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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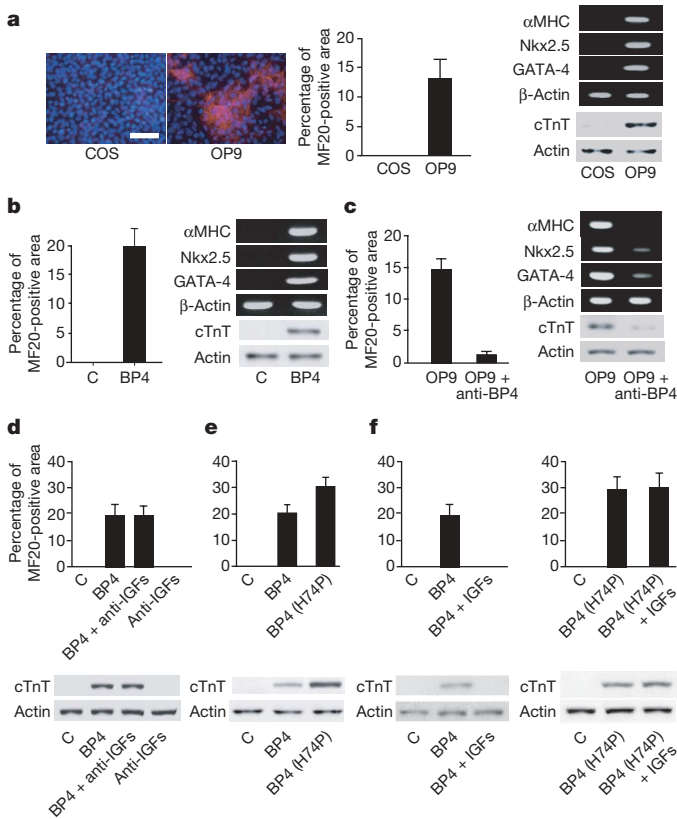
Figure 1 | IGFBP-4 promotes cardiomyocyte differentiation in an IGF-independent manner. a, Culture media conditioned by OP9 cells but not by COS7 cells induced cardiomyocyte differentiation of P19CL6 cells as assessed by MF20-positive area, cardiac marker-gene expression and cTnT protein expression. 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* (*XlGFBP-4*) mRNA alone had minimal effects on axis

formation. However, Xwnt8-induced or LRP6-induced secondary axis formation was efficiently blocked by coexpression of XlGFBP-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 Frz2CDR (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
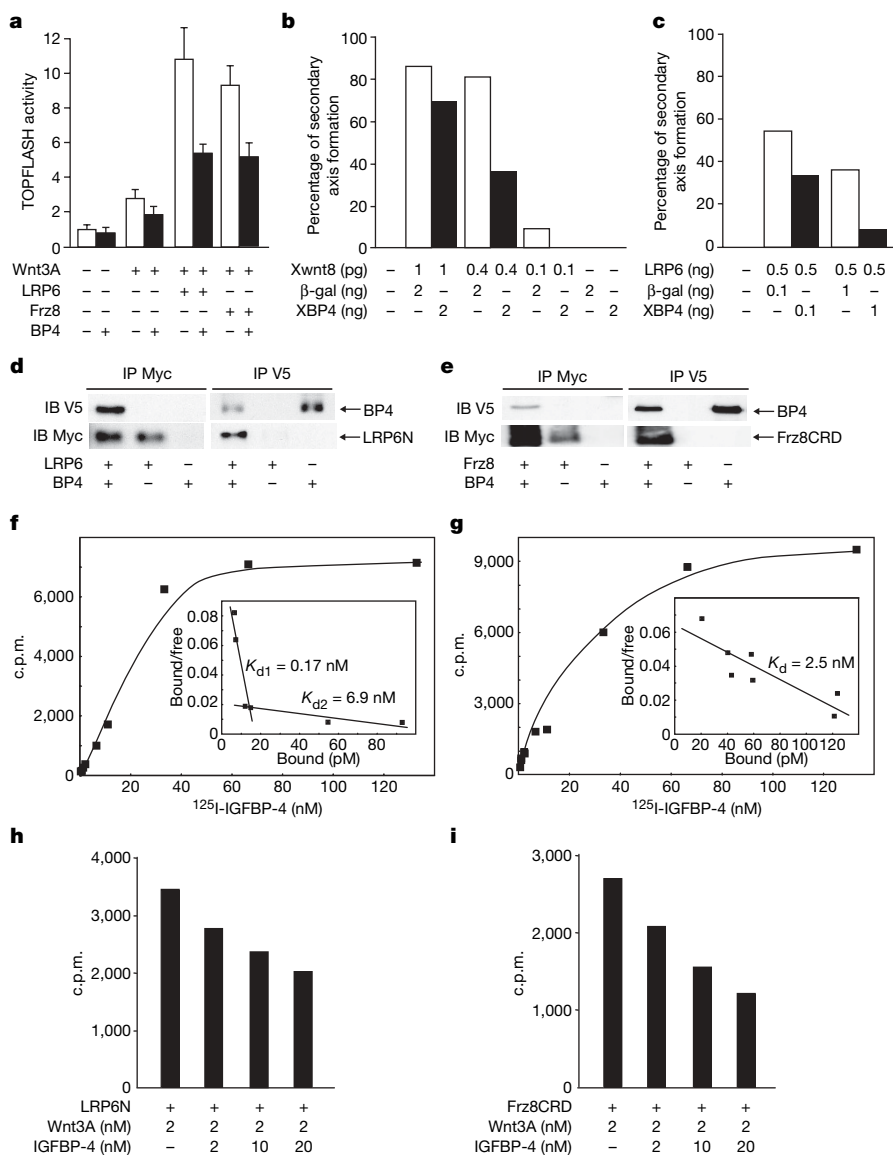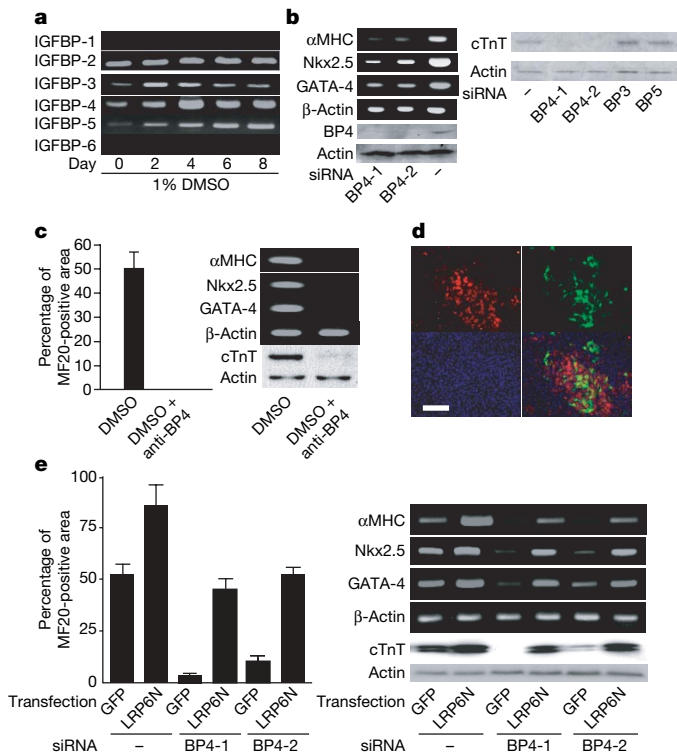

Figure 2 | IGFBP-4 inhibits Wnt/β-catenin signalling through direct interactions with Wnt receptors. a, IGFBP-4 attenuated β-catenin-dependent transcription in P19CL6 cells. P19CL6 cells were transfected with TOPFLASH reporter gene and expression vectors for LRP6 or Frz8, and then treated with Wnt3A 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.
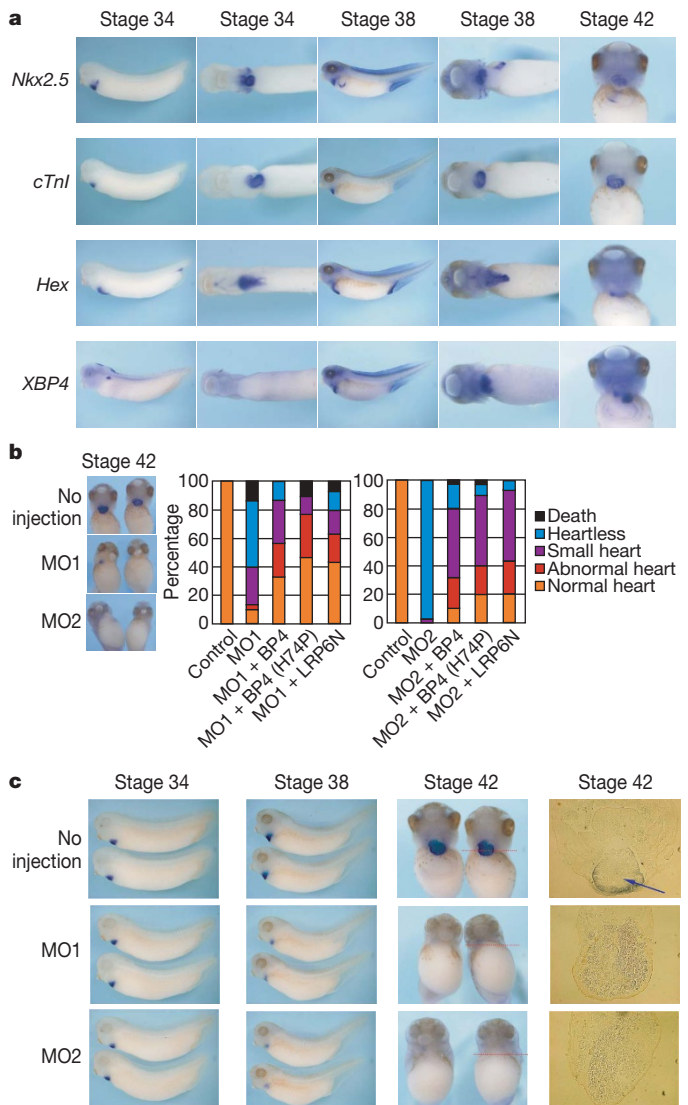
promotes cardiogenesis by maintaining the proliferation and/or survival of embryonic cardiomyocytes.

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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Author Contributions W.Z., I.S. and Y.I. contributed equally to this work. I.K. designed and supervised the research. W.Z., I.S., Y.I., Z.L., H.I., M.Y. and A.T.N. performed experiments, J.N., H.U., A.U., T.M., T.N., A.K. and M.A. contributed new reagents and/or analytical tools. W.Z., I.S., Y.I., A.K. and I.K. analysed data. W.Z., I.S., Y.I. and I.K. prepared the manuscript.

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METHODS

Plasmids and reagents. cDNA clones encoding mouse IGFBPs and *Xenopus* IGFBP-4 were purchased from Open Biosystems. XIGFBP-4-H74P mutant was generated with a QuickChange Site-Directed Mutagenesis kit (Stratagene). His-tagged human wild-type IGFBP-4 and mutant IGFBP-4-H74P (vectors provided by X. Qin)[8] were produced and purified with HisTrap HP Kit (Amersham). Full-length Frz8, Frz8CRD and LRP6N were provided by X. He[22,23]. Full-length LRP6, membrane-bound forms of LRP6 deletion mutants, and Dkk1 were from C. Niehrs[24]. pXwnt8 and pCSKA-Xwnt8 were from J. Christian[25]. pCS2–β-catenin was from D. Kimelman[26]. αMHC–GFP was from B. Fleischmann[27]. BRE–luc was from P. ten Dijke[28]. pCGN-Dvl-1 was described previously[29]. Soluble forms of LRP6 deletion mutants and probes for *in situ* hybridization analysis (Nkx2.5, cTnI and Hex) were generated by PCR. IGFBP-4, Wnt3A, IGF-I, IGF-II and BMP2 were from R&D. Neutralizing antibodies were from R&D (anti-IGFBP-4), Sigma (anti-IGF-I and anti-IGF-II), and Oncogene (anti-type-I IGF receptor). The antibodies used for immunoprecipitation, western blotting and immunostaining were from Invitrogen (anti-Myc, anti-V5), Santa Cruz (anti-cTnT, anti-IGFBP-4, anti-topoisomerase I (TOPO-I)), Sigma (anti-β-actin, anti-β-catenin, anti-FLAG (M2)) and Developmental Studies Hybridoma Bank (anti-sarcomeric myosin heavy chain (MF20)).

Cell culture experiments. 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. P19CL6 cells or ES cells stably transfected with αMHC promoter driven–GFP were generated by transfection of αMHC–GFP plasmid into P19CL6 cells or ht7 ES cells followed by G418 selection. Luciferase reporter gene assays, western blot analyses, immunostaining and RT–PCR were performed as described[10]. Reporter gene assays were repeated at least three times. PCR primers and PCR conditions are listed in Supplementary Table 1. For siRNA-mediated knockdown, siRNAs were expressed with pSIREN-RetroQ vector (Clontech). Oligonucleotide sequences used are listed in Supplementary Table 2. pSIREN-RetroQ vectors ligated with double-stranded oligonucleotides were transfected into P19CL6 cells or ES cells, and puromycin-resistant clones were isolated and expanded. For β-catenin stabilization assays, nuclear extracts of L cells were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). Data are shown as means and s.d.

IP/western blot analyses and binding assays. Conditioned media for IP/western blot analyses containing full-length or various deletion mutants of IGFBPs, LRP6, Frz8CRD and Dkk1 were produced with 293 cells. Binding reactions were performed overnight at 4 °C. Immunoprecipitation was performed with Protein G–Sepharose 4 Fast Flow (Amersham). ^{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]. In brief, conditioned media containing LRP6N–Myc or Frz8CRD–Myc were mixed with various concentrations of ^{125}I-labelled IGFBP-4 and incubated overnight at 4 °C. LRP6N–Myc or Frz8CRD–Myc was immunoprecipitated and the radioactivity of bound IGFBP-4 was measured after extensive washing of the Protein G–Sepharose

beads. For a competitive binding assay, conditioned media containing LRP6N–Myc or Frz8CRD–Myc were mixed with ^{125}I-labelled Wnt3A and unlabelled IGFBP-4, and incubated overnight at 4 °C. LRP6N–Myc or Frz8CRD–Myc was then immunoprecipitated and the radioactivity of bound Wnt3A was measured.

***Xenopus* experiments and mouse *in situ* hybridization analysis.** Axis duplication assays, animal cap assays and *in situ* hybridization analyses in *Xenopus* were performed essentially as described[20]. Two independent cDNAs for *XIGFBP-4*, presumably resulting from pseudotetraploid genomes, were identified by 5′ rapid amplification of cDNA ends (Supplementary Fig. 4a). Two different MOs targeting both of these two IGFBP-4 transcripts were designed (Gene Tools) (Supplementary Fig. 4a and Supplementary Table 2). MO-sensitive *XIGFBP-4* cDNA including a 41-base-pair 5′-untranslated region (UTR) was generated by PCR. MO-resistant *XIGFBP-4* cDNA (wild-type and H74P mutant) was generated by introducing five silent mutations in the MO1 target sequence and excluding the 5′-UTR (Supplementary Fig. 4a). To determine the specificity of MOs, MO-sensitive or MO-resistant *XIGFBP-4–myc* mRNA was injected into *Xenopus* embryos with or without MOs, and protein/mRNA expression was analysed. PCR primers and PCR conditions are listed in Supplementary Table 1. MOs and plasmid DNAs were injected at the eight-cell stage into the dorsal region of two dorsal–vegetal blastomeres fated to be heart and liver anlage. Electroporation of mRNA was performed essentially as described[21]. Injection of mRNA (5 ng in 5 nl of solution) into the vicinity of heart anlage and application of electric pulses were performed at stage 28. Whole-mount *in situ* hybridization analysis of murine IGFBP-4 was performed as described[30].

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CORRECTIONS & AMENDMENTS

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

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It has recently been brought to our attention that some of the lanes in the reverse-transcriptase-mediated polymerase chain reaction (RT–PCR) analyses of this Letter appear to be duplicated. Specifically, two β-actin bands in Fig. 1c, lanes 1, 4 and 6 of the αMHC bands and lanes 1 and 6 of the GATA4 bands in Fig. 3e, lane 1 of the Nkx2.5 band and lane 3 of the GATA4 band in Supplementary Fig. 3e, and lane 1 of the Nkx2.5 band and lane 1 of the β-actin band in Supplementary Fig. 3f might be duplicated. Lane 2 of the GATA4 band in Supplementary Fig. 3c might be 'pasted in'. Because the original raw data could not be located, we were unable to verify definitively whether the data in the original figures are correct or whether some of the lanes were indeed inadvertently duplicated. We therefore repeated the RT–PCR experiments shown in these figures. There is one difference in the experimental procedures between the original work and the repeated experiments. In the original work, knockdown of insulin-like growth-factor-binding protein 4 (IGFBP-4) in P19CL6 and embryonic stem (ES) cells was achieved by establishing stable cell lines that constitutively express IGFBP-4 small interfering RNAs (BP4-1 and BP4-2 in Fig. 3e and Supplementary Fig. 3e). In the repeated experiments, knockdown of IGFBP-4 was done by transient transfection of IGFBP-4 siRNAs. Figure 1 of this Corrigendum shows the corrected right-hand panels of the original Figs 1c and 3e. The Supplementary Information of this Corrigendum shows the corrected Supplementary Fig. 3c, e and f of the original Letter (in which the RT–PCR panels represent new and independent experiments); it also contains the raw data for all the corrected figures. Repeated experiments show that the results are reproducible and essentially the same as those of the original Letter. We thank Seitaro Nomura from the University of Tokyo, who generated data for the corrigendum. We apologize for any confusion these possible errors may have caused. The corrections described here do not alter the overall conclusion that IGFBP-4 promotes cardiogenesis by inhibiting canonical Wnt signalling.

Supplementary Information is available in the online version of this Corrigendum.

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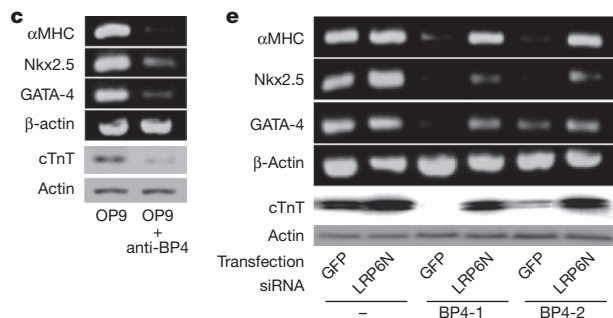
Figure 1 | This figure shows the corrected right-hand panels of Fig. 1c and Fig. 3e of the original Letter. In both figures, the RT–PCR panels represent new and independent experiments.