厚生労働科学研究費補助金

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新規拡張型心筋症モデルマウスを用いた拡張型心筋症発症機序の解明

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新規拡張型心筋症モデルマウスを用いた拡張型心筋症発症機序の解明

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研究要旨　拡張型心筋症の原因の約30％は遺伝子変異であるが、それらの遺伝子変異により拡張型心筋症を発症する機序に関しては不明である。我々は拡張型心筋症モデルマウスの確立と、拡張型心筋症発症機構の解明を目的に実験を行った。我々が確立した拡張型心筋症モデルマウスでは、カルシウム依存性脱リン酸化酵素 calcineurin とリン酸化酵素 CaMKIIδ の活性が亢進し、癌抑制遺伝子 p53の発現量が増加していた。calcineurin の阻害薬では心機能の改善は認めず、CaMKIIδ の阻害薬で心機能の改善および p53の発現量低下を認めた。また、p53遺伝子欠損マウスとの交配により拡張型心筋症モデルマウスの心機能は改善した。さらに、このマウスモデルの心筋においてはカルシウム感受性が低下していた。以上の結果から、カルシウム感受性低下に起因する CaMKIIδ 活性の上昇とそれに伴う p53の発現増加が遺伝子変異による拡張型心筋症の発症に重要であることが示唆された。

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A．研究目的

拡張型心筋症の予後は、非常に不良である。また、拡張型心筋症の最終的な治療法は現在のところ心臓移植しかなく、新たな治療法の確立が切望されている。拡張型心筋症の原因の約30％が遺伝子変異であることが明らかとなってきたが、それらの遺伝子変異により収縮機能不全をきたす機序は未だ不明である。新規治療法の開発のためには拡張型心筋症の発症機序解明が必要であると考え、マウスモデルを用いて拡張型心筋症の発症機序を明らかにすることを本研究の目的とした。

B．研究方法

これまで拡張型心筋症の発症機序が明らかにならなかった理由の一つに、拡張型心筋症を解析するための適切なモデル動物が存在しなかったことが挙げられる。一方ヒトでは拡張型心筋症の原因遺伝子は複数報告されており、そのひとつに心筋αアクチン遺伝子がある。そこでまず我々は、ヒトで報告されている変異型心筋αアクチン遺伝子（cardiac α-actin R312H）を心臓特異的に過剰発現させた遺伝子改変マウスを作成し、拡張型心筋症のモデルマウス作成を試みた。

次に、拡張型心筋症発症機序の解析のために、このモデルマウスにおけるカルシウム依

存性脱リン酸化酵素 calcineurin とリン酸化酵素 CaMKIIδ の活性、および、癌抑制遺伝子 p53の発現量について検討するとともに、これらの因子の拡張型心筋症における病態生理学的意義を検討するため、この拡張型心筋症モデルマウスに calcineurin と CaMKIIδ のそれぞれの阻害薬を投与した。また p53ヘテロノックアウトマウスと交配させ、その表現型について検討した。

一方、in vivo における calcineurin と CaMKIIδ の酵素活性の変化や p53の発現量の変化は、変異型心筋αアクチンの直接的な影響によって生じる場合と、心機能低下により二次的に誘導される場合の２つの可能性が考えられる。そこで培養心筋細胞を用いて、変異型心筋αアクチン遺伝子を培養心筋細胞に導入し、上記の変化が変異型心筋αアクチンの直接的な影響によるものかについて検討した。

さらに、拡張型心筋症モデルマウスから細胞膜除去筋繊維（スキンドファイバー）を単離し、カルシウム感受性について検討した。また、心筋を単離して心筋細胞内カルシウム濃度変化について検討した。

（倫理面への配慮）

実験動物を用いる研究については、千葉大学および大阪大学の動物実験指針に準拠して研究を実施した。動物愛護と動物福祉の観点から実験動物使用は、目的に合致した最小限にとどめた。また麻酔等の手段により苦痛を与えない等の倫理的配慮をおこない、実験者は、管理者と相互協力のもと適切な環境のもと飼育管理を行った。

C．研究結果

（１）拡張型心筋症モデルマウスの樹立

我々は拡張型心筋症モデルマウス作成を目的とし、ヒトで報告されているのと同様の変異を有する心筋αアクチン遺伝子（cardiac α-actin R312H）を心臓特異的に過剰発現させた遺伝子改変マウスを作成した。このマウスは胎児期の発生過程には特に異常を認めなかったが、生後５週齢から心拡大・左室壁菲薄化・収縮能低下をきたし、生後１年までに半数以上が心不全で死亡した。すなわち、このマウスの表現型はヒトでみられる拡張型心筋症にきわめて類似しており、ヒト拡張型心筋症のモデルマウスとして使用できるものと考えられた。

（２）癌抑制遺伝子 p53の拡張型心筋症発症における役割

癌抑制遺伝子である p53が心不全発症に重要であることを我々はすでに報告している。そこで、この拡張型心筋症モデルマウスにおける p53の発現について検討したところ、拡張型心筋症モデルマウスでは p53の蛋白量が野生型に比べて増加していることを見いだした。さらに、拡張型心筋症発症における p53の役割について検討するために、拡張型心筋症モデルマウスと p53ノックアウトマウスを交配したところ、p53遺伝子ヘテロ欠損により拡張型心筋症モデルマウスの心拡大が著明に抑制され、心機能も改善された。以上の結果は、p53の発現量増加が拡張型心筋症の病態形成に重要であることを示すものと考えられた。

p53はさまざまな機能を有するがそのひとつに細胞死誘導がある。そこで拡張型心筋症モデルマウスにおける心筋細胞死について検討した。αアクチン過剰発現マウスでは TUNEL 陽性心筋細胞が増加し、心臓における Bax や cleavaed caspase3の発現量も増加していることから、心筋細胞死が亢進しているものと考えられた。また、これらの変化は p53遺伝子ヘテロ欠損により著明に改善された。さらに、拡張型心筋症モデルマウスと心筋特

異的Bcl2過剰発現マウスを交配し心筋細胞における細胞死を抑制すると、p53遺伝子を欠損させた場合と同様に心拡大が著明に抑制され心機能の改善もみとめられた。以上の結果は、p53の発現量増加が心筋細胞死を誘導することにより拡張型心筋症の発症を引き起こすことを示すものと考えられた。

（3）脱リン酸化酵素calcineurinの拡張型心筋症発症における役割

calcineurinはカルシウム依存性脱リン酸化酵素であり、転写因子NFATを脱リン酸化することにより核内移行を促進し、心肥大形成や心不全発症に関与することが報告されている。そこでこの拡張型心筋症モデルマウスにおけるNFATの転写活性について検討するために、拡張型心筋症モデルマウスとNFAT依存性に心筋内ルシフェラーゼ活性が増加するNFAT-lucレポーターマウスを交配し心筋におけるNFAT活性を測定した。その結果、拡張型心筋症モデルマウスでは心筋内のNFAT転写活性が野生型に比べて増加していることを見いだした。また、calcineurinによって脱リン酸化されるtalinやcaspase12のcleaved formも拡張型心筋症モデルマウスの心筋で増加していた。以上の結果はこの拡張型心筋症モデルマウスの心筋においてcalcineurinが活性化していることを示すものと考えられた。

calcineurin活性化の拡張型心筋症発症における役割をさらに検討するために、その阻害薬であるFK506を拡張型心筋症モデルマウスに投与してその表現型を解析した。当初はFK506により拡張型心筋症の表現型が軽減されると予想したが、予想に反しFK506投与による心拡大抑制効果や心機能改善効果は認めなかった。以上の結果は、拡張型心筋症においてはcalcineurinの活性化が認められ、それが心筋における一部の変化に関与している可能性はあるものの、calcineurinの活性化自体が拡張型心筋症の病態発症において重要な役割を果たすものではないことを示すと考えられた。

（4）カルシウム依存性リン酸化酵素CaMKIIδの拡張型心筋症発症における役割

CaMKIIδは細胞内カルシウム濃度の上昇に伴いその酵素活性が上昇し標的蛋白をリン酸化するカルシウム依存性リン酸化酵素である。CaMKIIδの活性化が心肥大・心不全発症に関与するとの報告が以前よりなされている。そこでこの拡張型心筋症モデルマウスにおけるCaMKIIδの活性化について検討をおこなった。

拡張型心筋症モデルマウスにおいてCaMKIIδの蛋白量は増加しており、また、リン酸化CaMKIIδも増加していた。さらに、CaMKIIδの標的蛋白のひとつであるフォスフォランバンのリン酸化亢進がみとめられた。以上の結果はこの拡張型心筋症モデルマウスの心筋においてCaMKIIδが活性化していることを示すものと考えられた。

つぎに、CaMKIIδ活性化の拡張型心筋症発症における役割をさらに検討するために、その阻害薬であるKN-93を拡張型心筋症モデルマウスに投与してその表現型を解析したところ、KN-93投与により心拡大が著明に抑制され心機能の改善もみとめられた。CaMKIIδの抑制ペプチドであるAC3-Iを心筋特異的に過剰発現したAC3-Iマウスと拡張型心筋症モデルマウスを交配しCaMKIIδの作用を抑制した場合も同様に心拡大が著明に抑制され心機能の改善もみとめられた。さらに、CaMKIIδの作用を抑制することにより、p53の発現量が低下し、TUNEL陽性心筋細胞も減少していることが明らかになった。以上の結果は、CaMKIIδの活性化がp53の発現を介して心筋細胞死を誘導することにより拡張型心筋症を引き起こすことを示すものと考えられた。

（5）培養心筋細胞への変異型心筋αアクチ

ン遺伝子の導入

in vivo における CaMKIIδ の酵素活性の上昇や p53の発現量増加は、心機能低下により二次的に起っている可能性が考えられる。この可能性を否定し、変異型心筋αアクチンの直接的な影響によって CaMKIIδ や p53の変化が生じていることを示すために、変異型心筋αアクチンと野生型心筋αアクチンの発現プラスミドを作成し、それぞれ培養心筋細胞に遺伝子導入した。

CaMKIIδ と p53の蛋白量を検討したところ、培養心筋細胞への変異型心筋αアクチン遺伝子の導入により、CaMKIIδ と p53の蛋白量の増加がみとめられたが、野生型心筋αアクチンの遺伝子導入ではそのような変化はみとめられなかった。以上の結果から、拡張型心筋症モデルマウスでみられた CaMKIIδ の酵素活性の上昇や p53の発現量増加は、心機能低下に伴う二次的な現象ではなく、変異型心筋αアクチンの直接的な作用により生じたものであることが明らかになった。

（6）拡張型心筋症モデルマウス心筋におけるカルシウム動態

拡張型心筋症モデルマウス心筋におけるカルシウム動態を検討するために、カルシウム感受性について検討した。拡張型心筋症モデルマウスおよび野生型マウスからスキンドファイバーを作成し、カルシウム感受性を測定したところ、最大カルシウム張力には両者で変化がみられなかったが、カルシウム感受性は拡張型心筋症モデルマウス由来心筋繊維で低下していることが明らかになった。また、単離心筋における心筋細胞内カルシウム濃度変化について検討したところ、野生型マウスと拡張型心筋症モデルマウスでは差がみとめられなかった。拡張型心筋症モデルマウスの心筋において calcineurin が活性化していることとあわせて考えると、以上の結果は、拡張型心筋症モデルマウス心筋ではカルシウム感受性が低下し、その結果心筋全体ではなく局所のカルシウム濃度が変化し、calcineurin や CaMKIIδ が活性化しているものと考えられた。

D．考察

今回の研究において特筆すべき点は、ヒトで報告されている変異型心筋αアクチン遺伝子を心臓特異的に過剰発現することにより、拡張型心筋症のモデルマウスを確立することができたことである。これまで拡張型心筋症の発症機序が明らかにならなかった理由の一つに、拡張型心筋症を解析するための適切なモデル動物が存在しなかったことが挙げられる。今後このマウスを用いて、さらに詳細な病態生理の解析や、新たな治療法の開発がなされることが期待される。

また、今回の検討により、拡張型心筋症発症の分子機構の一端が明らかになったことも特筆すべき点である。すなわち、拡張型心筋症モデルマウスの心筋においてはカルシウム感受性が低下しており、おそらくそれが原因となって心筋局所のカルシウム濃度が変化し calcineurin や CaMKIIδ のようなカルシウム依存性の酵素活性が上昇することにより心筋症発症に至る可能性が考えられる。また、calcineurin および CaMKIIδ に対する阻害薬による検討から、心筋症の発症自体には CaMKIIδ の関与が大きく、calcineurin はこのマウスモデルにおいては相対的にその関与は少ないと考えられる。さらに、CaMKIIδ 阻害薬により p53の発現上昇が抑制されることや、p53ヘテロ遺伝子欠損あるいは Bcl2過剰発現により心筋症発症が抑制されることから、CaMKIIδ 活性化が心筋症をおこす機序としては、p53の発現上昇とそれにともなう心筋細胞死が考えられる。

今後の検討課題として、以下のような点が

挙げられる。今回の検討ではカルシウム感受性が低下する原因が明らかにされていない。また、カルシウム感受性の低下が本当に心筋症発症のプライマリーな原因であるのかについても確証は得られていない。さらに、CaMKIIδ活性化によるp53発現量増加の機序についても明らかではない。これらについては今後さらに検討を要するものと考えられる。

E. 結論

変異型心筋αアクチン遺伝子を心臓特異的に過剰発現することにより、ヒトにおける拡張型心筋症のモデルマウスを確立することができた。また、このマウスモデルにおける拡張型心筋症の発症機序としてCaMKIIδの活性化とp53の蛋白量の増加が重要であることが明らかとなった。今後、両因子を標的とした治療が拡張型心筋症の治療となり得る可能性が示唆された。

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G．知的財産権の出願・登録状況

1. 特許取得　　該当なし
2. 実用新案登録　該当なし
3. その他　　該当なし

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LETTERS

IGFBP-4 is an inhibitor of canonical Wnt signalling required for cardiogenesis

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Insulin-like growth-factor-binding proteins (IGFBPs) bind to and modulate the actions of insulin-like growth factors (IGFs)[1]. Although some of the actions of IGFBPs have been reported to be independent of IGFs, the precise mechanisms of IGF-independent actions of IGFBPs are largely unknown[1,2]. Here we report a previously unknown function for IGFBP-4 as a cardiogenic growth factor. IGFBP-4 enhanced cardiomyocyte differentiation *in vitro*, and knockdown of *Igfbp4* attenuated cardiomyogenesis both *in vitro* and *in vivo*. The cardiogenic effect of IGFBP-4 was independent of its IGF-binding activity but was mediated by the inhibitory effect on canonical Wnt signalling. IGFBP-4 physically interacted with a Wnt receptor, Frizzled 8 (Frz8), and a Wnt co-receptor, low-density lipoprotein receptor-related protein 6 (LRP6), and inhibited the binding of Wnt3A to Frz8 and LRP6. Although IGF-independent, the cardiogenic effect of IGFBP-4 was attenuated by IGFs through IGFBP-4 sequestration. IGFBP-4 is therefore an inhibitor of the canonical Wnt signalling required for cardiogenesis and provides a molecular link between IGF signalling and Wnt signalling.

The heart is the first organ to form during embryogenesis, and abnormalities in this process result in congenital heart diseases, the most common cause of birth defects in humans[3]. Molecules that mediate cardiogenesis are of particular interest because of their potential use for cardiac regeneration[4,5]. Previous studies have shown that soluble growth factors such as bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), Wnts and Wnt inhibitors mediate the tissue interactions that are crucial for cardiomyocyte specification[3,4]. We proposed that there might be additional soluble factors that modulate cardiac development and/or cardiomyocyte differentiation.

P19CL6 cells differentiate into cardiomyocytes with high efficiency in the presence of 1% dimethylsulphoxide (DMSO)[6]. We cultured P19CL6 cells with culture media conditioned by various cell types in the absence of DMSO, and screened the cardiogenic activity of the conditioned media. The extent of cardiomyocyte differentiation was assessed by the immunostaining with MF20 monoclonal antibody that recognizes sarcomeric myosin heavy chain (MHC). Among the several cell types tested, culture media conditioned by a murine stromal cell line OP9 induced cardiomyocyte differentiation of P19CL6 cells without DMSO treatment (Fig. 1a, left and middle panels). Increased MF20-positive area was accompanied by the induction of cardiac marker genes such as *αMHC*, *Nkx2.5* and *GATA-4*, and by the increased protein levels of cardiac troponin T (cTnT) (Fig. 1a, right panel). In contrast, culture media conditioned by COS7 cells, mouse embryonic fibroblasts, NIH3T3 cells, HeLa cells, END2 cells (visceral endoderm-like cells), neonatal rat cardiomyocytes and neonatal rat cardiac fibroblasts did not induce cardiomyocyte differentiation of P19CL6 cells in the absence of DMSO (Fig. 1a and data not shown). From these observations, we postulated that OP9 cells secrete one or more cardiogenic growth factors.

To identify an OP9-derived cardiogenic factor, complementary DNA clones isolated by a signal sequence trap method from an OP9 cell cDNA library[7] were tested for their cardiogenic activities by transient transfection. When available, recombinant proteins were also used to confirm the results. Among candidate factors tested, IGFBP-4 induced cardiomyocyte differentiation of P19CL6 cells, as demonstrated by the increase in MF20-positive area and the induction of cardiac markers (Fig. 1b). We also cultured P19CL6 cells with OP9-conditioned media pretreated with an anti-IGFBP-4 neutralizing antibody. The application of an anti-IGFBP-4 neutralizing antibody attenuated the efficiency of cardiomyocyte differentiation induced by OP9-conditioned media (Fig. 1c). These findings strongly suggest that IGFBP-4 is a cardiogenic factor secreted from OP9 cells.

Because IGFBPs have been characterized as molecules that bind to and modulate the actions of IGFs, we tested whether IGFBP-4 promotes cardiogenesis by either enhancing or inhibiting the actions of IGFs. We first treated P19CL6 cells with a combination of anti-IGF-I and IGF-II-neutralizing antibodies or a neutralizing antibody against type-I IGF receptor. If IGFBP-4 induces cardiomyocyte differentiation by inhibiting IGF signalling, treatment with these antibodies should induce cardiomyocyte differentiation and/or enhance the cardiogenic effects of IGFBP-4. In contrast, if IGFBP-4 promotes cardiogenesis by enhancing IGF signalling, treatment with these antibodies should attenuate IGFBP-4-mediated cardiogenesis. However, treatment with these antibodies did not affect the efficiency of IGFBP-4-induced cardiomyocyte differentiation (Fig. 1d and data not shown). Treatment of P19CL6 cells with IGF-I and IGF-II also did not induce cardiomyocyte differentiation (data not shown). Furthermore, treatment with an IGFBP-4 mutant (IGFBP-4-H74P; His 74 replaced by Pro)[8] that is unable to bind IGFs induced cardiomyocyte differentiation of P19CL6 cells even more efficiently than wild-type IGFBP-4 (Fig. 1e). This is presumably due to the sequestration of wild-type IGFBP-4 but not mutant IGFBP-4-H74P by endogenous IGFs. In agreement with this idea, exogenous IGFs attenuated wild-type IGFBP-4-induced but not IGFBP-4-H74P-induced cardiogenesis (Fig. 1f). Taken together, these observations indicate

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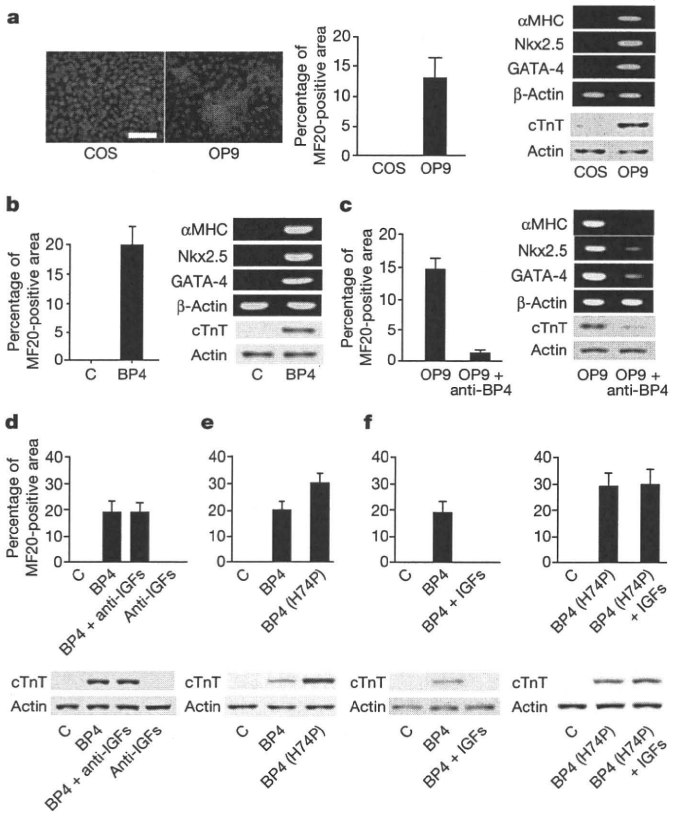

Figure 1 | IGFBP-4 promotes cardiomyocyte differentiation in an IGF-independent manner. **a**, Culture media conditioned by OP9 cells but not by COS7 cells induced cardiomyocyte differentiation of P19CL6 cells as assessed by MF20-positive area, cardiac marker-gene expression and cTnT protein expression. Scale bar, 100 μm. Error bars show s.d. **b**, Treatment with IGFBP-4 (1 μg ml^{-1}) induced cardiomyocyte differentiation of P19CL6 cells in the absence of DMSO. Error bars show s.d. **c**, Treatment with a neutralizing antibody against IGFBP-4 (anti-BP4; 40 μg ml^{-1}) attenuated cardiomyocyte differentiation of P19CL6 cells induced by OP9-conditioned media. Error bars show s.d. **d**, Treatment with neutralizing antibodies against IGF-I and IGF-II (anti-IGFs; 5 μg ml^{-1} each) had no effect on IGFBP-4-induced cardiomyocyte differentiation of P19CL6 cells. Error bars show s.d. **e**, Mutant IGFBP-4 (BP4(H74P)) that is incapable of binding to IGFs retained cardiomyogenic activity. Error bars show s.d. **f**, IGFs (100 ng ml^{-1} each) attenuated wild-type IGFBP-4-induced but not mutant IGFBP-4-H74P-induced cardiomyocyte differentiation of P19CL6 cells. Error bars show s.d.

that IGFBP-4 induces cardiomyocyte differentiation in an IGF-independent fashion.

To explore further the mechanisms by which IGFBP-4 induces cardiomyogenesis, we tested the hypothesis that IGFBP-4 might modulate the signals activated by other secreted factors implicated in cardiogenesis. It has been shown that canonical Wnt signalling is crucial in cardiomyocyte differentiation[3,4]. In P19CL6 cells, Wnt3A treatment activated β-catenin-dependent transcription of the TOPFLASH reporter gene, and this activation was attenuated by IGFBP-4 (Fig. 2a). Wnt/β-catenin signalling is transduced by the cell-surface receptor complex consisting of Frizzled and low-density-lipoprotein receptor (LDLR)-related protein 5/6 (LRP5/6)[9] and IGFBP-4 attenuated TOPFLASH activity enhanced by the expression of LRP6 or Frizzled 8 (Frz8) (Fig. 2a). As a control, IGFBP-4 did not alter BMP-mediated activation of a BMP-responsive reporter BRE–luc (Supplementary Fig. 1b). These findings suggest that IGFBP-4 is a specific inhibitor of the canonical Wnt pathway. To examine this possibility *in vivo*, we performed axis duplication assays in *Xenopus* embryos. Injection of *Xwnt8* or *Lrp6* mRNA caused secondary axis formation, and injection of *Xenopus IGFBP-4* (*XIGFBP-4*) mRNA alone had minimal effects on axis formation. However, Xwnt8-induced or LRP6-induced secondary axis formation was efficiently blocked by coexpression of XIGFBP-4 (Fig. 2b, c), indicating that IGFBP-4 inhibits canonical Wnt signalling *in vivo*. To explore the mechanisms of Wnt inhibition by IGFBP-4, *Xenopus* animal cap assays and TOPFLASH reporter gene assays were performed. In animal cap assays, IGFBP-4 inhibited LRP6-induced but not β-catenin-induced Wnt-target gene expression (Supplementary Fig. 1c). Similarly, IGFBP-4 attenuated Wnt3A-induced or LRP6-induced TOPFLASH activity but did not alter Dishevelled-1 (Dvl-1)-induced, LiCl-induced or β-catenin-induced TOPFLASH activity (Supplementary Fig. 1d, e). These findings suggest that IGFBP-4 inhibits canonical Wnt signalling at the level of cell-surface receptors. To examine whether IGFBP-4 antagonizes Wnt signalling via direct physical interaction with LRP5/6 or Frizzled, we produced conditioned media containing the Myc-tagged extracellular portion of LRP6 (LRP6N–Myc), the Myc-tagged cysteine-rich domain (CRD) of Frz8 (Frz8CRD–Myc), and V5-tagged IGFBP-4 (IGFBP-4–V5). Immunoprecipitation (IP)/western blot experiments revealed that IGFBP-4 interacted with LRP6N (Fig. 2d) and Frz8CRD (Fig. 2e). A liquid-phase binding assay with ^{125}I-labelled IGFBP-4 and conditioned media containing LRP6N–Myc or Frz8CRD–Myc demonstrated that the interaction between IGFBP-4 and LRP6N or Frz8CRD was specific and saturable (Fig. 2f, g). A Scatchard plot analysis revealed two binding sites with different binding affinities for LRP6N (Fig. 2f, inset) and a single binding site for Frz8CRD (Fig. 2g, inset). A similar binding assay with ^{125}I-labelled Wnt3A demonstrated that IGFBP-4 inhibited Wnt3A binding to LRP6N (Fig. 2h) and Frz8CRD (Fig. 2i), and a Lineweaver–Burk plot revealed that IGFBP-4 was a competitive inhibitor of the binding of Wnt3A to Frz8CDR (Supplementary Fig. 2a). IP/western blot analyses with various deletion mutants of LRP6 and IGFBP-4 revealed that IGFBP-4 interacted with multiple domains of LRP6 and that the carboxy-terminal thyroglobulin domain of IGFBP-4 was required for IGFBP-4 binding to LRP6 or Frz8CRD (Supplementary Fig. 2b–f). It has been shown that inhibition of canonical Wnt signalling promotes cardiomyocyte differentiation in embryonic stem (ES) cells and in chick, *Xenopus* and zebrafish embryos[4,10,11]. These results therefore collectively suggest that IGFBP-4 promotes cardiogenesis by antagonizing the Wnt/β-catenin pathway through direct interactions with Frizzled and LRP5/6.

Next we investigated the role of endogenous IGFBP-4 in P19CL6 cell differentiation into cardiomyocytes. Reverse transcriptase-mediated polymerase chain reaction (RT–PCR) analysis revealed that the expression of *Igfbp4* was upregulated during DMSO-induced P19CL6 cell differentiation (Fig. 3a). Expression of *Igfbp3* and *Igfbp5* was also upregulated in the early and the late phases of differentiation, respectively. Expression of *Igfbp2* was not altered, and that of *Igfbp1* or *Igfbp6* was not detected. When IGFBP-4 was knocked down by two different small interfering RNA (siRNA) constructs, DMSO-induced cardiomyocyte differentiation was inhibited in both cases (Fig. 3b). In contrast, knockdown of *Igfbp3* or *Igfbp5* did not inhibit DMSO-induced cardiomyocyte differentiation (Fig. 3b, right panel). Treatment with an anti-IGFBP-4 neutralizing antibody also blocked DMSO-induced cardiomyocyte differentiation (Fig. 3c). Secretion of endogenous IGFBP-4 is therefore required for the differentiation of P19CL6 cells into cardiomyocytes. Immunostaining for IGFBP-4 revealed that cardiac myocytes were surrounded by the IGFBP-4-positive cells, suggesting that a paracrine effect of IGFBP-4 on cardiomyocyte differentiation is predominant (Fig. 3d). Essentially the same results were obtained in ES cells (Supplementary Fig. 3d–g). To investigate whether IGFBP-4 promotes the differentiation of P19CL6 cells into cardiomyocytes by the inhibition of the canonical Wnt pathway, we expressed dominant-negative LRP6 (LRP6N) in P19CL6 cells. Expression of LRP6N enhanced cardiomyocyte differentiation of P19CL6 cells and reversed the inhibitory effect of *Igfbp4*

knockdown on cardiomyogenesis (Fig. 3e). These observations suggest that endogenous IGFBP-4 is required for cardiomyocyte differentiation of P19CL6 cells and ES cells, and that the cardiogenic effect of IGFBP-4 is mediated by its inhibitory effect on Wnt/β-catenin signalling.

The role of endogenous IGFBP-4 in cardiac development *in vivo* was also examined with *Xenopus* embryos. Whole-mount *in situ* hybridization analysis revealed that strong expression of *XIGFBP-4* was detected at stage 38 in the anterior part of the liver adjacent to the heart (Fig. 4a). Knockdown of *XIGFBP-4* by two different morpholino (MO) constructs resulted in cardiac defects, with more than 70% of the embryos having a small heart or no heart (Fig. 4b). The specificity of MO was confirmed by the observation that simultaneous injection of MO-resistant *XIGFBP-4* cDNA rescued the MO-induced cardiac defects (Fig. 4b, Supplementary Fig. 4c). Coexpression of IGF-binding-defective XIGFBP-4 mutant (XIGFBP-4-H74P) or dominant-negative LRP6 (LRP6N) also rescued the cardiac defects induced by *XIGFBP-4* knockdown (Fig. 4b), whereas overexpression of Xwnt8 in the heart-forming region resulted in cardiac defects similar to those induced by *XIGFBP-4* knockdown (Supplementary Fig. 4d–f), supporting the notion that the cardiogenic effect of IGFBP-4 is independent of IGFs but is mediated by inhibition of the Wnt/β-catenin pathway. The temporal profile of cardiac defects induced by *XIGFBP-4* knockdown was also examined by *in situ* hybridization with *cardiac troponin I* (*cTnI*) (Fig. 4c). At stage 34, morphology of the heart was comparable between control embryos and MO-injected embryos. However, at stage 38, when *XIGFBP-4* starts to be expressed in the anterior part of the liver, the expression of *cTnI* was markedly attenuated in MO-injected embryos; expression of *cTnI* was diminished and no heart-like structure was observed at stage 42. Thus, the heart is initially formed but its subsequent growth is perturbed in the absence of XIGFBP-4, suggesting that IGFBP-4

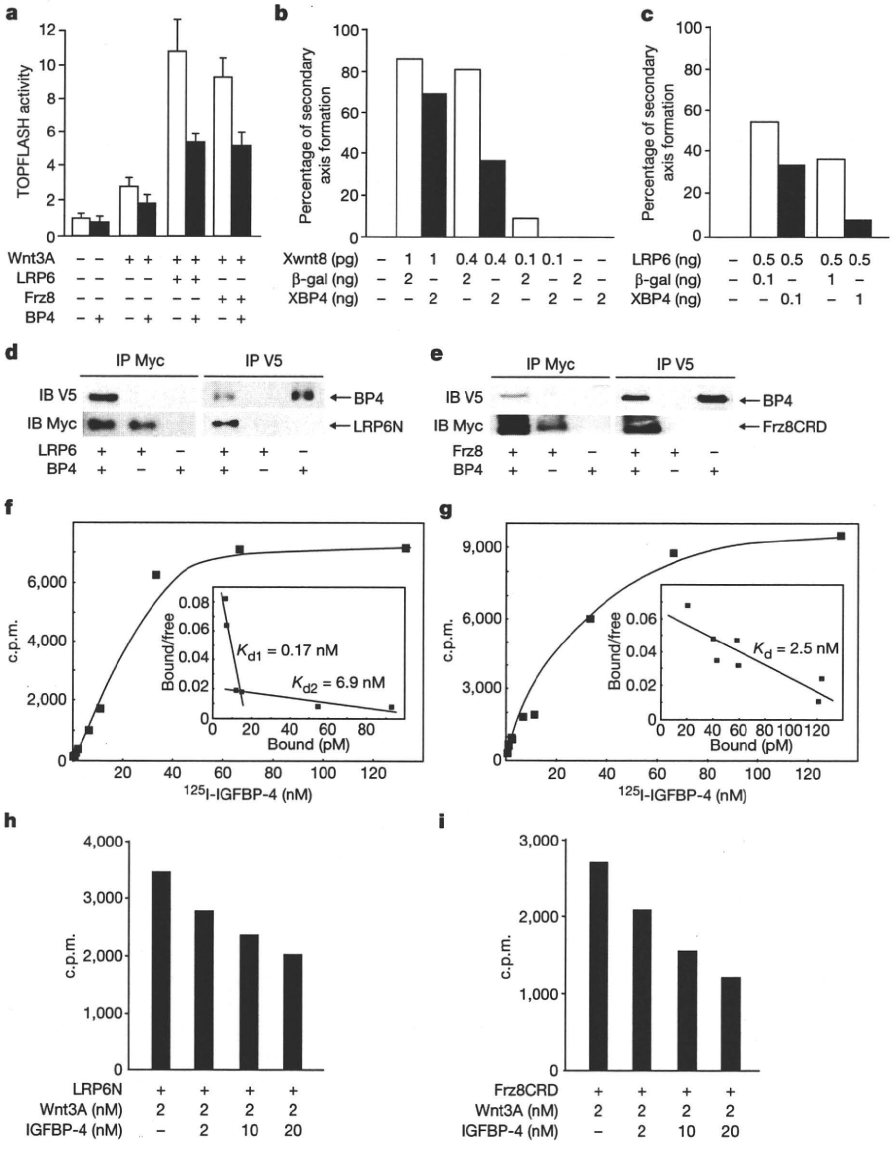

Figure 2 | IGFBP-4 inhibits Wnt/β-catenin signalling through direct interactions with Wnt receptors. **a**, IGFBP-4 attenuated β-catenin-dependent transcription in P19CL6 cells. P19CL6 cells were transfected with TOPFLASH reporter gene and expression vectors for LRP6 or Frz8, and then treated with Wnt3A or Wnt3A plus IGFBP-4; luciferase activities were then measured. Error bars show s.d. **b**, XIGFBP-4 (XBP4) inhibited Xwnt8-induced secondary-axis formation in *Xenopus* embryos ($n = 20$ for each group). **c**, IGFBP-4 inhibited LRP6-induced secondary-axis formation in *Xenopus* embryos ($n = 30$ for each group). **d**, **e**, IGFBP-4 interacted directly with LRP6N (**d**) and Frz8CRD (**e**). IB, immunoblotting; IP, immunoprecipitation. **f**, A binding assay between ^{125}I-labelled IGFBP-4 and LRP6N. The inset is a Scatchard plot showing two binding sites with different binding affinities. **g**, A binding assay between ^{125}I-labelled IGFBP-4 and Frz8CRD. The inset is a Scatchard plot showing a single binding site. **h**, **i**, IGFBP-4 inhibited Wnt3A binding to LRP6N (**h**) or Frz8CRD (**i**). ^{125}I-labelled Wnt3A binding to LRP6N or Frz8CRD was assessed in the presence of increasing amounts of IGFBP-4.

promotes cardiogenesis by maintaining the proliferation and/or survival of embryonic cardiomyocytes.

It has been shown that canonical Wnt signals inhibit cardiogenesis in chick and frog embryos, and that Wnt antagonists such as Dkk1 and Crescent secreted from the anterior endoderm or the organizer region counteract the Wnt-mediated inhibitory signals and induce cardiogenesis in the anterior lateral mesoderm[4]. However, IGFBP-4-mediated Wnt inhibition is required at later stages of development, when the heart is already formed at the ventral portion and starts to grow and remodel to maintain embryonic circulation. It has been shown that Wnt/β-catenin signalling has time-dependent effects on cardiogenesis in ES cells: canonical Wnt signalling in the early phase of ES-cell differentiation promotes cardiomyogenesis, whereas it inhibits cardiomyocyte differentiation in the late phase[10–12]. In agreement with this notion, IGFBP-4 promoted cardiomyocyte differentiation of ES cells only when IGFBP-4 was applied in the late phase after embryoid body formation (Supplementary Fig. 3a–c). Similar time-dependent effects of Wnt/β-catenin signalling on cardiogenesis has been shown in zebrafish embryos[11]. Moreover, several recent reports suggest that Wnt/β-catenin signalling is a positive regulator of cardiac progenitor-cell proliferation in the secondary heart field[13]. It therefore seems that canonical Wnt signalling has divergent effects on cardiogenesis at multiple stages of development: first, canonical Wnt signalling promotes cardiogenesis at the time of gastrulation or mesoderm specification; second, it inhibits cardiogenesis at the time when cardiac mesoderm is specified in the anterior lateral mesoderm; third, it promotes the expansion of cardiac progenitors in the secondary heart field; and fourth, it inhibits cardiogenesis at later stages when the embryonic heart is growing. It is interesting to note that IGFBP-4 is expressed predominantly in the liver. Mouse IGFBP-4 is

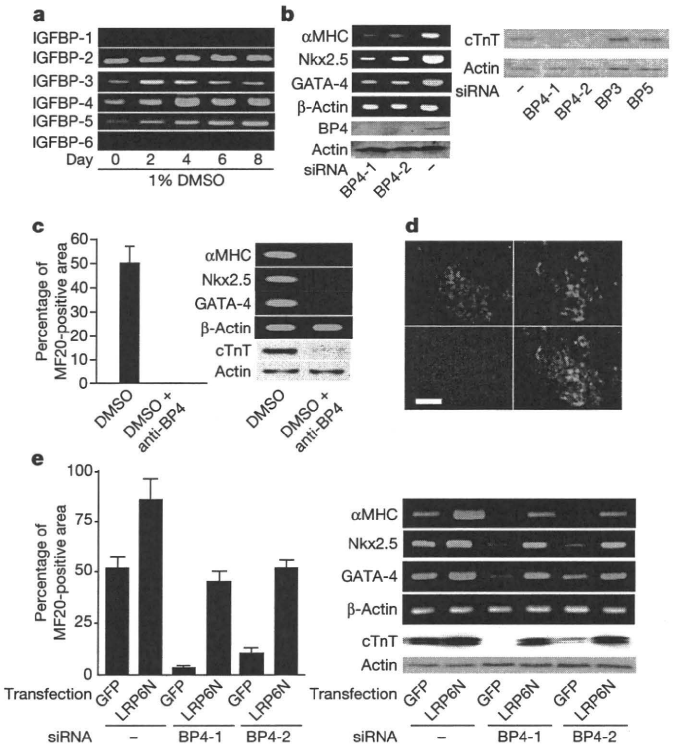

Figure 3 | IGFBP-4 is required for the differentiation of P19CL6 cells into cardiomyocytes. **a**, Expression analysis of IGFBP family members by RT–PCR during DMSO-induced cardiomyocyte differentiation of P19CL6 cells (from day 0 to day 8). **b**, Left: knockdown of *Igfbp4* in P19CL6 cells attenuated cardiac marker expression in response to treatment with DMSO. BP4-1 and BP4-2 represent two different siRNAs for IGFBP-4. Right: knockdown of *Igfbp3* or *Igfbp5* had no effect on cTnT expression in response to DMSO treatment. **c**, Treatment with a neutralizing antibody against IGFBP-4 (anti-BP4; 40 μg ml^{-1}) attenuated DMSO-induced cardiomyocyte differentiation of P19CL6 cells. Error bars show s.d. **d**, IGFBP-4 immunostaining during DMSO-induced differentiation of P19CL6 cells stably transfected with αMHC–green fluorescent protein (GFP) reporter gene. Top left, IGFBP-4 staining (red); top right, GFP expression representing differentiated cardiomyocytes; bottom left, nuclear staining with DAPI (4′,6-diamidino-2-phenylindole); bottom right, a merged picture. Scale bar, 100 μm. **e**, Attenuated cardiomyocyte differentiation of P19CL6 cells by *Igfbp4* knockdown was rescued by inhibiting Wnt/β-catenin signalling. Control and *Igfbp4*-knocked-down P19CL6 cells were transfected with an expression vector for GFP or LRP6N (a dominant-negative form of LRP6) and induced to differentiate into cardiomyocytes by treatment with DMSO. LRP6N overexpression rescued the attenuated cardiomyocyte differentiation induced by *Igfbp4* knockdown as assessed by MF20-positive area (left panel), cardiac marker-gene expression and cTnT protein expression (right panel). Error bars show s.d.

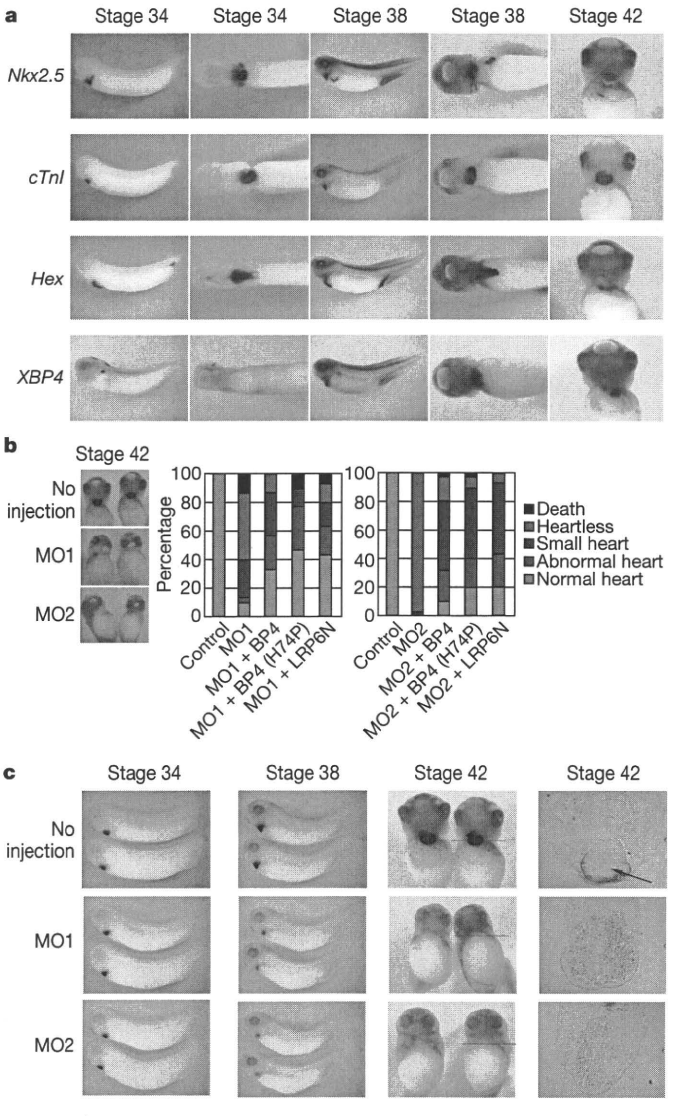

Figure 4 | IGFBP-4 is required for the maturation of the heart in *Xenopus* embryos. **a**, *In situ* hybridization analysis of *Nkx2.5* (an early cardiac marker), *cTnI* (a mature cardiac marker), *Hex* (a liver marker), and *XIGFBP-4* (*XBP4*) mRNA expression at stages 34, 38 and 42. **b**, Knockdown of *XIGFBP-4* by two different morpholinos (MO1 and MO2) resulted in severe cardiac defects as assessed by *cTnI in situ* hybridization at stage 42 (left). These cardiac defects were rescued by simultaneous injection of MO-resistant wild-type XIGFBP-4, mutant XIGFBP-4-H74P (BP4(H74P) and LRP6N ($n = 30$ for each group). **c**, Temporal profile of cardiac defects induced by *XIGFBP-4* knockdown. Morphology of the heart as assessed by cTnI *in situ* hybridization was almost normal at stage 34 but was severely perturbed at stages 38 and 42. The right column shows sections of control and MO-injected embryos. The arrow indicates the heart in control embryos. No heart-like structure was observed in MO-injected embryos.

also strongly expressed in the tissues adjacent to the heart such as pharyngeal arches and liver bud at embryonic day (E)9.5 (Supplementary Fig. 3h). These observations and the results of IGFBP-4 immunostaining in P19CL6 cells and ES cells suggest that IGFBP-4 promotes cardiogenesis in a paracrine fashion. Together with a previous report showing that cardiac mesoderm secretes FGFs and induces liver progenitors in the ventral endoderm[14], these observations suggest that there exist reciprocal paracrine signals between the heart and the liver that coordinately promote the development of each other.

IGFBPs are composed of six members, IGFBP-1 to IGFBP-6. Reporter gene assays and β-catenin stabilization assays revealed that IGFBP-4 was the most potent canonical Wnt inhibitor and that IGFBP-1, IGFBP-2 and IGFBP-6 also showed modest activity in Wnt inhibition, whereas IGFBP-3 and IGFBP-5 had no such activity (Supplementary Fig. 5a–c). In agreement with this, IP/western blot analyses demonstrated that IGFBP-1, IGFBP-2, IGFBP-4 and IGFBP-6 but not IGFBP-3 or IGFBP-5 interacted with LRP6 or Frz8CRD (Supplementary Fig. 5d, e). Thus, the lack of cardiac phenotypes in IGFBP-4-null mice or IGFBP-3/IGFBP-4/IGFBP-5 triple knockout mice[15] may be due to genetic redundancies between IGFBP-4 and other IGFBPs such as IGFBP-1, IGFBP-2 and/or IGFBP-6.

The identification of IGFBP-4 as an inhibitor of Wnt/β-catenin signalling may also have some implications for cancer biology[16]. It was shown that treatment with IGFBP-4 reduces cell proliferation in some cancer cell lines *in vitro*, and that overexpression of IGFBP-4 attenuates the growth of prostate cancer *in vivo*. Decreased serum levels of IGFBP-4 are associated with the risk of breast cancer. Because the activation of Wnt signalling is implicated in several forms of malignant tumours[17,18], it is possible that the inhibitory effect of IGFBP-4 on cell proliferation is mediated in part by the inhibition of canonical Wnt signalling.

METHODS SUMMARY

Cell culture. P19CL6 cells and ES cells were cultured and induced to differentiate into cardiomyocytes essentially as described[6,10]. P19CL6 cells (2,000 cells per 35-mm dish) were treated with various conditioned media for screening of their cardiogenic activities. For siRNA-mediated knockdown, pSIREN-RetroQ vectors (Clontech) ligated with double-stranded oligonucleotides were transfected into P19CL6 cells or ES cells, and puromycin-resistant clones were selected.

IP/western blot analyses and binding assays. Conditioned media for IP/western blot analyses were produced by using 293 cells. Binding reactions were performed overnight at 4 °C. ^{125}I-labelling of IGFBP-4 and Wnt3A was performed with IODO-BEADS Iodination Reagent (Pierce). A liquid-phase binding assay was performed essentially as described[19].

***Xenopus* experiments.** Axis duplication assays, animal cap assays, and *in situ* hybridization analyses in *Xenopus* were performed essentially as described[20]. Electroporation of mRNA was performed at stage 28 essentially as described[21].

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Role of Heat Shock Transcriptional Factor 1 and Heat Shock Proteins in Cardiac Hypertrophy

Haruhiro Toko, Tohru Minamino, and Issei Komuro*

Cardiac hypertrophy is an independent risk factor for cardiovascular disease. Initially, cardiac hypertrophy is an adaptive response to increased wall stress, but sustained stress leads to heart failure. It remains unclear how the transition from adaptive cardiac hypertrophy to maladaptive cardiac hypertrophy occurs. It has been postulated that there are two forms of cardiac hypertrophy, which are physiologic and pathologic cardiac hypertrophy. Unlike pathologic cardiac hypertrophy caused by chronic pressure or volume overload, cardiac hypertrophy induced by exercise is associated with less fibrosis and better systolic function, suggesting that adaptive mechanisms may be involved in exercise-induced cardiac hypertrophy. Therefore, elucidation of the molecular differences between these two types of cardiac hypertrophy may provide insights into the mechanisms underlying the transition from adaptive cardiac hypertrophy to heart failure. By comparing the two types of cardiac hypertrophy, we have identified heat shock transcription factor 1 and its target heat shock proteins as key factors involved in the adaptive mechanism of cardiac hypertrophy. In this review, we summarize the protective role of heat shock transcription factor 1 and heat shock proteins in cardiovascular disease.
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• Introduction

Heart failure is the final outcome of various heart diseases, and cardiac hypertrophy is one of the main causes of heart failure. The Framingham Heart Study revealed that there is a relationship between the severity of cardiac hypertrophy and the incidence of cardiovascular events, and that cardiac hypertrophy is an independent risk factor for heart failure, arrhythmia, myocardial infarction, and sudden death (Levy et al. 1990, Behar et al. 1992, Haider et al. 1998, Verdecchia et al. 2001). Therefore, it is important to develop therapeutic strategies for this condition, but the precise mechanisms underlying the transition from cardiac hypertrophy to heart failure are still largely unknown.

Cardiac hypertrophy is induced by various pathologic or physiologic stimuli. For example, acute pressure overload initially induces adaptive cardiac hypertrophy that is associated with normal cardiac function, but systolic and diastolic dysfunction occur in the setting of chronic pressure overload, resulting in heart failure. Thus, chronic pressure overload is thought to cause pathologic or maladaptive cardiac hypertrophy. On the other hand, regular exercise can induce cardiac hypertrophy without causing systolic or diastolic dysfunction (Pluim et al. 2000). Because exercise-induced cardiac hypertrophy does not progress to heart failure, it is thought to be physiologic or adaptive cardiac hypertrophy. Although it has been reported that these two types of cardiac hypertrophy are morphologically (Richey and Brown 1998, Iemitsu et al. 2001, McMullen and Jennings 2007), functionally, and molecularly distinct from each other, the precise mechanism underlying these differences remains unclear. What are the exact differences between pathologic and physiologic cardiac hypertrophy? Why is cardiac function preserved in physiologic cardiac hypertrophy? Why does sustained pressure overload cause heart failure? Answering these questions will provide insights into novel therapeutic options for both cardiac hypertrophy and heart failure.

• Pathologic and Physiologic Cardiac Hypertrophy

The differences between these two conditions include the stimuli inducing cardiac hypertrophy, their duration of action, and the signaling pathways involved. Pathologic cardiac hypertrophy is induced by persistent stress, such as pressure overload and volume overload caused by hypertension or valvular heart disease. On the other hand, physiologic cardiac hypertrophy is induced by intermittent stress such as exercise. Thus, the manifestations of cardiac hypertrophy caused by various stimuli may depend on their duration and intensity. In a recent study, Perrino et al. (2006) applied intermittent pressure overload to the heart and investigated the role of the duration of stress in the development of cardiac failure. Despite only developing mild cardiac hypertrophy, the hearts exposed to intermittent pressure overload displayed various pathologic changes, including diastolic dysfunction and histologic abnormalities.

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