作用を明らかにした (Matsumura, Suzuki et al., 2005)。具体的には、KLF5が脱アセチル化酵素HDAC1と相互作用し、その結果前者のDNA結合活性ならびに転写活性を阻害することを示した。さら、KLF5との相互作用において、HDAC1とp300が競合することを示した。p300とTAF-Iによる協調的な制御と併せて、複数のクロマチン構造変換因子によるDNA結合転写因子への協調的制御をはじめて明らかにした。

これらの新規のクロマチンレベルでの遺伝子発現の制御に関わる分子メカニズムに対して創薬を試みた。まず、前述のアデノウイルスを用いた実験を通して、遺伝子治療の可能性を示した。相互作用を制御すっる化合物をスクリーニングし、現在も進行中である。また、DNA結合型転写因子Sp/KLFとクロマチン構造変換酵素TAF-Iの相互作用の物理化学的な基盤を明らかにし、ピンポイント制御薬を開発する目的で、TAF-Iの結晶構造を解析した。その結果、2.8オングストロームの解像度のX線回折像を得た(Muto et al., in press)。最終的には、複合体解析を行い、相互作用の作用点をピンポイントで制御するコンパウンドをデザインする。

D. 考察

我々は、クロマチン構造変換酵素とDNA結合型転写因子が相互作用することにより特定のプロモーターへ誘導されるという真核遺伝子転写の選択性、特異性を説明しうる新しいメカニズムを提唱してきた。今まで

に、我々の仮説に基づき、DNA結合蛋白とクロマチン構造変換因子の相互作用及び制御の機能的な意義を明らかにしてきた。アセチル化酵素とDNA結合蛋白の相互作用及び、相互間の制御さらにDNA結合蛋白によるアセチル化酵素活性の制御を世界ではじめて明らかにした研究実績に立脚し、今回新たにATP非依存クロマチン構造変換因子との相互作用を明らかにした。

E．結論

本研究の基礎となるクロマチン構造変換酵素とDNA結合型転写因子の協調的な相互作用のメカニズムは我々が世界に先駆けて見出したことであり(Suzuki et al., 2000)、このメカニズムの応用である本計画は極めて独創性が高く、また内外において他に研究されていない状況にある。

本計画の特徴のひとつは、クロマチン構造変換因子の酵素活性に着目した点にある。すなわち、クロマチン構造変換因子は化学修飾酵素（アセチル化、メチル化、燐酸化、ユビキチン化）及びATP依存（SWI/SNF等）とATP非依存（ヒストンシャペロンASF/CIA/RCAF、TAF-1等）のクロマチン構造変換因子の３群に大別されるが、これらの因子の酵素活性の機能ならびに構造は十分に明らかにされていない。しかしながら、酵素活性がクロマチン構造変換にとって必須であるため、活性制御がクロマチン構造変換の制御の鍵になると考えられる。そのため、酵素活性の制御（増減）を通したクロマチンへのアクセスの調節の視点からの研究を進めることは、真核転写を解明し、さらに調節を可能にするうえで重要と考えられる。

F．研究発表

１．論文発表

Shindo T, Manabe I, Fukushima Y, Tobe K, Aizawa K, Miyamoto S, Kawai-Kowase K, Moriyama N, Imai Y, Kawakami H, Nishimatsu H, Ishikawa T, Suzuki T, Morita H, Maemura K, Sata M, Hirata Y, Komukai M, Kagechika H, Kadowaki T, Kurabayashi M, Nagai R. Kruppel-like zinc-finger transcription factor KLF5/BTEB2 is a target for angiotensin II signaling and an essential regulator of cardiovascular remodeling. Nat Med 2002;8:856-863.

Suzuki T, Horikoshi M, Nagai R. Regulation of the Sp/KLF-family of transcription factors: focus on post-transcriptional modification and protein-protein interaction in the context of chromatin. Gene Ther Mol Biol 2003:7;91-97.

Suzuki T, Muto S, Miyamoto S, Aizawa K, Horikoshi M, Nagai R.Functional interaction of the DNA-binding transcription factor Sp1 through its DNA-binding domain with the histone chaperone TAF-I. J Biol Chem 2003;278:28758-28764.

Miyamoto S, Suzuki T, Muto S, Aizawa K, Kimura A, Mizuno Y, Nagino T, Imai Y, Adachi N, Horikoshi M, Nagai R. Positive and negative regulation of the cardiovascular transcription factor KLF5 by p300 and the oncogenic regulator SET through interaction and acetylation on the DNA-binding domain. Mol Cell Biol 2003;23:8528-41.

Muto S, Senda M, Adachi N, Suzuki T, Nagai R, Horikoshi M. Purification, crystallization and preliminary X-ray diffraction analysis of human oncoprotein SET/TAF-1β. Acta Crystallogr D Biol Crystallogr. 2004;60:712-4.

Matsumura T, Suzuki T, Aizawa K, Munemasa Y, Muto S, Horikoshi M, Nagai R. The deacetylase HDAC1 negatively regulates the cardiovascular transcription factor Kruppel-like factor 5 through direct interaction. J Biol Chem. 2005 in press.

２．学会発表

・第７５回日本生化学会大会（平成１４年

１０月１６日、京都）
鈴木　亨、宮本索、宗政歓子、武藤真祐、堀越正美、永井良三。転写調節因子のDNA結合ドメインを介するクロマチン転写調節制御の新規モデル

・アメリカ心臓病学会　American Heart Association（平成１４年１１月１２日、米国シカゴ）
Suzuki T, Miyamoto S, Aizawa K, Muto S, Horikoshi M, Nagai R. Identification of a transcriptional repressor of the transcription factor KLF5/BTEB2

・Zinc Signals 4th Annual International Conference（平成１５年５月２日ー同９日、イギリス領ケイマン諸島）
Suzuki T, Nagai R. Functional Roles of SP/KLF-family Zinc-finger Transcription Factors: Focus On Role of KLF5/BTEB2 in the Cardiovascular System

・アメリカ心臓病学会　American Heart Association（平成１５年１１月９日ー同１２日、米国オーランド）
Shindo T, Manabe I, Suzuki T, Aizawa K, Miyamoto S, Nagai R. Kruppel-Like Zinc-Finger Transcription Factor KLF5/BTEB2 is an Essential Regulator of Cardiovascular Remodeling.

・日本分子生物学会（平成１５年１２月１０日ー同１３日、神戸）
武藤真祐、梅原崇、葛原隆、鈴木亨、永井良三、堀越正美。ヒストンシャペロンによる抗サイレンシング反応の分子機構

・第６８回日本循環器学会（平成１６年３月２７日ー同２９日、東京）
Takayoshi Matsumura, Toru Suzuki, Kenichi Aizawa, Yoshiko Mizuno, Tomoko Nagino, Yoshiko Munemasa, Nanae Kada, Kana Sasaki, Ryozo Nagai. The Cardiovascular Remodeling Transcription Factor KLF5 Is Negatively Regulated by the Histone Deacetylase HDAC1 through Direct Interaction

Toru Suzuki, Saku Miyamoto, Shinsuke Muto, Kenichi Aizawa, Akatsuki Kimura, Yoshiko Mizuno, Tomoko Nagino, Yasushi Imai, Naruhiko Adachi, Takayoshi Matsumura, Kana Sasaki, Nanae Kada, Yoshiko Munemasa, Masami Horikoshi, Ryozo Nagai. Positive and negative regulation of the cardiovascular transcription factor KLF5 by p300 and SET through interaction and acetylation.

・アメリカ心臓病学会　American Heart Association（平成１６年１１月９日ー同１３日、米国ニューオーリンズ）

Takayoshi Matsumura, Toru Suzuki, Kenichi Aizawa, Yoshiko Munemasa, Kana Iimuro, Ryozo Nagai. The Cardiovascular Remodeling Transcription Factor KLF5 is Regulated by the Histone Deacetylase HDAC1 through Direct Interaction.

・第１０回放射光医学研究会講演会（平成１７年１月２２日、大阪）
鈴木亨、武藤真祐、千田俊哉、堀越正美、永井良三。心血管転写因子KLF5及びそれの相互作用制御因子の結晶構造解析を通したピンポイント創薬

G．知的財産権の出願・登録状況（予定を含む）。

１．特許取得
なし

２．実用新案登録
なし

３．その他
なし

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
無し							

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
Shindo T, Manabe I, Fukushima Y, Tobe K, Aizawa K, Miyamoto S, Kawai-Kowase K, Moriyama N, Imai Y, Kawakami H, Nishimatsu H, Ishikawa T, Suzuki T, Morita H, Maemura K, Sata M, Hirata Y, Komukai M, Kagechika H, Kadowaki T, Kurabayashi M, Nagai R.	Kruppel-like zinc-finger transcription factor KLF5/BTEB2 is a target for angiotensin II signaling and an essential regulator of cardiovascular remodeling	Nat Med	8	856-863	2002
Suzuki T, Horikoshi M, Nagai R.	Regulation of the Sp/KLF-family of transcription factors: focus on post-transcriptional modification and protein-protein interaction in the context of chromatin.	Gene Ther Mol Biol	7	91-97	2003
Suzuki T, Muto S, Miyamoto S, Aizawa K, Horikoshi M, Nagai R	Functional interaction of the DNA-binding transcription factor Sp1 through its DNA-binding domain with the histone chaperone TAF-I	J Biol Chem	278	28758-28764	2003
Miyamoto S, Suzuki T, Muto S, Aizawa K, Kimura A, Mizuno Y, Nagino T, Imai Y, Adachi N, Horikoshi M, Nagai R	Positive and negative regulation of the cardiovascular transcription factor KLF5 by p300 and the oncogenic regulator SET through interaction and acetylation on the DNA-binding domain	Mol Cell Biol	23	8528-8541	2003
Muto S, Senda M, Adachi N, Suzuki T, Nagai R, Horikoshi M	Purification, crystallization and preliminary X-ray diffraction analysis of human oncoprotein SET/TAF-1β	Acta Crystallogr D Biol Crystallogr	60	712-714	2004
Matsumura T, Suzuki T, Aizawa K, Munemasa Y, Muto S, Horikoshi M, Nagai R	The deacetylase HDAC1 negatively regulates the cardiovascular transcription factor Kruppel-like factor 5 through direct interaction	J Biol Chem		in press	2005

Krüppel-like zinc-finger transcription factor KLF5/BTEB2 is a target for angiotensin II signaling and an essential regulator of cardiovascular remodeling

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We recently isolated a Krüppel-like zinc-finger transcription factor 5 (KLF5; also known as BTEB2 and IKLF), which is markedly induced in activated vascular smooth-muscle cells and fibroblasts. Here we describe our analysis of the *in vivo* function of KLF5 using heterozygous KLF5-knockout mice (*Klf5*$^{+/-}$). In response to external stress, *Klf5*$^{+/-}$ mice showed diminished levels of arterial-wall thickening, angiogenesis, cardiac hypertrophy and interstitial fibrosis. Also, angiotensin II induced expression of KLF5, which in turn activated platelet-derived growth factor-A (PDGF-A) and transforming growth factor-β (TGF-β) expression. In addition, we determined that KLF5 interacted with the retinoic-acid receptor (RAR), that synthetic RAR ligands modulated KLF5 transcriptional activity, and that *in vivo* administration of RAR ligands affected stress responses in the cardiovascular system in a KLF5-dependent manner. KLF5 thus seems to be a key element linking external stress and cardiovascular remodeling.

In response to metabolic and/or mechanical stress such as hypertension and hyperlipidemia, the heart and vasculature undergo structural remodeling involving cellular hypertrophy and hyperplasia, interstitial fibrosis and angiogenesis, all of which underlie the pathogenesis of heart failure and atherosclerosis[1,2]. Locally expressed growth factors have key roles in these processes; however, little is known about the transcriptional regulatory mechanisms underlying them.

We recently identified Krüppel-like zinc-finger transcription factor 5 (KLF5; also known as BTEB2 and IKLF) as a transcription factor for SMemb/non-muscle myosin heavy chain-B (NMHC-B), which is a molecular marker of phenotypically modulated smooth-muscle cells[3–5]. KLF5 is a member of the Krüppel-like transcription factor family, which has diverse functions during cell differentiation and embryonic development[7–9]. Normally, KLF5 is abundantly expressed in developing blood vessels, but is downregulated in adult vessels[6]. However, its expression is strongly upregulated in activated smooth-muscle cells and fibroblasts (myofibroblasts) within vascular lesions[6,10]. In addition, we have shown that phorbol-12-myristate-13-acetate (PMA) induces *Klf5* expression through the mitogen-activated protein-kinase pathway[11]. To better understand the involvement of KLF5 in cardiovascular disease, we generated heterozygous KLF-knockout (*Klf5*$^{+/-}$) mice, which were then used to analyze the *in vivo* function of KLF5.

Homozygote lethality in KLF5-knockout mice

Targeting strategy and analysis of homologous recombination are shown in Fig. 1*a* and *b*. The number of live births was significantly reduced when *Klf5*$^{+/-}$ mice were intercrossed. Analysis of the embryos from timed *Klf5*$^{+/-}$ intercrosses showed that *Klf5*$^{-/-}$ homozygotes died before embryonic day (E) 8.5.

Reduced responses to injury and angiogenesis in *Klf5*$^{+/-}$ mice

Unlike their homozygous *Klf5*$^{-/-}$ littermates, *Klf5*$^{+/-}$ mice survived until adulthood and were apparently normal and fertile, though expression of KLF5 was reduced to about half that in wild-type mice (Fig. 1*c* and *d*). Closer observation revealed abnormal thinning of the medial and adventitial layers of the aortic wall in *Klf5*$^{+/-}$ mice (media: wild-type, 39.3 ± 1.3 μm; *Klf5*$^{+/-}$, 34.0 ± 4.1 μm; $P < 0.05$; adventitia: wild-type, 17.0 ± 1.0 μm; *Klf5*$^{+/-}$, 13.0 ± 1.3 μm; $P < 0.05$; $n = 6$) (Fig. 1*e–h*).

KLF5 is upregulated in the neointima within vascular lesions[6–11], so we compared the structural changes in the walls of

femoral arteries between wild-type and *Klf5*$^{+/-}$ mice injured using a polyethylene tube cuff. In wild-type mice, a thick layer of granulation tissue, containing numerous microvessels and inflammatory cells and a large amount of extracellular matrix, developed around the cuff (Fig. 2*a* and *c*). In contrast, the *Klf5*$^{+/-}$ mice developed markedly less granulation tissue and showed less angiogenesis (Fig. 2*b* and *d*). Moreover, within the cuffs, the arteries of the heterozygotes were thin-walled and dilated (Fig. 2*f*), which was in contrast to the wild-type animals, which showed thickened medial and intimal layers and high proliferation of smooth-muscle cells (Fig. 2*e*). Areas of the neointima (wild-type, 11650 ± 1426 μm^2; *Klf5*$^{+/-}$, 1020 ± 160 μm^2; $P < 0.01$; $n = 6$ in each) and the granulation tissue around the cuff (wild-type, 1.052 ± 0.100 mm^2; *Klf5*$^{+/-}$, 0.209 ± 0.038 mm^2; $P < 0.01$; $n = 5$ in each) were both smaller in *Klf5*$^{+/-}$ mice (Fig. 2*g* and *h*). Responses were similarly attenuated in *Klf5*$^{+/-}$ mice in the wire-injured femoral artery model (data not shown). Apparently, activation and proliferation of smooth-muscle cells and fibroblasts in response to vascular injury was impaired in *Klf5*$^{+/-}$ mice, as were inflammatory responses and angiogenesis.

Next, we found that *Klf5*$^{+/-}$ mice showed impaired angiogenic activity in a hind-limb ischemia model, in which the femoral arteries were ablated (data not shown), and that angiogenic responses to implanted tumors were markedly attenuated (Fig. 2*i* and *j*). Thus, KLF5 seems to be involved in several facets of vascular remodeling, including mesenchymal-cell activation, development of interstitial fibrosis and angiogenesis.

Reduced cardiac hypertrophy and fibrosis in *Klf5*$^{+/-}$ mice

The reduced vascular remodeling observed in *Klf5*$^{+/-}$ mice prompted us to examine the role of KLF5 in responses elicited by angiotensin II, a potent growth factor known to affect both cardiac hypertrophy and vascular remodeling[12–14]. Following a continuous, 14-day infusion of angiotensin II, the hearts of wild-type mice were significantly heavier than those of *Klf5*$^{+/-}$ mice (Fig.3*a*,*b*). Interstitial and perivascular fibrosis was much reduced in Klf5$^{+/-}$ mice (Fig.3*c*,*d*).The ratios of heart weight (left ventricle + right ventricle) to body weight and perivascular fibrosis area to lumen area were both significantly lower in *Klf5*$^{+/-}$ mice (heart weight: body weight: wild-type, 5.54 ± 0.36 mg/g; *Klf5*$^{+/-}$, 4.52 ± 0.16 mg/g; $P < 0.05$; $n = 5$ in each; perivascular fibrosis area:lumen area ratio: wild-type, 1.36 ± 0.29; *Klf5*$^{+/-}$, 0.43 ± 0.07; $P < 0.01$; $n = 6$ in each) (Fig. 3*e* and *f*). Echocardiographic analysis of ventricular-wall thickness (intraventricular septal (IVS) and posterior-wall (PW) thickness) following angiotensin II infusion revealed that hearts of *Klf5*$^{+/-}$ mice had comparatively thin ventricular walls (IVS:

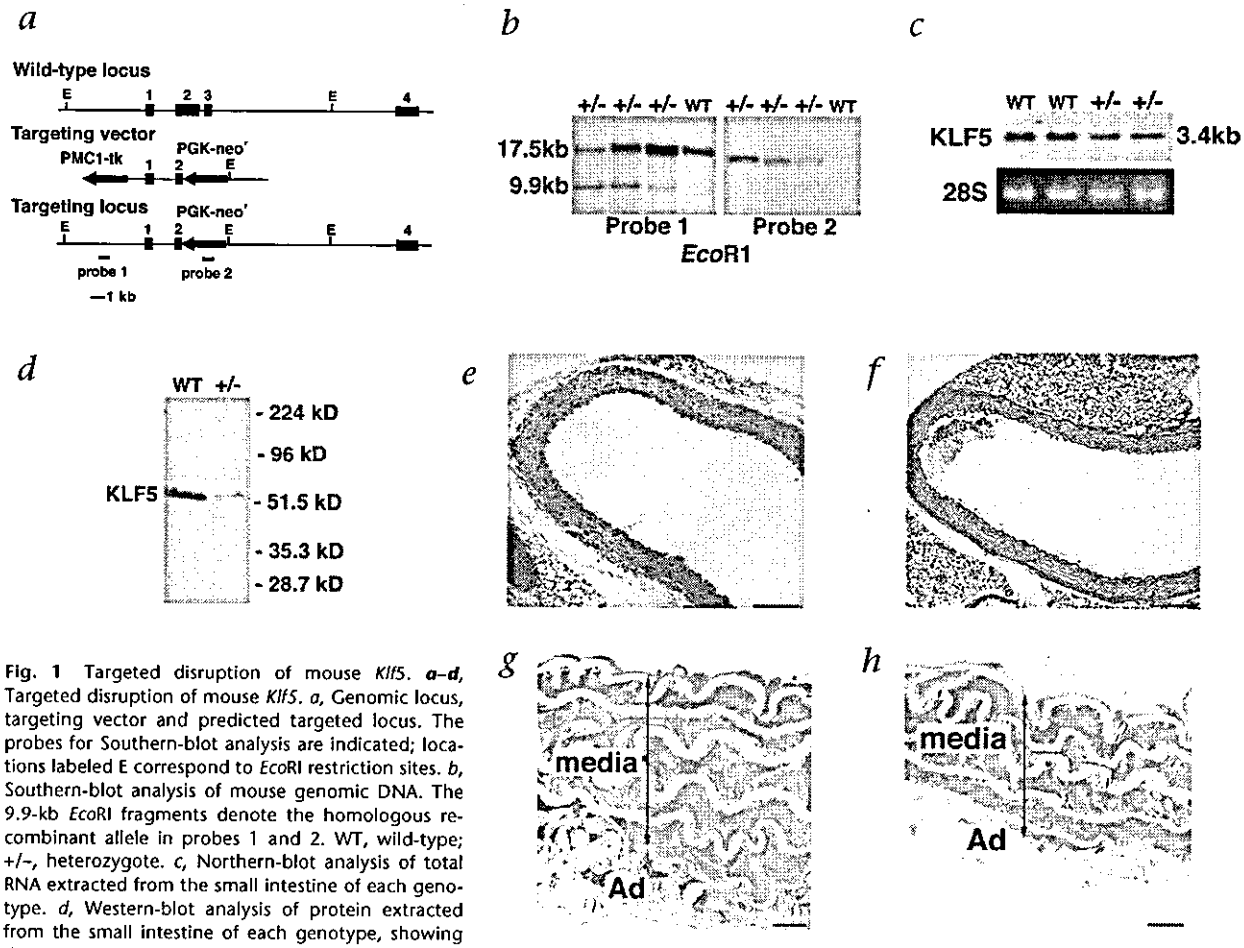

Fig. 1 Targeted disruption of mouse *Klf5*. ***a–d***, Targeted disruption of mouse *Klf5*. *a*, Genomic locus, targeting vector and predicted targeted locus. The probes for Southern-blot analysis are indicated; locations labeled E correspond to *Eco*RI restriction sites. *b*, Southern-blot analysis of mouse genomic DNA. The 9.9-kb *Eco*RI fragments denote the homologous recombinant allele in probes 1 and 2. WT, wild-type; +/–, heterozygote. *c*, Northern-blot analysis of total RNA extracted from the small intestine of each genotype. *d*, Western-blot analysis of protein extracted from the small intestine of each genotype, showing decreased levels of KLF5 protein in the heterozygote. ***e–h***, Vascular structure of wild-type (*e* and *g*) and *Klf5*$^{+/-}$ (*f* and *h*) mice. *e* and *f*, H&E staining of cross-sections of thoracic aorta. *g* and *h*, Transmission electron micrographs of the aortic wall. Ad: adventitia. Scale bars, 100 μm (*e* and *f*); 10 μm (*g* and *h*).

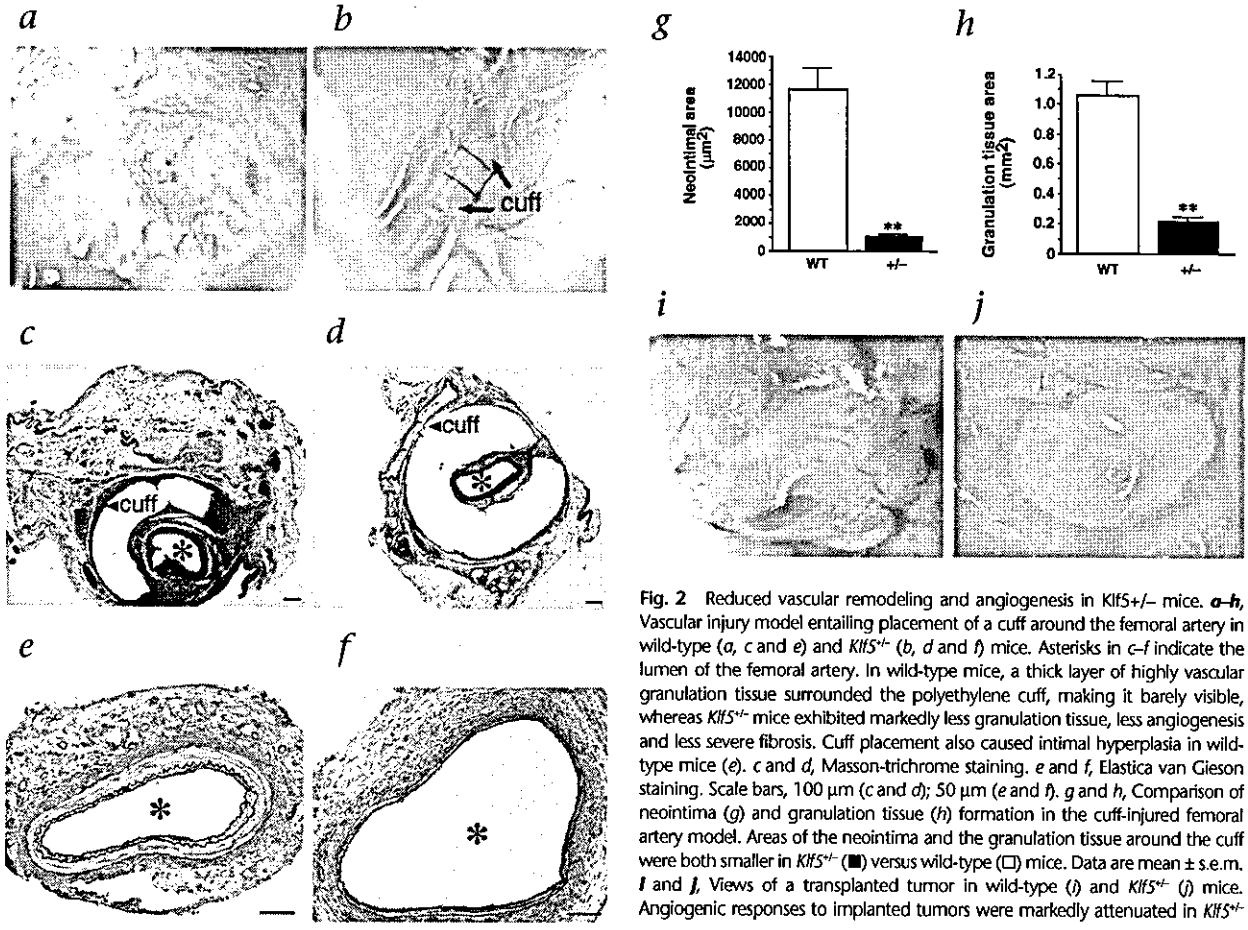

Fig. 2 Reduced vascular remodeling and angiogenesis in Klf5+/– mice. ***a–h***, Vascular injury model entailing placement of a cuff around the femoral artery in wild-type (*a*, *c* and *e*) and *Klf5*$^{+/-}$ (*b*, *d* and *f*) mice. Asterisks in *c–f* indicate the lumen of the femoral artery. In wild-type mice, a thick layer of highly vascular granulation tissue surrounded the polyethylene cuff, making it barely visible, whereas *Klf5*$^{+/-}$ mice exhibited markedly less granulation tissue, less angiogenesis and less severe fibrosis. Cuff placement also caused intimal hyperplasia in wild-type mice (*e*). *c* and *d*, Masson-trichrome staining. *e* and *f*, Elastica van Gieson staining. Scale bars, 100 μm (*c* and *d*); 50 μm (*e* and *f*). *g* and *h*, Comparison of neointima (*g*) and granulation tissue (*h*) formation in the cuff-injured femoral artery model. Areas of the neointima and the granulation tissue around the cuff were both smaller in *Klf5*$^{+/-}$ (■) versus wild-type (□) mice. Data are mean ± s.e.m. *I* and *j*, Views of a transplanted tumor in wild-type (*i*) and *Klf5*$^{+/-}$ (*j*) mice. Angiogenic responses to implanted tumors were markedly attenuated in *Klf5*$^{+/-}$

wild-type, 0.147 ± 0.003 cm; *Klf5*$^{+/-}$, 0.119 ± 0.004 cm; $P < 0.01$; $n = 8$; PW: wild-type, 0.162 ± 0.012 cm; *Klf5*$^{+/-}$, 0.102 ± 0.006 cm; $P < 0.01$; $n = 6$) (Fig. 3*g* and *h*). The reduced cardiac hypertrophy and fibrosis further indicates that KLF5 is one of the transcription factors mediating angiotensin II-induced cardiovascular remodeling.

Angiotensin II-induced expression of *Klf5* and *Pdgfa*

To test whether KLF5 acts by regulating the expression of various growth factors, we next examined the molecular mechanisms by which KLF5 controls cardiovascular remodeling. Because platelet-derived growth factor-A (PDGF-A) is a well-known growth factor involved in mesenchymal-cell activation, angiogenesis and tissue remodeling[12,15], we analyzed angiotensin II–stimulated upregulation of KLF5 and PDGF-A in cultured cardiac fibroblasts. Upregulation of KLF5 was detected within two hours after the start of angiotensin II stimulation, was sustained for more than four hours, and was followed by upregulation of PDGF-A (Fig. 4*a*). Similar induction of PDGF-A expression by angiotensin II also occurred in cultured smooth-muscle cells (data not shown). Immunohistochemical analysis revealed that levels of PDGF-A expression were significantly greater in angiotensin II-treated hearts and cuff-injured arteries of wild-type mice (Fig. 4*b* and *d*) than those of *Klf5*$^{+/-}$ mice (Fig. 4*c* and *e*). Furthermore, overexpression of KLF5 markedly increased PDGF-A promoter activity in transiently transfected cells (Fig. 4*f*).

To further confirm that KLF5 controls the *Pdgfa* gene promoter in response to angiotensin II, we carried out a series of chromatin immunoprecipitation (ChIP) assays using anti-KLF5 antibody. This enabled us to directly assess KLF5 binding to target sites on endogenous genes within chromatin in living cells. When cultured cardiac fibroblasts treated with 1 μmol/L angiotensin II for 3.5 hours and untreated cells were formaldehyde-fixed and subjected to ChIP analysis, angiotensin II increased the binding of KLF5 to the promoter region of *Pdgfa* (Fig. 4*g*). However, KLF5 did not bind to either the last exon of *Pdgfa*, which does not contain KLF5-binding sites, or to the β-globin gene promoter, which is silent in fibroblasts. It thus seems that KLF5 induced by angiotensin II directly controls PDGF-A transcription *in vivo*.

Gastrointestinal abnormalities in *Klf5*$^{+/-}$ mice

KLF5 is also abundantly expressed in the gastrointestinal tract, and we observed misshapen villi and reductions in the number of mesenchymal cells and the amount of extracellular matrix in the gastrointestinal tracts of *Klf5*$^{+/-}$ mice (Fig. 5*a* and *b*). Notably, the gastrointestinal phenotypes of *Klf5*$^{+/-}$ mice were very similar to those of *Pdgfa*$^{-/-}$ mice[16], confirming that KLF5 and PDGF-A occur in the same signaling pathway. In fact, PDGF-A expression was significantly diminished in the gastrointestinal tract of *Klf5*$^{+/-}$ mice at both the mRNA (Fig. 5*e*) and protein levels (Fig. 5*c* and *d*).

Diminished type IV collagen expression in *Klf5*$^{+/-}$ mice

By regulating production of extracellular matrix components, including various collagen forms, transforming growth factor β (TGF-β) also has a role in tissue remodeling. Following angiotensin II infusion, the level of TGF-β expression was significantly lower in hearts of *Klf5*$^{+/-}$ mice than in those of wild-type mice (data not shown), suggesting that TGF-β also lies downstream of KLF5. Indeed, immunostaining of collagen type IV, a TGF-β target[17], showed that its expression is very much diminished in the subendothelial basal membrane of aortas from *Klf5*$^{+/-}$ mice (Fig. 5*f* and *g*). Thus, attenuation of cardiovascular remodeling in *Klf5*$^{+/-}$ mice likely reflects reduced expression of both PDGF-A and TGF-β.

Effects of retinoids on KLF-5 dependent transcription

Given the results above, we reasoned that it should be possible to control cardiovascular remodeling by modulating the activity of KLF5. We therefore screened a number of compounds for their ability to modulate KLF5 activity using a PDGF-A promoter reporter construct cotransfected with the KLF5 expression vector. We found that LE135, a synthetic retinoic-acid receptor (RAR) antagonist[18,19], enhanced PDGF-A promoter activity in cells overexpressing KLF5 (Fig. 6*a*), but had no effect in the absence of KLF5 overexpression (data not shown). Conversely, Am80, a synthetic RAR agonist[18,19], reduced PDGF-A promoter activity in cells overexpressing KLF5 and RAR-α (Fig. 6*b*), but did not significantly affect PDGF-A promoter activity if either KLF5 or RAR-α was not overexpressed (data not shown). These results suggest that RAR ligands affect KLF5 transcriptional activity, an idea substantiated by our subsequent finding that KLF5 coimmunoprecipitates with RAR (Fig. 6*c*), and indicating direct physical interaction between KLF5 and RAR.

To test whether the synthetic RAR ligands have effects on cardiovascular remodeling *in vivo*, we administered LE135 or Am80 to wild-type and *Klf5*$^{+/-}$ mice. When administered to *Klf5*$^{+/-}$ mice with cuffed femoral arteries, LE135 enhanced development of granulation tissue and the neointima (Fig. 6*e*, *g* vs. *d*,*f*) to a point where they approximated those seen in wild-type mice. In addition, LE135 increased the mesenchymal components and the thickness of villi in the gastrointestinal tracts of *Klf5*$^{+/-}$ mice, making the structure of the gastrointestinal mucosa similar to that in wild-type mice. PDGF-A expression was also increased in LE135-treated mice (data not shown). Conversely, Am80 reduced development of granulation tissue and the neointima in wild-type mice (Fig. 6*i*, *k* vs. *h*,*j*) until they approximated those seen in *Klf5*$^{+/-}$ mice and suppressed angiotensin II-induced cardiac hypertrophy (Fig. 6*l* and *m*). LE135 and Am80 thus appear to modulate KLF5 function both *in vitro* and *in vivo*

Discussion

Cardiovascular remodeling is a complex process involving activation of mesenchymal cells, production of extracellular matrix, and angiogenesis, all of which contribute to the pathogenesis of atherosclerosis and heart failure. Following initial activation of cells of mesenchymal origin (for example, smooth-muscle cells and fibroblasts) by an external stress, several immediate early-response genes are activated during the acute phase of the stress response[12]; remodeling processes are then sustained over longer periods of time by an autocrine/paracrine loop involving a number of humoral factors[20].

We cloned KLF5 from a smooth-muscle cell cDNA library as a transcription factor for SMemb/NMHC-B (ref. 6). Therefore, KLF5 is thought to have a role in the phenotypic modulation and pro-

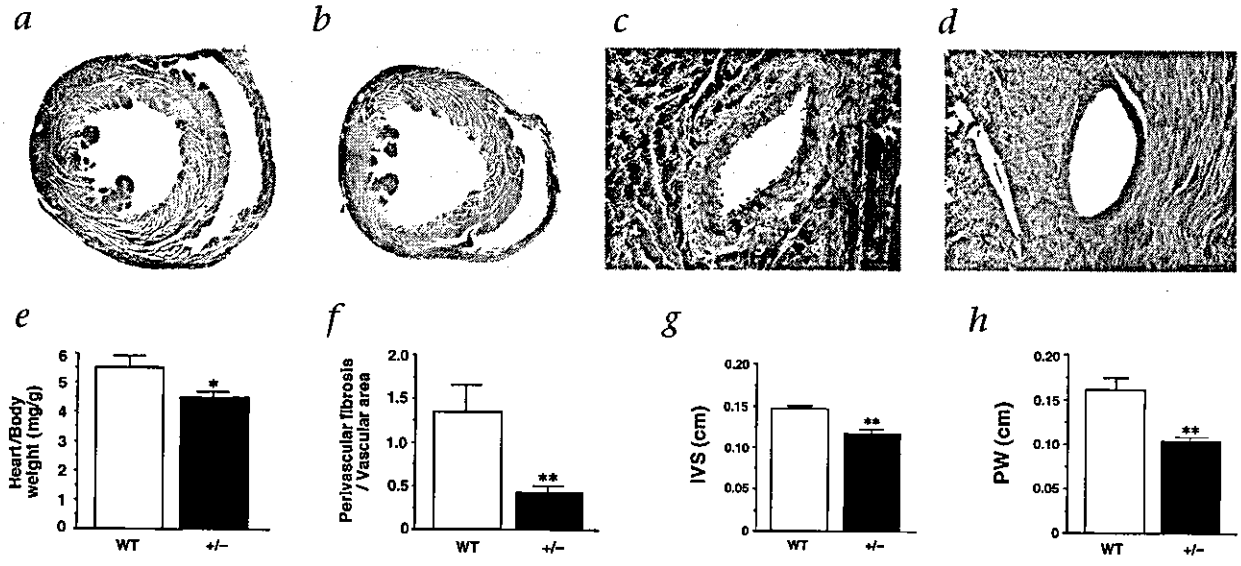

Fig. 3 Reduced angiotensin II-induced cardiac hypertrophy and fibrosis in Klf5$^{+/-}$ mice. ***a–d***, Masson-trichrome staining of cross-sections of heart after 14 d of angiotensin II infusion. Substantial perivascular and interstitial fibrosis were observed in wild-type mice (*a* and *c*), but such changes were less severe or almost undetectable in *Klf5*$^{+/-}$ mice (*b* and *d*). Scale bars, 50 μm (*c* and *d*). ***e*** and ***f***, Comparison of heart weights (*e*) and the areas of perivascular fibrosis around the coronary artery (*f*) after 14 d of angiotensin II infusion. Heart-weight (left ventricle + right ventricle):body-weight and perivascular fibrosis-area:lumen-area ratios were both significantly lower in *Klf5*$^{+/-}$ mice. ***g*** and ***h***, Echocardiographic analysis comparing intraventricular septal (IVS) and posterior (PW) wall thickness after angiotensin II infusion. The hearts of *Klf5*$^{+/-}$ mice possessed a comparatively thin ventricular wall. □, Wild type; ■, *Klf5*$^{+/-}$. *, $P < 0.05$; **, $P < 0.01$. *e*; $n=5$; *f–n*; $n=6$; data are mean ± s.e.m

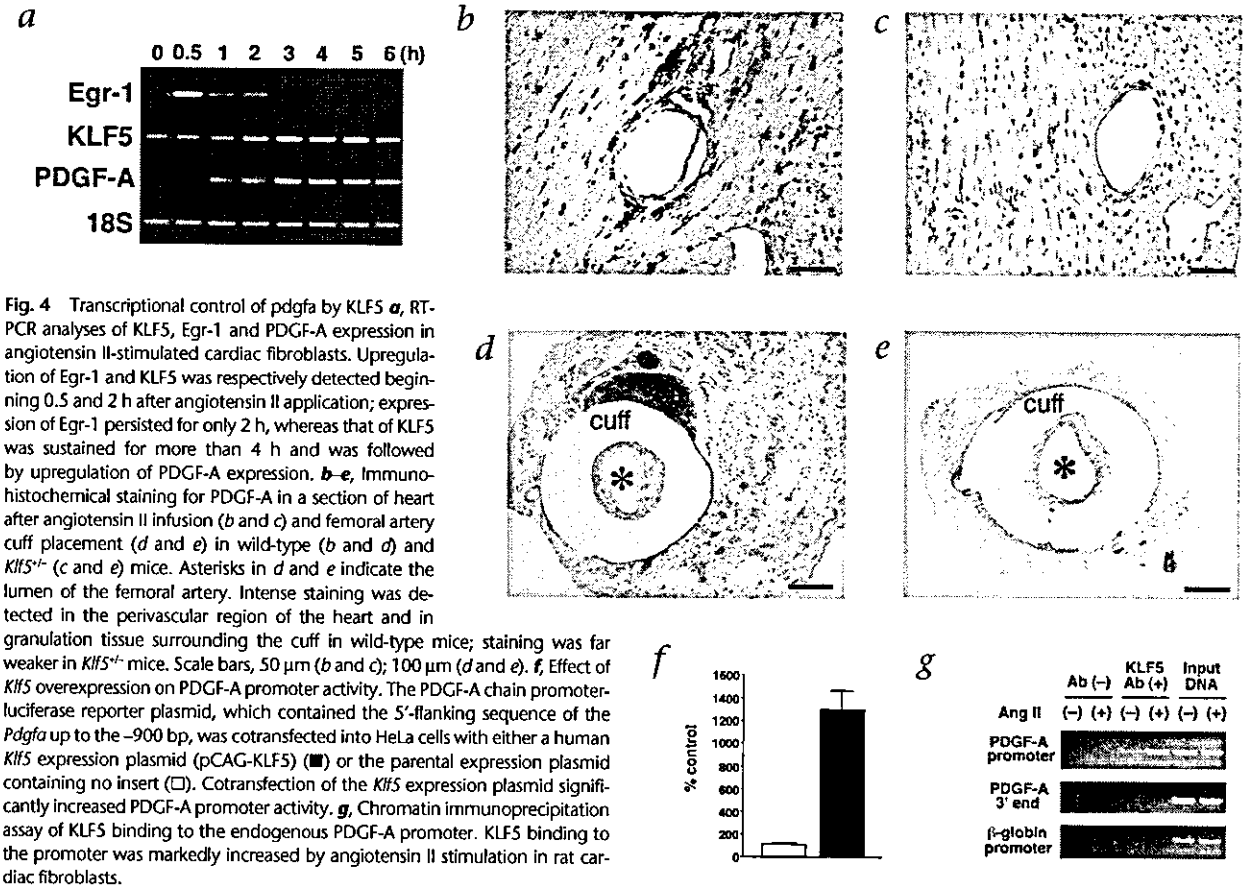

Fig. 4 Transcriptional control of pdgfa by KLF5 *a*, RT-PCR analyses of KLF5, Egr-1 and PDGF-A expression in angiotensin II-stimulated cardiac fibroblasts. Upregulation of Egr-1 and KLF5 was respectively detected beginning 0.5 and 2 h after angiotensin II application; expression of Egr-1 persisted for only 2 h, whereas that of KLF5 was sustained for more than 4 h and was followed by upregulation of PDGF-A expression. *b–e*, Immunohistochemical staining for PDGF-A in a section of heart after angiotensin II infusion (*b* and *c*) and femoral artery cuff placement (*d* and *e*) in wild-type (*b* and *d*) and *Klf5*$^{+/-}$ (*c* and *e*) mice. Asterisks in *d* and *e* indicate the lumen of the femoral artery. Intense staining was detected in the perivascular region of the heart and in granulation tissue surrounding the cuff in wild-type mice; staining was far weaker in *Klf5*$^{+/-}$ mice. Scale bars, 50 μm (*b* and *c*); 100 μm (*d* and *e*). *f*, Effect of *Klf5* overexpression on PDGF-A promoter activity. The PDGF-A chain promoter-luciferase reporter plasmid, which contained the 5′-flanking sequence of the *Pdgfa* up to the –900 bp, was cotransfected into HeLa cells with either a human *Klf5* expression plasmid (pCAG-KLF5) (■) or the parental expression plasmid containing no insert (□). Cotransfection of the *Klf5* expression plasmid significantly increased PDGF-A promoter activity. *g*, Chromatin immunoprecipitation assay of KLF5 binding to the endogenous PDGF-A promoter. KLF5 binding to the promoter was markedly increased by angiotensin II stimulation in rat cardiac fibroblasts.

liferation of dedifferentiated smooth-muscle cells. As we expected, neointimal formation was markedly attenuated in *Klf5*$^{+/-}$ mice, which also showed attenuated interstitial and perivascular fibrosis in cardiac hypertrophy and reduced adventitial thickening in cuff-injured arteries, where activated fibroblasts have a critical role. In addition, *Klf5*$^{+/-}$ mice showed impaired angiogenic activity, which is consistent with our observation that KLF5 is expressed in activated endothelial cells. It is apparent from these data that KLF5 functions not only in vascular smooth-muscle cells, but also in activated fibroblasts and endothelial cells, affecting several facets of cardiovascular remodeling.

Given that KLF5 is induced following activation of immediate early response genes (for example, Egr-1)[11] and that, in turn, it controls expression of various growth factors (for example, PDGF-A and TGF-β), KLF5 may be in a position to mediate between the acute response to an external stress and tissue remodeling. Notably, other transcription factors activated during the immediate early phase of stress responses, including Egr-1, nuclear factor-κB (NF-κB) and signal transducer and activator of transcription 3 (Stat3), have not been clearly shown to be involved in the structural remodeling of the blood vessels and heart *in vivo*[21–23]. By contrast, heterozygous knockout of KLF5 results in marked attenuation of stress responses *in vivo*.

Sixteen members of the Krüppel-like transcription factor family have now been identified; moreover, KLF1, KLF2 and KLF4 have critical roles in development and pathogenesis *in vivo*. All KLFs recognize similar GC-rich elements, which are found in the regulatory regions of various mammalian genes, including a number of growth factor genes[9]. Many of these GC-rich elements were thought to be target sites for Sp1 as both KLFs and Sp1 bind to identical GC-rich elements *in vitro*, the so-called Sp1 sites[7]. We showed, for example, that the KLF5-binding element in the *Smemb* gene also binds Sp1 *in vitro*[6]. Unfortunately, this apparent lack of strict selectivity in *in vitro* binding has hindered identification of *in vivo* targets of KLFs. Here we used ChIP assays to identify the endogenous PDGF-A promoter as the target of KLF5. Given the lack of *in vitro* binding selectivity, it will be important to examine target genes of KLFs and Sp1 family factors using methods such as ChIP assays, which allow direct identification of transcription factors bound to target elements within intact chromatin. It will also be important to clarify how KLFs and Sp1 family members discriminate similar binding sites. One possible mechanism is that the interactions between KLFs and other factors, including coactivators involved in chromatin modification, may differentiate target sequences[7].

In summary, our results provide strong evidence that KLF5 is a crucial determinant of the cellular response to cardiovascular injury, playing a key role in mediating tissue remodeling. The finding that KLF5 interacts with the RAR suggests that RAR ligands may exert protective effects against cardiovascular remodeling via transrepression of KLF5 (ref. 24), and provides a clear basis for the development of drugs modulating KLF5 function to control cardiovascular remodeling and angiogenesis.

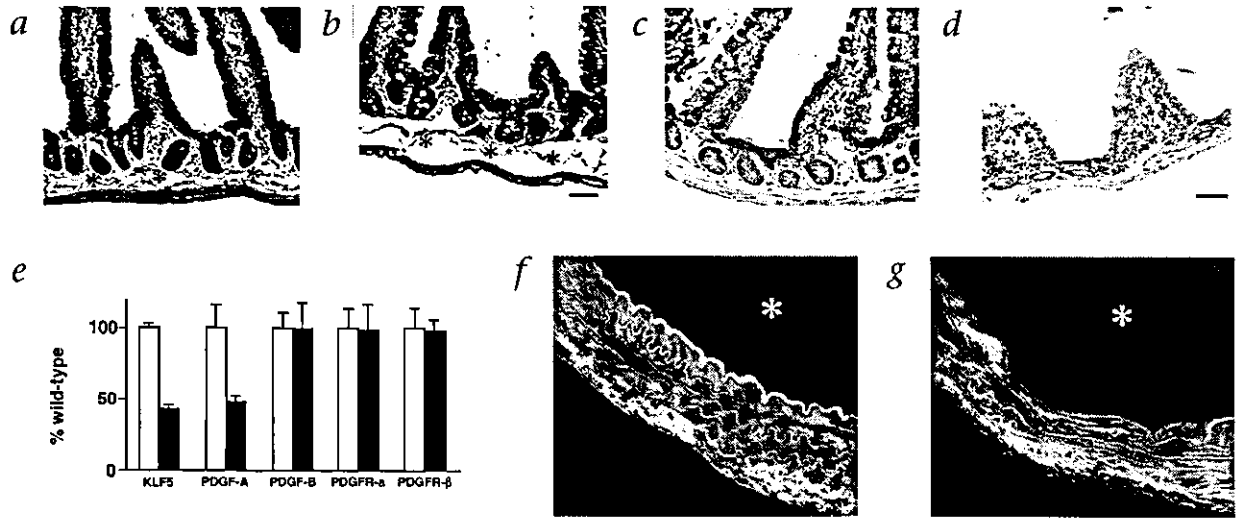

Fig. 5 Reduced growth factor expression in Klf5$^{+/-}$ mice *a* and *b*, Masson-trichrome staining of sections from jejunum in wild-type (*a*) and *Klf5*$^{+/-}$ (*b*) mice. The submucosal mesenchyme (asterisks) was sparser with less connective tissue in *Klf5*$^{+/-}$ mice. *c* and *d*, Immunohistochemical staining using anti-mouse PDGF-A antibody in sections of jejunum from wild-type (*c*) and *Klf5*$^{+/-}$ (*d*) mice. Intense staining was detected in epithelial and mesenchymal cells in the intestinal villi of wild-type mice, but was much weaker in *Klf5*$^{+/-}$ mice. Scale bars, 50 μm (*a–d*). *e*, Real-time PCR analysis of *Klf5*, PDGF-A and -B, and PDGFR-α and -β, in the small intestine of wild-type and *Klf5*$^{+/-}$ mice. The level of expression of each gene was normalized to that of the 18s ribosomal RNA. Error bars show means ± s.e.m. □, wild type; ■, *Klf5*$^{+/-}$. *f* and *g*, Immunostaining for type IV collagen in thoracic aorta from wild-type (*f*) and *Klf5*$^{+/-}$ (*g*) mice: green, immunohistochemical staining using anti-mouse type IV collagen antibody; red, phalloidin (used for labeling of actin); blue, TO-PRO-3 iodide (used for labeling of nuclei). Asterisks in *f* and *g* indicate the luminal side of the aorta. Staining for type IV collagen is apparently reduced in the subendothelial basement structure and in the adventitia of *Klf5*$^{+/-}$ mice.

Methods

Generation of *Klf5*$^{+/-}$ mice. Knockout mice were generated as described[25–27]. Briefly, a plasmid-targeting vector was constructed to replace the 3.3-kb fragment encompassing exons 2 and 3 of *Klf5* with the neomycin-resistance gene (Fig. 1*a*), after which it was linearized and introduced into 129/Sv-derived embryonic stem cells by electroporation. Homologous recombinants were identified and 2 independently targeted clones were injected into C57BL/6 blastocysts to generate chimeric mice. Male chimeras were crossbred with C57BL/6 females, and germline transmission was verified by Southern-blot analysis (Fig. 1*b*). All experiments were performed in accordance with the Declaration of Helsinki and were approved by the University of Tokyo Ethics Committee for Animal Experiments.

Vascular injury by cuff placement. Cuff-placement surgery was carried out on 10–12-wk-old male mice as described[28,29], with some modification. After isolating the right femoral artery from the surrounding tissues, a polyethylene tube (2-mm PE-50; inner diameter, 0.56 mm; outer diameter, 0.965 mm; Becton-Dickinson, San Jose, California) was opened longitudinally, loosely placed around the artery and then closed with sutures. After the experimental period, the mice were killed with an overdose of anesthetic and perfused first with PBS and then with 10% neutral buffered formalin at 100 mmHg. Thereafter, the cuffed artery was removed. The tissue was fixed in 10% neutral buffered formalin overnight, dehydrated and embedded in paraffin. The middle segment of the artery was cut into subserial 5-μm cross sections with an interval of 200 μm between. The sections were stained with Elastica van Gieson or Masson-trichrome stain.

Tumor transplantation. A sarcoma 180 (S180) murine transplantable tumor cells were transplanted subcutaneously into the bilateral axillae of the mice at a dose of 2×10^6 cells at 0.2 ml per mouse.

Angiotensin II infusion. An incision was made in the midscapular region under sterile conditions, and osmotic minipumps (Alzet model 2002, Alza Corp, Mountain View, California) containing angiotensin II (Wako, Osaka, Japan) dissolved in 0.15 mol/L NaCl and 1 mmol/L acetic acid were implanted. Thereafter, angiotensin II was delivered for 14 d at a rate of 3.2 mg/kg/d. The loading dose was determined from an earlier study[30]. Sham-treated mice underwent an identical surgical procedure, except that an osmotic minipump containing 0.15 mol/L NaCl and 1 mmol/L acetic acid was implanted.

Cell culture. Primary rat cardiac fibroblasts were obtained from newborn rats as described[31] and grown to confluence in DMEM supplemented with 10% FBS. To reversibly growth-arrest the cells, they were placed in serum-free, defined medium for 4 d before angiotensin II stimulation[32].

Quantitative reverse transcriptase PCR. Total RNA was purified from the cells using an RNeasy kit (Qiagen). Total RNA was extracted from mouse tissues using Trizol (Nippongene, Toyama, Japan) and then further purified using RNeasy columns. The methods for reverse transcription of RNA and quantitative relative PCR have been described[33]. For quantification of transcript levels within tissues, real-time PCR was performed using LightCycler (Roche) and QuantiTect SYBR green PCR kits (Qiagen). The sequences of the PCR primers were: *Klf5*, 5′-GGTTGCACAAAAGTTTATAC-3′ and 5′-GGCTTGGCGCCCGTGTGCTTCC-3′; PDGF-A, 5′-CAGCATC-CGGGACCTCCAGCGACTC-3′ and 5′-TCGTAAATGACCGTCCTGGTCTT GC-3′; PDGF-B, 5′-TGAGTGAGAGTCACCCTGCCAATGG-3′ and 5′-AC-CACGGTGACCTCCTGCGAATCTC-3′; PDGFR-α, 5′-TGTGCCGTCTTTAA-CAACGAGGTC-3′ and 5′-TTGTCCTTCAGCCACGATATCCTG-3′; PDGFR-β, 5′-CCGAGGACCTGTTCATTTTTGTCAC-3′ and 5′- CATTGGAAGTTCACCA-CATCATTGC-3′.

Chromatin immunoprecipitation assays. Quiescent cardiac fibroblasts were treated with or without 1 μmol/L angiotensin II and then fixed in 1% formaldehyde. The fixed chromatin samples were subjected to immunoprecipitation as described[33], with minor modifications. Anti-KLF5 antibody was raised against recombinant KLF5 protein, and its specificity was thoroughly examined by western-blot analyses (Supplementary Fig. A online). Protein G (Roche) was used to preclear samples and for immunoprecipitation instead of the protein A used in the original protocol[33].

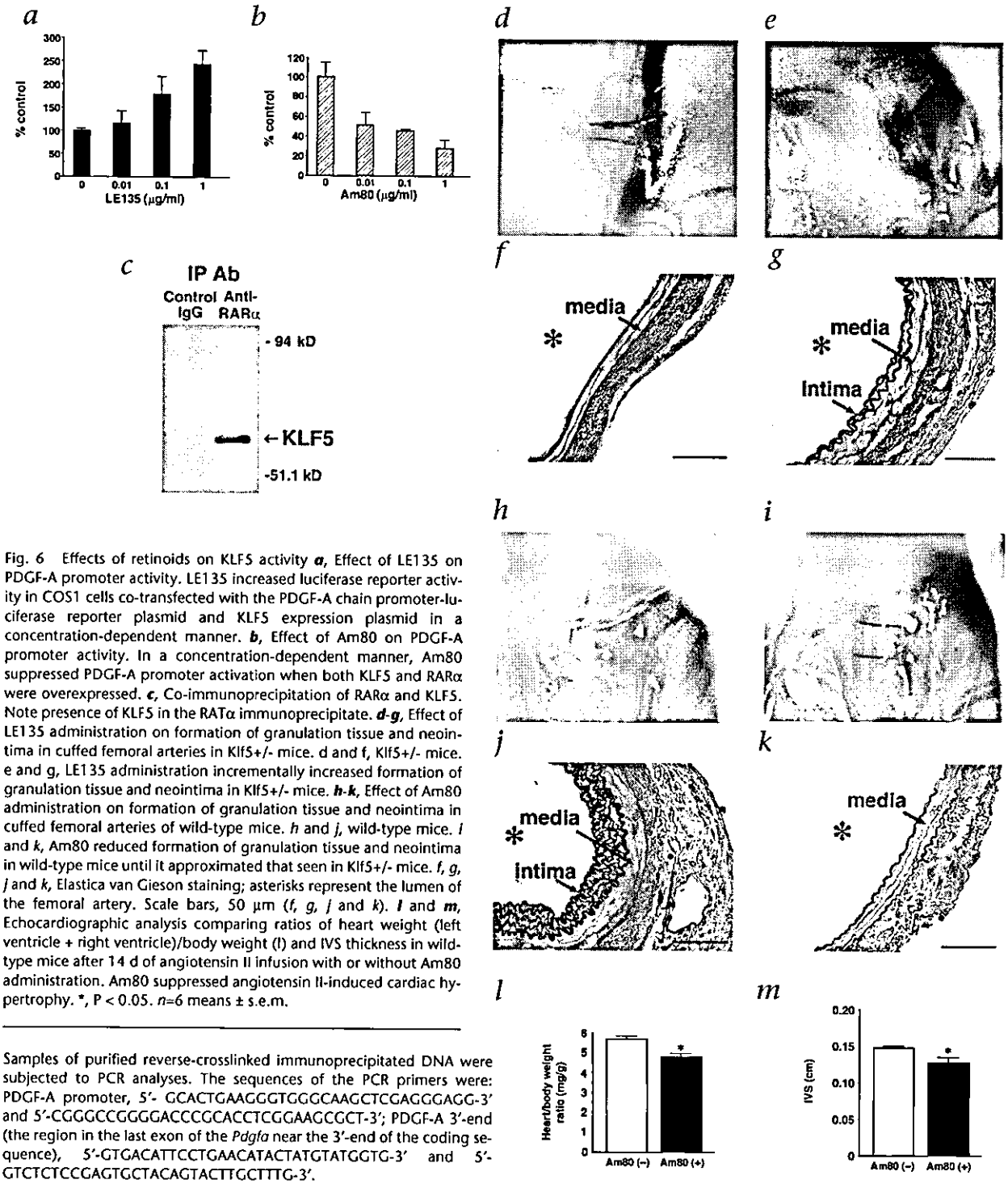

Fig. 6 Effects of retinoids on KLF5 activity ***a***, Effect of LE135 on PDGF-A promoter activity. LE135 increased luciferase reporter activity in COS1 cells co-transfected with the PDGF-A chain promoter-luciferase reporter plasmid and KLF5 expression plasmid in a concentration-dependent manner. ***b***, Effect of Am80 on PDGF-A promoter activity. In a concentration-dependent manner, Am80 suppressed PDGF-A promoter activation when both KLF5 and RARα were overexpressed. ***c***, Co-immunoprecipitation of RARα and KLF5. Note presence of KLF5 in the RATα immunoprecipitate. ***d-g***, Effect of LE135 administration on formation of granulation tissue and neointima in cuffed femoral arteries in Klf5+/- mice. d and f, Klf5+/- mice. e and g, LE135 administration incrementally increased formation of granulation tissue and neointima in Klf5+/- mice. ***h-k***, Effect of Am80 administration on formation of granulation tissue and neointima in cuffed femoral arteries of wild-type mice. *h* and *j*, wild-type mice. *i* and *k*, Am80 reduced formation of granulation tissue and neointima in wild-type mice until it approximated that seen in Klf5+/- mice. *f*, *g*, *j* and *k*, Elastica van Gieson staining; asterisks represent the lumen of the femoral artery. Scale bars, 50 μm (*f*, *g*, *j* and *k*). ***l*** and ***m***, Echocardiographic analysis comparing ratios of heart weight (left ventricle + right ventricle)/body weight (l) and IVS thickness in wild-type mice after 14 d of angiotensin II infusion with or without Am80 administration. Am80 suppressed angiotensin II-induced cardiac hypertrophy. *, $P < 0.05$. n=6 means ± s.e.m.

Samples of purified reverse-crosslinked immunoprecipitated DNA were subjected to PCR analyses. The sequences of the PCR primers were: PDGF-A promoter, 5'- GCACTGAAGGGTGGGCAAGCTCGAGGGAGG-3' and 5'-CGGGCCGGGGACCCGCACCTCGGAAGCGCT-3'; PDGF-A 3'-end (the region in the last exon of the *Pdgfa* near the 3'-end of the coding sequence), 5'-GTGACATTCCTGAACATACTATGTATGGTG-3' and 5'-GTCTCTCCGAGTGCTACAGTACTTGCTTTG-3'.

Co-immunoprecipitation assay. To prepare extracts for immunoprecipitation, HeLa S3 cells were first lysed by sonication. The lysates were centrifuged at 18,000*g* for 10 min, and the protein concentration in the extracts was adjusted to 1 mg/ml. One mg of anti-RAR antibody (Santa Cruz Biotechnology, Santa Cruz, California) or 1 mg of control rat IgG was bound to 10 ml of protein G sepharose (Amersham Pharmacia Biotechnology, Piscataway, New Jersey), after which the antibody-bound protein G sepharose was stirred with 1 ml of HeLa S3 extract for 6 h. The resultant immunoprecipitate was washed 10 times with RIPA buffer (50 mM Tris-HCl, pH 7.9, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml leupeptin and 1 mg/ml pepstatin A) and then subjected to SDS–PAGE and immunoblotted with anti-KLF5 antibody.

Note: Supplementary information is available on the Nature Medicine website.

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Competing interests statement

The authors declare that they have no competing financial interests.

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1. Carmeliet, P. & Collen, D. Transgenic mouse models in angiogenesis and cardiovascular disease. *J. Pothol.* **190**, 387–405 (2000).
2. Libby, P. Changing concepts of atherogenesis. *J. Intern. Med.* **247**, 349–358 (2000).
3. Kuro-o, M. *et al.* Developmentally regulated expression of vascular smooth muscle myosin heavy chain isoforms. *J. Biol. Chem.* **264**, 18272–82725 (1989).
4. Manabe, I. *et al.* Isolation of the embryonic form of smooth muscle myosin heavy chain (SMemb/NMHC-B) gene and characterization of its 5'-flanking region. *Biochem. Biophys. Res. Commun.* **239**, 598–605 (1997).
5. Aikawa, M. *et al.* Phenotypic modulation of smooth-muscle cells during progression of human atherosclerosis as determined by altered expression of myosin heavy chain isoforms. *Ann. NY Acad. Sci.* **748**, 578–585 (1995).
6. Watanabe, N. *et al.* BTEB2, a Kruppel-like transcription factor, regulates expression of the SMemb/nonmuscle myosin heavy chain B (SMemb/NMHC-B) gene. *Circ. Res.* **85**, 182–191 (1999).
7. Bieker, J.J. Kruppel-like factors: Three fingers in many pies. *J. Biol. Chem.* **276**, 34355–34358 (2001).
8. Dang, D.T., Pevsner, J. & Yang, V.W. The biology of the mammalian Kruppel-like family of transcription factors. *Int. J. Biochem. Cell Biol.* **32**, 1103–1121 (2000).
9. Black, A.R., Black, J.D. & Azizkhan-Clifford, J. Sp1 and kruppel-like factor family of transcription factors in cell growth regulation and cancer. *J. Cell Physiol.* **188**, 143–160 (2001).
10. Hoshino, Y. *et al.* Regulated expression of the BTEB2 transcription factor in vascular smooth-muscle cells: Analysis of developmental and pathological expression profiles shows implications as a predictive factor for restenosis. *Circulation* **102**, 2528–2534 (2000).
11. Kawai-Kowase, K., Kurabayashi, M., Hoshino, Y., Ohyama, Y. & Nagai, R. Transcriptional activation of the zinc finger transcription factor BTEB2 gene by Egr-1 through mitogen-activated protein kinase pathways in vascular smooth-muscle cells. *Circ. Res.* **85**, 787–795 (1999).
12. Berk, B.C. Vascular smooth muscle growth: Autocrine growth mechanisms. *Physiol. Rev.* **81**, 999–1030 (2001).
13. Matsusaka, T., Katori, H., Inagami, T., Fogo, A. & Ichikawa, I. Communication between myocytes and fibroblasts in cardiac remodeling in angiotensin chimeric mice. *J. Clin. Invest.* **103**, 1451–1458 (1999).
14. Weir, M.R. & Dzau, V.J. The renin-angiotensin-aldosterone system: A specific target for hypertension management. *Am. J. Hypertens.* **12**, 205S–213S (1999).
15. Powell, D.W. *et al.* Myofibroblasts. I. Paracrine cells important in health and disease. *Am. J. Physiol.* **277**, C1–9 (1999).
16. Karlsson, L., Lindahl, P., Heath, J.K. & Betsholtz, C. Abnormal gastrointestinal development in PDGF-A and PDGFR-α deficient mice implicates a novel mesenchymal structure with putative instructive properties in villus morphogenesis. *Development* **127**, 3457–66 (2000).
17. Zdunek, M., Silbiger, S., Lei, J. & Neugarten, J. Protein kinase CK2 mediates TGF-β1-stimulated type IV collagen gene transcription and its reversal by estradiol. *Kidney Int.* **60**, 2097–2108 (2001).
18. Umemiya, H. *et al.* Regulation of retinoidal actions by diazepinylbenzoic acids. Retinoid synergists which activate the RXR-RAR heterodimers. *J. Med. Chem.* **40**, 4222–34 (1997).
19. Eyrolles, L. *et al.* Retinobenzoic acids. 6. Retinoid antagonists with a heterocyclic ring. *J. Med. Chem.* **37**, 1508–17 (1994).
20. Friedman, S.L. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *J. Biol. Chem.* **275**, 2247–50 (2000).
21. Silverman, E.S. & Collins, T. Pathways of Egr-1-mediated gene transcription in vascular biology. *Am. J. Pathol.* **154**, 665–70 (1999).
22. Valen, G., Yan, Z.Q. & Hansson, G.K. Nuclear factor κ-B and the heart. *J. Am. Coll. Cardiol.* **38**, 307–14 (2001).
23. Yamauchi-Takihara, K. & Kishimoto, T. A novel role for STAT3 in cardiac remodeling. *Trends Cardiovasc. Med.* **10**, 298–303 (2000).
24. Glass, C.K. & Rosenfeld, M.G. The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev.* **14**, 121–41 (2000).
25. Shindo, T. *et al.* ADAMTS-1: A metalloproteinase-disintegrin essential for normal growth, fertility, and organ morphology and function. *J. Clin. Invest.* **105**, 1345–52 (2000).
26. Shindo, T. *et al.* Vascular abnormalities and elevated blood pressure in mice lacking adrenomedullin gene. *Circulation* **104**, 1964–71 (2001).
27. Oh-hashi, Y. *et al.* Elevated sympathetic nervous activity in mice deficient in αCGRP. *Circ. Res.* **89**, 983–90 (2001).
28. Moroi, M. *et al.* Interaction of genetic deficiency of endothelial nitric oxide, gender, and pregnancy in vascular response to injury in mice. *J. Clin. Invest.* **101**, 1225–32 (1998).
29. Akishita, M. *et al.* Estrogen inhibits cuff-induced intimal thickening of rat femoral artery: Effects on migration and proliferation of vascular smooth-muscle cells. *Atherosclerosis* **130**, 1–10 (1997).
30. Wang, H.D. *et al.* Role of NADPH oxidase in the vascular hypertrophic and oxidative stress response to angiotensin II in mice. *Circ. Res.* **88**, 947–53 (2001).
31. Komuro, I. *et al.* Stretching cardiac myocytes stimulates protooncogene expression. *J. Biol. Chem.* **265**, 3595–8 (1990).
32. Kihm, A.J., Hershey, J.C., Haystead, T.A., Madsen, C.S. & Owens, G.K. Phosphorylation of the rRNA transcription factor upstream binding factor promotes its association with TATA binding protein. *Proc. Natl. Acad. Sci. USA* **95**, 14816–20 (1998).
33. Manabe, I. & Owens, G.K. Recruitment of serum response factor and hyperacetylation of histones at smooth muscle-specific regulatory regions during differentiation of a novel P19-derived *in vitro* smooth muscle differentiation system. *Circ. Res.* **88**, 1127–1134 (2001).

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Regulation of the Sp/KLF-family of transcription factors: focus on post-transcriptional modification and protein-protein interaction in the context of chromatin

Review Article

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Summary

The Sp1- and Krüppel-like zinc finger transcription factor family is a rapidly expanding and highlighted group of factors given important biological roles. Understanding specific regulation is important to dissect individual functions. In this collective review, the regulation of this family of transcription factors with a particular focus on post-transcriptional modification and protein-protein interaction in the context of chromatin will be discussed. Studies by ourselves and others show that the zinc finger DNA-binding domain region of these factors mediates important regulatory interactions and modifications which may explain at least in part their specific regulation. Their possible implications in gene therapy are discussed.

I. Introduction

The zinc finger motif (paired cysteine and histidine type) was discovered approximately two decades ago (Diakun et al, 1986). Since then, we have learnt that this is one of the major motifs for proteins in the cell ranging from enzymes to transcription factors. Recent analysis of the human genome showed that transcription factors with this zinc finger motif have evolved in cascading magnitude as shown by their increased genomic complexity in eukaryotes (Tupler et al, 2001). At present, the paired-cysteine and histidine-type (C_2H_2-type) zinc finger transcription factors are thought to be one of the most important type of regulatory transcription factor in the eukaryotic cell. Among these factors, the Sp/KLF (for Sp1- and Krüppel-like factor) family of transcription factors has received recent attention due to important roles in development, differentiation, and oncogenic processes (Philipsen and Suske, 1999; Turner and Crossley, 1999; Dang et al, 2000; Bieker, 2001; Black et al, 2001; Bouwman and Philipsen, 2002; Kaczynski et al, 2003).

DNA-binding activators/repressors bind in a sequence-specific manner to their cognate binding sites in enhancers/silencers and core promoter regions and activate/repress transcription of genes through combinatorial effects with the general transcription machinery (Horikoshi et al. 1988a, b; Zawel and Reinberg 1995). The DNA-binding transcription factor has been classically shown to possess modular functional regions consisting of an activation/regulatory domain which regulates transcription through interactions with basal transcription machinery and the DNA-binding domain (DBD) which specifies the target promoter gene (Ptashne and Gann, 1990; Zawel and Reinberg, 1995).

The DNA-binding transcription factor is regulated at multiple steps. Presence as dictated by spatial expression (e.g. ubiquitous versus restricted expression) in addition to temporal regulation (e.g. constitutive versus inducible expression) plays a primary regulatory role. Sequence-

specific DNA-binding is further critically important for dictating gene-specific actions. DNA-binding transcription factors with common DNA-binding domains often bind similar DNA sequences (e.g. basic helix-loop-helix proteins bind E-boxes, homeoproteins bind A/T-rich sites) but additional regulatory steps must be present as the complexity of these factors in undertaking specific functions cannot be readily explained by their expression patterns and sequence-specific DNA binding properties alone. Regulation through differential protein-protein interactions and/or chemical modifications (e.g. phosphorylation, acetylation) further contribute to their differential functions. In the present review, the regulation of the Sp/KLF-family of transcription factors with a particular focus on post-transcriptional modification and protein-protein interactions in the context of chromatin will be discussed.

II. Basic classification of Sp/KLF factors

factors

The Sp/KLF family of zinc-finger transcription factors are comprised of over 20 mammalian family members which have in common three contiguous C_2H_2-type zinc fingers at the carboxyl-terminus which comprises the DNA-binding domain (Philipsen and Suske, 1999; Turner and Crossley, 1999; Dang et al, 2000; Bieker, 2001; Black et al, 2001; Bouwman and Philipsen, 2002; Kaczynski et al, 2003). Sp/KLF family members can be classified into Sp- and KLF-subsets based on their similarities. The Sp-subtype is based on the founding ubiquitous factor Sp1 (Dynan and Tjian, 1983), and the KLF-subtype is based on the Drosophila Krüppel gene (Preiss et al, 1985). The first systematic classification used to distinguish mammalian Krüppel-like factors was demonstrated in a distinction with the GLI subgroup, which defined the consensus amino acid finger sequence for the Krüppel subgroup to be [Y/F]XCX2CX3FX5LX2HXRXHTGEKP (Ruppert et al, 1988). The Sp subgroup is based on similarity to the founding factor Sp1. Among the KLFs are erythroid differentiation factor EKLF/KLF1 (Miller and Bieker, 1993) and the tumor suppressor gene KLF6/GBF/Zf9/COPEB which we and others identified as a cellular factor possibly involved in HIV-1 transcription (Koritschoner et al, 1997; Suzuki et al, 1998; Narla et al, 2001). We have recently shown by gene knockout studies that the protooncogene KLF5/BTEB2/IKLF (Sogawa et al, 1993; Shi et al, 1999) is important for cardiovascular remodeling in response to stress (Shindo et al, 2002).

At present, the annotation of this family of factors uses a numbering system in order of identification in accordance with an international collaboration to unify the nomenclature. Factors of the Sp-subset have six to eight members, whereas the KLF-subset have approximately 15 members, and are still increasing in numbers. Contrary to initial expectations that this family of factors would likely have redundant functions, they in fact have important individual biological functions as shown by gene knockout studies (e.g. EKLF/KLF1, LKLF/KLF2, KLF5). However, the underlying mechanisms governing their specific functions and regulation are poorly understood.

III. Differential regulation of Sp/KLF factors

The mechanisms underlying specificity of this family of factors have been the topic of great interest among concerned researchers to understand the basis for their individual functions. As the paired cysteine-histidine type zinc finger is a DNA-binding motif, initial studies began by investigations of DNA-binding characteristics. One of the hallmark features of the Sp/KLF factors is that they bind to similar GC-rich sites and/or CACC-boxes. Well studied crystal structure analyses of DNA-binding zinc finger transcription factors have allowed the prediction of the cognate DNA-binding sequence from the primary amino acid structure (Klevit, 1991; Suzuki et al, 1994). Amino acids which contact DNA reside in the α-helical region of the zinc finger. As these critical amino acids are highly conserved in Sp/KLF zinc finger transcription factors, it is tempting to assume that they likely share similar DNA binding properties.

Closer examination of this zinc finger region, however, shows discrete yet distinct differences. For instance, the third amino acid critical for DNA binding of the third zinc finger, and in the amino acids N-terminal adjacent to the first amino acid critical for DNA binding and the third amino acid critical for DNA binding in each of the zinc fingers differ (Suzuki et al, 1998). The relevance of these differences in the context of DNA-binding specificity or affinity remains to be clarified. The optimal cognate binding sequence of selected factors have been shown experimentally which showed that Sp1 binds the sequence 5'-GGGGCGGGGT-3' (Thiesen et al, 1990) and KLF4/GKLF binds the sequence 5'-G/AG/AGGC/TGC/T-3' (Shields and Yang, 1998) which is a derivative of the CACC-box and BTE-element (which is a GC-rich site which binds BTEB1). Collectively, it is generally thought that this family of factors bind similar GC-rich sequences in a sequence-specific manner with a binding selectivity which does not allow individual factors to be clearly discriminated based on their DNA-binding characteristics alone.

It is important to note here, however, that DNA-binding characteristics likely differ in the context of chromatin DNA as separate from the naked DNA-state often used for biochemical experiments. One important example using transgenic mice showed that EKLF/KLF1 preferentially binds the beta-globin locus site *in vivo* which had been shown to bind both EKLF and Sp1 in biochemical studies (Gillemans et al, 1998).

We too had been interested in understanding whether there is specific binding of factors to GC-rich sites *in vivo* which are not reflected in biochemical studies *in vitro*. For this, we used a yeast one-hybrid assay using the GC-rich sites of the HIV-1 core promoter which have been shown to bind Sp1 to investigate what factors actually bind this site. The binding site probe used for the assay was