CHAPTER 3

Insulin-like growth factor promotes cardiac lineage induction *in vitro* by selective expansion of early mesoderm

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Adapted from: Stem Cells. 2014;32:1493-1502.

Abstract

A thorough understanding of the developmental signals that direct pluripotent stem cells (PSCs) toward a cardiac fate is essential for translational applications in disease modeling and therapy. We screened a panel of 44 cytokines/signaling molecules for their ability to enhance Nkx2.5^{+} cardiac progenitor cell (CPC) formation during *in vitro* embryonic stem cell (ESC) differentiation. Treatment of murine ESCs with insulin or insulin-like growth factors (IGF1/2) during early differentiation increased mesodermal cell proliferation and, consequently, CPC formation. Furthermore, we show that downstream mediators of IGF signaling (e.g., phospho-Akt and mTOR) are required for this effect. These data support a novel role for IGF family ligands to expand the developing mesoderm and promote cardiac differentiation. Insulin or IGF treatment could provide an effective strategy to increase the PSC-based generation of CPCs and cardiomyocytes for applications in regenerative medicine.

Introduction

Despite the availability of many treatment options, heart disease remains the leading cause of death worldwide, prompting the need for more innovative therapeutic strategies such as cell-based therapy.[1] The ability to produce patient-specific induced pluripotent stem cells (iPSCs) holds great promise for such regenerative applications.[2,3] A pivotal challenge in translating the potential of iPSCs into effective cardiac therapy is to generate sufficient quantities of functional cardiomyocytes to replace the large numbers of cells that are lost after myocardial injury.[4] Although *in vitro* cardiac differentiation protocols for pluripotent stem cells (PSCs) are readily available, the yield for most PSC lines remains modest and highly variable.[5,6] To improve the efficiency of cardiac differentiation, it is critical to understand the molecular mechanism of pluripotent cell commitment toward mesoderm during early development.[7]

In vitro differentiation of embryonic stem cells (ESCs) has been used to model early cardiac development due to the limited number of cells available when working with early stage embryos. When provided with appropriate cues, ESCs have been shown to faithfully recapitulate developmental gene expression patterns.[8] During ESC differentiation, a gastrulation-like step takes place resulting in the commitment of some cells into ectodermal lineage and another set of cells into the mesendodermal lineage. A portion of the latter cells gives rise to the Brachyury$^+$ mesodermal cell population. Some of these Brachyury$^+$ cells become the first committed cardiac progenitor cells (CPCs) as defined by their expression of two key cardiac transcription factors, Isl-1 and Nkx2.5.[9,10] CPCs are multipotent at this stage and can give rise to cardiomyocytes, smooth muscle cells, and endothelial cells.[11,12]

Cardiogenic commitment is driven by the activation of a number of highly conserved signaling pathways. For example, the transforming growth factor β superfamily members Activin A, bone morphogenetic protein 4 (BMP4), and Nodal as well as members of the fibroblast growth factor (i.e., FGF2) and Wnt (*i.e.*, Wnt3a) families of signaling molecules have been shown to enhance or inhibit cardiac differentiation in a spatial- and temporal-specific fashion.[5,13-19] To comprehensively evaluate signaling pathway activation during early cardiac lineage induction, we systematically screened a panel of 44 candidate cytokines/signaling molecules for their ability to enhance CPC formation. Consistent with previous findings, Wnt3a treatment during early differentiation enhanced mesodermal commitment leading to increased Nkx2.5$^+$ CPC formation.[20,21] Surprisingly, treatment with insulin and insulin-like growth factors (IGFs) positively regulated selective expansion of the mesendodermal cell population resulting in greater CPC formation. These ligands act through phosphorylation and activation of downstream targets such as Akt and mTOR and synergize with Wnt3a and FGF2.

Mechanistically, IGF induces selective expansion of the mesodermal cell population through increased proliferation. This study reveals a role for IGFs and insulin as regulators of *in vitro* mesodermal expansion and provides a strategy to significantly enhance the generation of PSC-derived CPCs.

Materials and methods

Growth factor screening

A previously described ESC line in which a cardiac-specific enhancer and base promoter of the murine Nkx2.5 locus drive enhanced green fluorescent protein (eGFP) gene expression was used for all experiments.[11] Nkx2.5-eGFP ESCs were cultured as previously described.[11] For the *in vitro* screening studies, cells were cultured in differentiation media containing 2% fetal bovine serum (FBS lot 894969; Life Technologies, Grand Island, NY, http://www.lifetech.com) and seeded at 4,000 cells per well in gelatin-coated 96-well plates (Corning Life Sciences, Tewksbury, MA, http://www.corning.com/lifesciences). Growth factors and signaling molecules were obtained from R&D Systems (Minneapolis, MN, http://www.rndsystems.com) as lyophilized powder and reconstituted as recommended by the manufacturer. A complete list of the compounds used for screening with the screening concentration range can be found in Supporting Information Table 1. Growth factors/signaling molecules were added to the cell culture at day 3 of differentiation at 1:2 dilutions in a dose range 4–1,000 μg/mL (i.e., 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1000 μg/mL). The concentration with the greatest effect on GFP+ signal was considered for our screening results. Cells were assayed on day 6 of differentiation using a FACSCalibur high-throughput screening platform for 96-well plates (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com). Data were analyzed with FlowJo software (Tree Star, Ashland, OR). The percentage of Nkx2.5-eGFP$^+$ cells was assessed for each treatment group and compared to that of solvent-exposed control cells. Each experiment was performed in triplicate, and three independent experiments were performed for each condition. A hit was determined as having a p value <.05 using two-tailed Student's t test. For all subsequent experiments, optimized treatment timing and cytokine concentrations were used (*Figure 1D; Supporting Information Figure 1*).

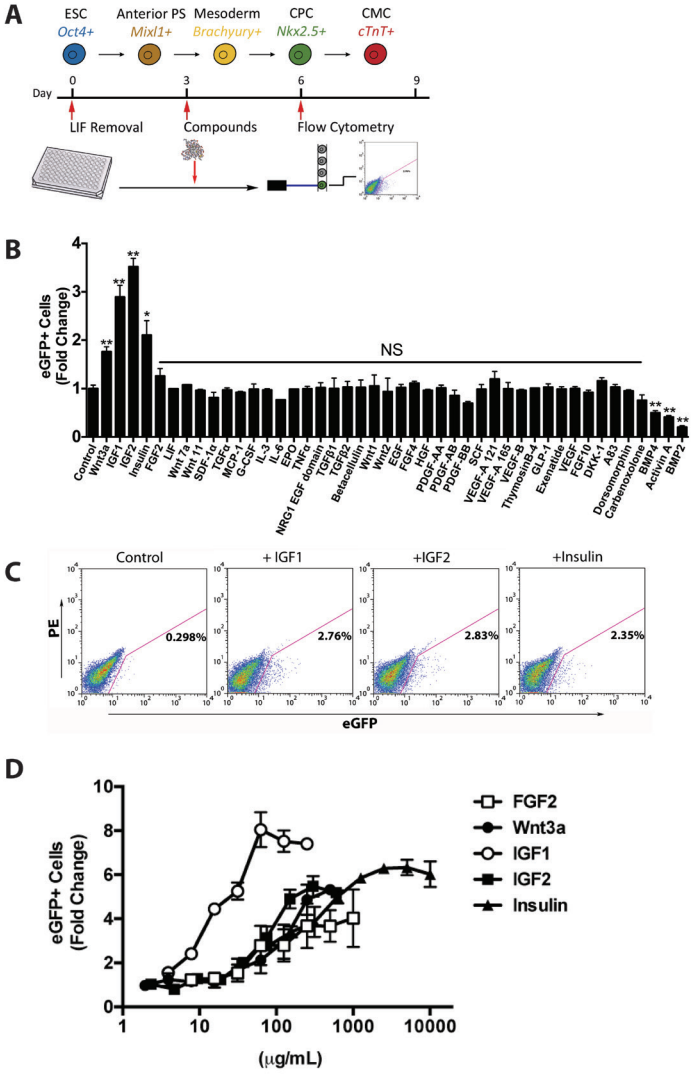

Figure 1. A murine ESC-based high throughput screen to identify growth factors/signaling molecules regulating Nkx2.5$^+$ CPC formation. (*A*) Schematic diagram of the high-throughput growth factor screen. Candidate growth factors/signaling molecules were added on day 3 of differentiation and cells were harvested on day 6 for flow cytometric analysis. (*B*) Quantification of eGFP$^+$ CPCs following growth factor treatment of Nkx2.5-eGFP ESCs. Values represent fold change in eGFP$^+$ cell count by growth factor treatment. **, $p < .01$ vs. untreated control cells; NS, non-significant difference with untreated control cells. (*C*) Representative flow cytometry dot plots of day 6 Nkx2.5-eGFP ESCs after treatment with IGF1, IGF2, or insulin on day 3. (*D*) Dose responses of Nkx2.5-eGFP ESCs to treatment with FGF2, Wnt3a, IGF1, IGF2, or insulin. Values represent fold increase of eGFP$^+$ cells as compared to untreated control cells. Mean ± SEM of triplicate experiments is shown. Abbreviations: BMP, bone morphogenetic protein; CPC, cardiac progenitor cell; CMC, cardiomyocyte; ESC, embryonic stem cell; eGFP, enchaned green fluorescent protein; FGF, fibroblast growth factor; IGF-1, insulin-like growth factor; LIF, leukemia inhibitor factor; PS, primitive streak; PE, phycoerythrin; TGF-β, transforming growth factor.

Immunofluorescence microscopy

Nkx2.5-eGFP ESCs were differentiated as previously described[11] and treated with growth factors at day 1 or 2 of differentiation. Cells were cultured on 0.1%-gelatin-coated coverslips until assay or beating embryoid bodies (EBs) were manually collected and transferred to Nunc Lab-Tek II Chamber Slides (Thermo Fisher Scientific, Waltham, MA, http://www.fishersci.com). Cells were then fixed with 4% paraformaldehyde solution (Thermo Fisher Scientific) and permeabilized with Triton X-100 (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com). After washing three times with phosphate-buffered saline (PBS)/10%FBS/0.1% Tween-20 (Sigma-Aldrich), cells were incubated overnight at 4°C with mouse monoclonal antibodies directed against sarcomeric α-actinin (clone EA-53; Sigma-Aldrich; 1:250 dilution), affinity-purified goat-anti-Brachyury IgG (R&D Systems, Minneapolis, MN, http://www.rndsystems.com/; 1:100 dilution), rabbit anti-Ki67 IgG (clone D3B5; Cell Signaling Technology, Danvers, MA; 1:100 dilution), and rabbit anti-phospho-Akt (Thr308) IgG (clone C31E5E; Cell Signaling Technology, 1:1600 dilution). Next, cells were washed again and incubated for 60 minutes at RT (room temperature) with Alexa Fluor dye-conjugated secondary antibodies (Life Technologies;http://www.lifetechnologies.com, Grand Island, NY, diluted 1:500 in PBS/10%FBS/0.1% Tween-20). Immunostained samples were mounted using VECTASHIELD mounting medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA,http://www.vectorlabs.com). For Draq5 labeling, Draq5 reagent (Cell Signaling Technology, Danvers, MA, http://www.cellsignal.com/, 1:5,000 dilution) was added to the culture media and incubated for 5 minutes at RT. Live cells were immediately imaged after staining. Images were acquired with an LSM 510 META inverted laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany, http://microscopy.zeiss.com) or a digital color camera-equipped fluorescence microscope (Nikon Eclipse 80i; Nikon Instruments Europe, Amstelveen, The Netherlands, http://www.nikon.com/) and processed using Fiji software (www.fiji.sc) and Adobe Photoshop version 5 (Adobe Systems, San Jose, CA, http://www.adobe.com/).

Quantitative reverse transcription-polymerase chain reaction

Nkx2.5-eGFP ESCs were seeded in 24-well plates (Corning Life Sciences) and treated with growth factors at day 1 or 2 of culture. At different time points after growth factor addition (i.e., day 0, 2, 4, 6, and 8), cells were lysed in TRIzol Reagent (Life Technologies) and total RNA was isolated using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands, http://www1.qiagen.com). Reverse transcription was done with iScript cDNA Synthesis Kit (Bio-Rad, Hercules CA, http://www.bio-rad.com). Gene expression levels were assayed using HotStart-IT SYBR Green quantitative polymerase chain reaction (qPCR) Master Mix (Affymetrix, Santa Clara, CA, http://www.affymetrix.com), and the primer pairs were specified in Supporting Information Table 2. PCR amplifications were performed in a CFX96 Touch Real-Time PCR Detection System (Bio-

Rad). Each condition was tested in three independent experiments using three samples per experiment.

Flow cytometry

Nkx2.5-eGFP ESCs were cultured in 24-well plates and treated at day 1 or 2 with growth factors. At the time point(s) of interest, cells were dissociated using trypsin/EDTA (Life Technologies) and 10 mg/mL collagenase A/B (Roche Applied Science, Indianapolis, IN, http://www.roche-applied-science.com) solutions in Hank's buffered salt solution (HBSS; Invitrogen)/20% FBS, fixed in 4% paraformaldehyde solution and permeabilized in ice-cold 100% methanol (Thermo Fisher Scientific). After washing two times with PBS, cells were incubated with primary antibodies for 1 hour at RT, washed again, and incubated for 1 hour at RT with appropriate Alexa Fluor dye-conjugated secondary antibodies. Following additional washings, cells were suspended in HBSS (Invitrogen) supplemented with 10% FBS and analyzed using a FACSCalibur flow cytometer (BD Biosciences). The following primary antibodies were used: cardiac troponin T (cTnT; clone 13-11; Thermo Fisher Scientific; 1:100 dilution in HBSS/10% FBS) and affinity-purified goat-anti-Brachyury IgG (R&D Systems; 1:100 dilution), mouse anti-GATA4 IgG_1 (clone L97-56; BD Biosciences; 1:200 dilution, San Jose, CA, http://www.bdbiosciences.com), mouse anti-Nestin IgG_{2a} (clone 307501; R&D Systems; 1:200 dilution), and rabbit anti-Ki67 IgG (clone D3B5; Cell Signaling Technology, Danvers, MA; 1:250 dilution). Appropriate dilutions were determined by comparison with undifferentiated ESCs and growth-inhibited mouse embryonic fibroblasts as negative controls. Data analysis was done using FlowJo software. Each condition was tested in three experiments using three independent samples per experiment.

Flow cytometric analysis of phospho-Akt

Nkx2.5-eGFP ESCs were differentiated as described previously.[11] EBs were collected at day 4. Next, the cells were dissociated, serum-starved for 2 hours, and exposed to growth factors for 15 minutes at 37°C in a humidified 5% CO_2 atmosphere. Cells were immediately fixed and stained with rabbit anti-phospho-Akt (Thr308) IgG (clone C31E5E; Cell Signaling Technology, diluted as specified by the manufacturer) as described previously.[22] The presence of phospho-Akt was determined using a FACSCalibur flow cytometer and FlowJo software for data analysis.

Small-molecule inhibition studies

Validated small-molecule inhibitors of downstream IGF signaling pathway targets were added to Nkx2.5-eGFP ESCs simultaneously with insulin, IGF1, or IGF2 at day 1 or 2. Cells were analyzed by flow cytometry for eGFP expression as previously described. A dose titration was performed to determine the optimal concentration of each compound. The following small molecules were used: MK-2206 dihydrochloride (Selleckchem, Houston, TX, http://www.selleckchem.

com/), PI 103 hydrochloride (R&D Systems), PP 242 (R&D Systems), and KU-0063794 (Stemgent, Cambridge, MA, https://www.stemgent.com/).

Statistical methods

A two-tailed Student's *t* test was performed using GraphPad Prism software version 6 (GraphPad Software, La Jolla, CA) or Microsoft Excel (Microsoft, Redmond, WA). Results were considered significant at p values <.05.

Results

IGFs and insulin promote cardiac differentiation of murine ESCs

To identify novel signaling pathways involved in early cardiac lineage commitment, murine Nkx2.5-eGFP ESCs were differentiated *in vitro* by leukemia inhibitory factor (LIF) withdrawal and treated with cytokines/signaling molecules on day 3 of differentiation. On day 6 of differentiation, which coincides with the onset of Nkx2.5$^+$ CPC formation,[11] the frequency of eGFP$^+$ cells was quantified by automated flow cytometry (*Figure 1A*). Four of the 44 factors that were screened (i.e., IGF1, IGF2, insulin, and Wnt3a) significantly increased CPC formation (*Figure 1B and C*). Interestingly, treatment with Activin A, BMP2, or BMP4 at day 3 of differentiation decreased CPC formation. The remaining 37 factors showed no significant effect. We validated the effects of the positive hits by demonstrating a dose-dependent increase in eGFP$^+$ cell formation following treatment with IGF1, IGF2, insulin or, as a positive control, Wnt3a, which had been previously implicated in early cardiogenesis (*Figure 1D*).[21] Although FGF2 treatment did not reach statistical significance in our initial screening assay, it showed a clear dose-dependent increase in the number of eGFP$^+$ cells when the timing of the treatment was optimized (*Supporting Information Figure S1*). Also the effect of IGFs and insulin on CPC formation is time/differentiation stage-specific (*Supporting Information Figure S1*). Accordingly, no increase in cell quantity was observed after treatment of FACS-sorted Nkx2.5-eGFP$^+$ cells (CPCs) with IGF/insulin compared to untreated controls (*Supporting Information Figure S2*). Furthermore, a synergistic effect on CPC formation was observed between IGF1, IGF2 or insulin and either Wnt3a or FGF2. No synergy was seen between FGF2 and Wnt3a or between members of the IGF family of ligands (*Supporting Information Figure S3*).

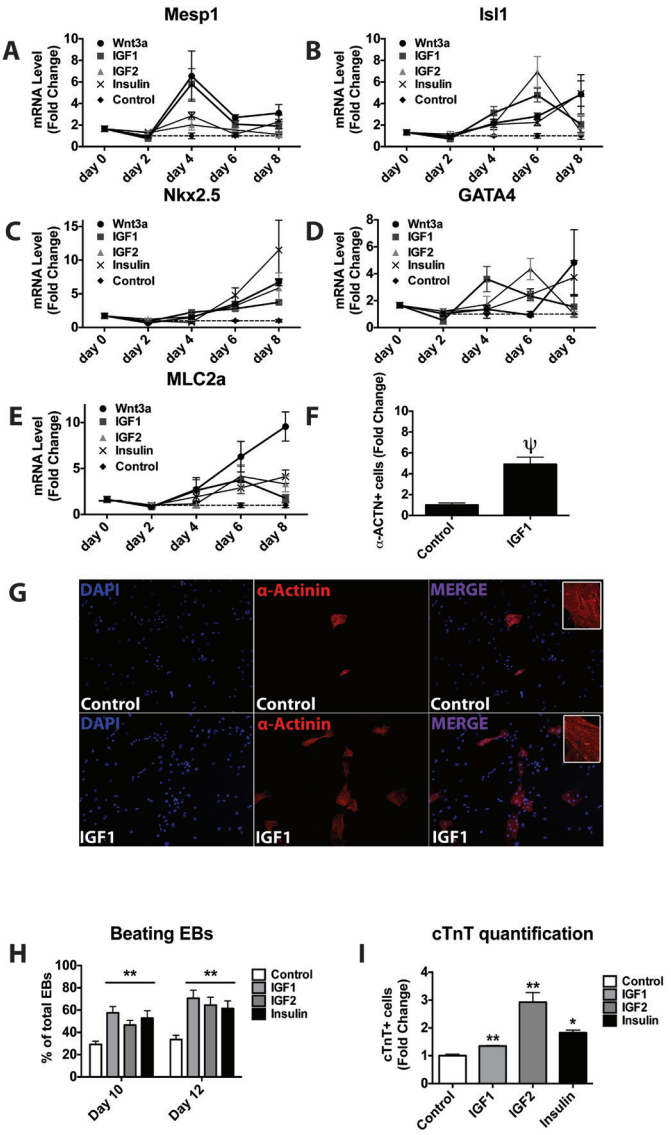

Figure 2. IGF/insulin treatment of murine embryonic stem cells enhances Nkx2.5+ cardiac progenitor cell (CPC) formation. Comparison by quantitative reverse transcriptase polymerase chain reaction of the expression levels of CPC genes in cells treated with 125 µg/mL Wnt3a, 125 µg/mL IGF1, 300 µg/mL IGF2, or 10 mg/mL insulin (*A*) Mesp1 (*B*) Isl1 (*C*) Nkx2.5 (*D*) GATA4, and (*E*) MLC2a. (*F*) Quantification of sarcomeric α-actinin^{+} cells of untreated controls and IGF1-treated cells. (*G*) Representative microscopic images of untreated controls and of IGF1-treated cells (day 12) following immunostaining for sarcomeric α-actinin. DAPI-stained DNA (DAPI; blue), sarcomeric α-actinin (α-Actinin; red). (*H*) Quantification of beating EBs as % of total EBs (days 10 and 12 of differentiation). (*I*) Quantification of cTnT^{+}cells by flow cytometry (day 12). Mean ± SEM of triplicate experiments is shown. NS, nonsignificant; *, $p < .05$; **, $p < .01$; ***, $p < .001$; ψ, $p < .0001$. Abbreviations: cTnT, cardiac troponin T; DAPI, 4′,6-diamidino-2-phenylindole; EBs, embryoid bodies; IGF-1, insulin-like growth factor.

Confirmation of IGF/insulin-mediated increase in CPC formation in differentiating murine ESCs

To validate the ability of IGFs and insulin to enhance cardiogenesis, we measured the expression of cardiac lineage genes in growth factor-treated differentiating cells. Treatment with IGF1, IGF2, or insulin increased the expression of cardiac mesoderm- and CPC-specific genes such as Mesp1, Isl-1, Nkx2.5, GATA4, and MLC2a compared with that in untreated control cells (*Figure 2A–E*). The ability of IGF/insulin-induced CPCs to produce cardiomyocyte-like cells with sarcomeric architecture was demonstrated by immunocytological staining for and sarcomeric α-actinin (*Figure 2F*). Cells treated with IGF1 also were able to differentiate more readily into cardiomyocytes compared to untreated controls (*Figure 2G*). Accordingly, IGF-stimulated cells were able to differentiate into Nkx2.5-eGFP$^+$ cells (*Supporting Information Figure S4*) and showed an increased frequency of beating EBs (*Figure 2H*). These findings were supported by flow cytometric quantification of the number of cTnT$^+$ cells (*Figure 2I*).

IGF/insulin treatment induces mesendoderm formation

To investigate the mechanism involved in the stimulation of CPC formation by IGFs and insulin, we examined the expression of developmental stage- and lineage-specific genes. No significant difference in the expression of pluripotency markers such as Pou5f1 (Oct 4) (*Figure 3A*) and Nanog (*Figure 3B*) was found between control and IGF/insulin-treated cells. This suggests that IGF/insulin treatment does not elicit a greater degree of overall differentiation from a pluripotent state. The expression levels of mesoderm- and endoderm-specific genes such as Eomes, Brachyury, GATA4, Goosecoid, alpha fetal protein, hepatocyte nuclear factor-1b, HNF3b, and SOX17 were all significantly increased by IGF/insulin treatment (*Figure 3C-D*). On the other hand, the expression of ectoderm-specific genes, such as Nestin, GBX2, FGF5, and Pax6 were unchanged or slightly decreased (*Figure 3E*). These results were corroborated by flow cytometric analysis of Brachyury (*Figure 4A-B*) and GATA4 expression (*Figure 4C-D*). Consistently, the number of Nestin$^+$ ectodermal cells decreased after IGF/insulin treatment (*Figure 4E-F*). Taken together, these data support the ability of IGF1, IGF2, and insulin to stimulate the formation of Brachyury$^+$mesoderm or GATA4$^+$ mesendoderm at the expense of ectoderm.

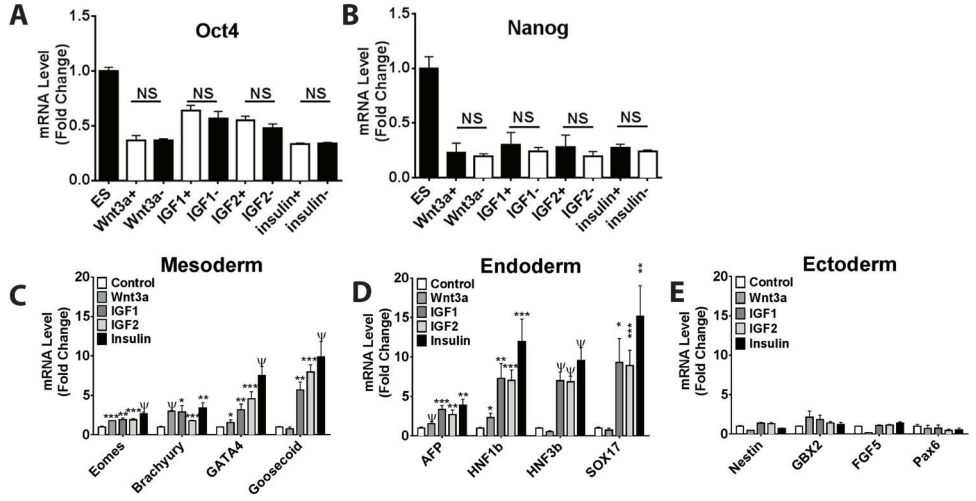

Figure 3. IGF/insulin stimulation of murine ESCs induces expression of mesendodermal genes. Comparison by reverse transcriptase polymerase chain reaction of the expression levels at day 4 of (*A*) Oct4, (*B*) Nanog, (*C*) mesodermal genes, (*D*) endodermal genes, and (*E*) ectodermal genes in untreated cells and in cells exposed to Wnt3a (125 µg/mL), IGF1 (125 µg/mL), IGF2 (300 µg/mL), or insulin (10 mg/mL). Mean ± SEM of triplicate experiments is shown. NS, nonsignificant; *, $p < .05$; **, $p < .01$; ***, $p < .001$; ψ, $p < .0001$. Abbreviations: ESC, embryonic stem cell; IGF, insulin-like growth factor.

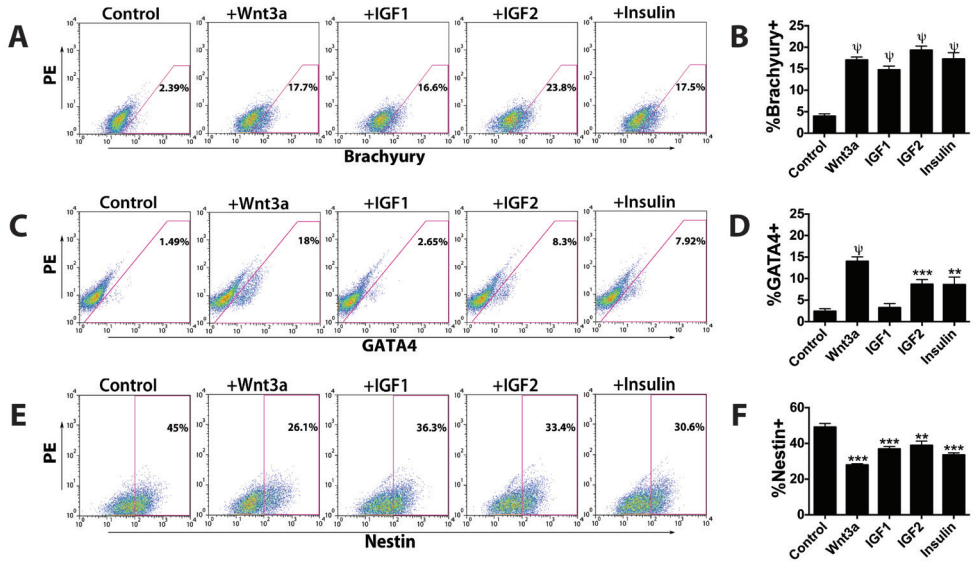

Figure 4. IGF/insulin treatment of murine embryonic stem cells gives rise to early mesodermal cells. Flow cytometric analysis of the expression at day 4 of the germ layer markers (*A*, *B*) Brachyury, (*C*, *D*) GATA4, and (*E*, *F*) Nestin in untreated cells and in cells exposed to Wnt3a (125 µg/mL), IGF1 (125 µg/mL), IGF2 (300 µg/mL), or insulin (10 mg/mL). Mean ± SEM of triplicate experiments is shown. **, $p < .01$; ***, $p < .001$; ψ, $p < .0001$. Abbreviations: IGF, insulin-like growth factor; PE, phycoerythrin.

IGFs and insulin stimulate proliferation of Brachyury$^+$ mesodermal cells

Since IGFs have been implicated in cardiomyocyte proliferation,[23-26] we studied the ability of IGFs and insulin to induce proliferation within the mesodermal Brachyury$^+$ cell population. Coimmunostaining for Brachyury and the proliferation marker Ki-67 revealed a significant increase in the percentage of Brachyury$^+$/Ki-67$^+$-double-positive cells after IGF1/2 or insulin treatment, but not after Wnt3a or FGF2 treatment (Brachyury+/Ki-67+: 22.6±4.4%) (*Figure 5A-B, E-F*). Interestingly, there was no significant increase in Ki-67 staining within the Brachyury-negative cell population after IGF/insulin treatment (*Figure 5C-D*). These data demonstrate that IGFs and insulin selectively promote mesodermal cell proliferation.

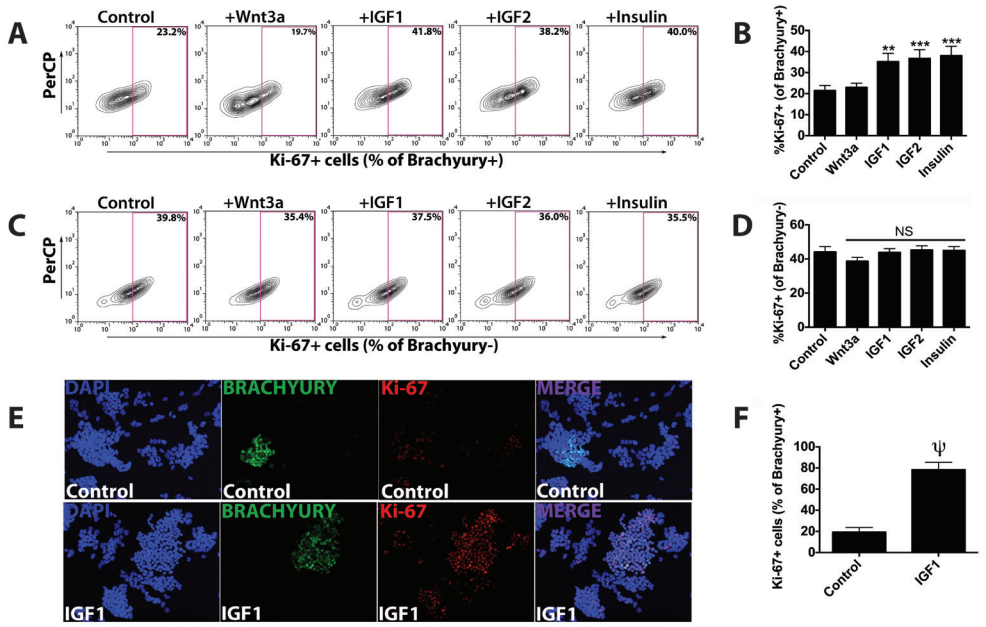

Figure 5. IGF/insulin treatment selectively increases proliferation within the Brachyury$^+$ cell population during murine embryonic stem cell differentiation. Flow cytometry analysis of the expression at day 4 of the proliferation marker Ki-67 in Brachyury$^+$ or Brachyury$^-$ cells in untreated controls and in cells exposed to Wnt3a (125 µg/mL), IGF1 (125 µg/mL), IGF2 (300 µg/mL), or insulin (10 mg/mL). (*A*, *B*) Ki-67 expression in Brachyury$^+$ cells. (*C*, *D*) Ki-67 expression in Brachyury$^-$ cells. Mean ± SEM of triplicate experiments is shown. NS, nonsignificant; **, $p < .01$; ***; $p < .001$. (*E*) Representative microscopy images (×200) of untreated controls and IGF1-treated cells after immunostaining for DAPI (blue), Brachyury (green), Ki-67 (red). (*F*) Quantification of Ki-67$^+$ cells in Brachyury$^+$ cells of untreated controls and IGF1-treated cells. Abbreviation: IGF, insulin-like growth factor.

IGF/insulin treatment causes rapid Akt phosphorylation in Brachyury$^+$ mesodermal cells

It has been established that Akt phosphorylation is one of the mediators of IGF signaling.[27] To examine whether Akt phosphorylation in mesodermal cells is involved in IGF/insulin-mediated induction of CPC formation, differentiating Nkx2.5-eGFP ESCs were treated with IGF1, IGF2 or insulin and subjected to flow cytometric analysis to investigate the occurrence of Akt phosphorylation in Brachyury$^+$ cells. We found a significant increase in the percentage of Brachyury$^+$/phospho-Akt$^+$ cells following IGF/insulin treatment (*Figure 6A-D*). The effect of IGFs and insulin on Akt phosphorylation appears specific since treatment with FGF2 or Wnt3a showed no such effect (*Supporting Information Figure S5*). These data indicate that IGFs and insulin indeed signal through downstream Akt phosphorylation in the Brachyury$^+$ cell population.

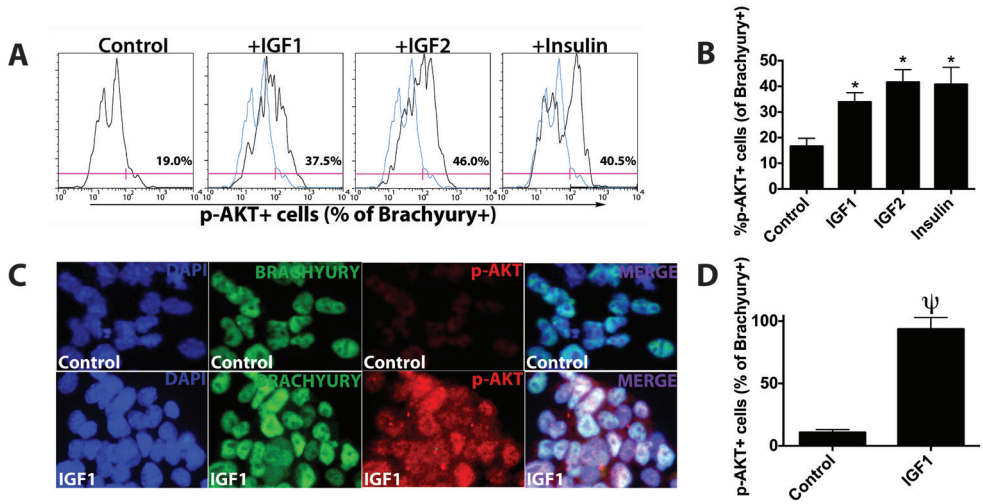

Figure 6. IGF/insulin treatment promotes Akt phosphorylation in murine embryonic stem cell (ESC)-derived Brachyury$^+$ cells. (*A*) Flow cytometric analysis of p-AKT expression in Brachyury$^+$ cells following treatment of Nkx2.5-eGFP ESCs with IGF1 (125 μg/mL), IGF2 (300 μg/mL), or insulin (10 mg/mL) and untreated control Nkx2.5-eGFP ESCs. (*B*) Quantification of p-AKT expression in Brachyury$^+$ cells. Mean ± SEM of triplicate experiments is shown. *, $p < .05$; ψ, $p < .0001$. (*C*) Representative microscopic images (×1,000) of untreated controls and IGF1-treated cells after immunostaining for DAPI (blue), Brachyury (green), and p-AKT (red). (*D*) Quantification of p-AKT$^+$cells in Brachyury$^+$ cells of untreated controls and IGF1-treated cells. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; IGF, insulin-like growth factor; p-Akt, phospho-AKT.

Akt and mTOR signaling are required for IGF/insulin-mediated expansion of the CPC pool

To further examine downstream mediators of IGF signaling responsible for its enhancement of CPC formation, differentiating Nkx2.5-eGFP ESCs were coincubated with IGF1, IGF2, or insulin and selective small-molecule inhibitors of PI3K, Akt, and mTOR. IGF/insulin-induced formation of eGFP$^+$ CPCs was dose-dependently reduced when differentiating Nkx2.5-eGFP ESCs were

treated with specific inhibitors of phosphoinositol-3 kinase (PI3K) (PI 103), Akt (MK-2206), and mammalian target of rapamycin (mTOR) (KU-0063794 and PP 242) (*Figure 7A-D*). Furthermore, after treatment of IGF/insulin-stimulated Nkx2.5-eGFP ESCs with MK-2206, the frequency of Brachyury$^+$/Ki-67$^+$-double-positive cells was significantly lower compared to mock-treated cells (*Supporting Information Figure S6*). These data demonstrate that IGF signaling through PI3K, Akt, and mTOR enhances proliferation of mesodermal cells resulting, ultimately, in increased CPC formation.

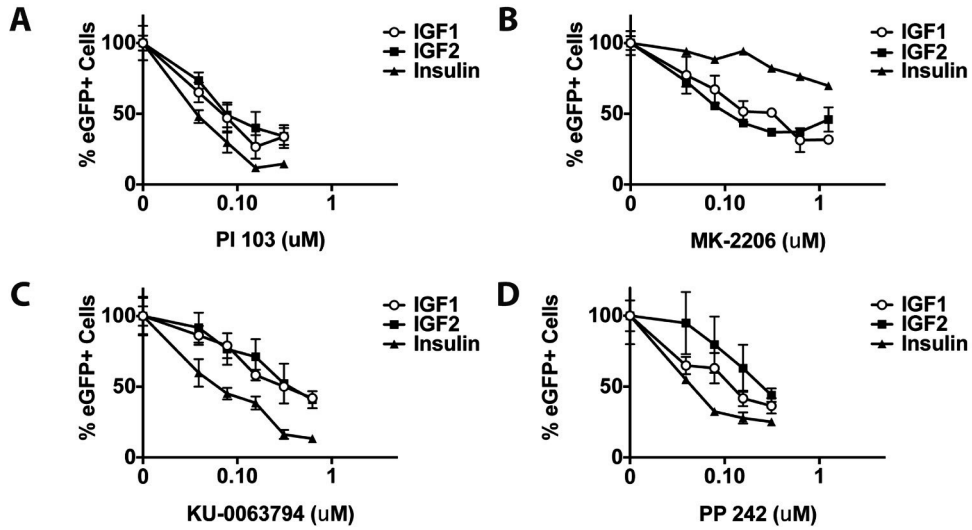

Figure 7. Selective inhibition of PI3K, Akt, or mTOR abolishes IGF-induced cardiac differentiation of murine embryonic stem cells (ESCs). Flow cytometric analysis of eGFP expression in IGF1- (125 µg/mL), IGF2- (125 µg/mL), or insulin (10 mg/mL)-treated Nkx2.5-eGFP ESCs in the presence of selective small molecule inhibitors of the PI3K/Akt/mTOR signaling pathway. Dose responses were determined for (*A*) PI 103 (inhibitor of PI3K), (*B*) MK-2206 (inhibitor of Akt), (*C*) KU-0063794 (inhibitor of mTOR), and (*D*) PP 242 (inhibitor of mTOR). Abbreviations: eGFP, enchanced green fluorescent protein; IGF, insulin-like growth factor.

Discussion

In this study, we performed a growth factor screen to identify mediators of early mesoderm induction that can give rise to cardiac lineage induction. The key findings of this study are: (a) IGF1, IGF2, insulin, Wnt3a, and FGF2 treatment of murine ESCs significantly increases Nkx2.5$^+$ CPC formation, (b) IGF and insulin selectively promote Brachyury$^+$ mesodermal cell proliferation, (c) IGF treatment leads to an increase in Akt phosphorylation within the Brachyury$^+$ cell population, and (d) activation of PI3K, Akt, and mTOR signaling is required for

the ability of IGFs and insulin to enhance CPC formation. These data demonstrate a role for IGFs and insulin in early germ layer development *in vitro*, by their ability to selectively enhance the proliferation of mesodermal cells, leading to increased cardiac lineage differentiation.

Signaling pathways involved in cardiac differentiation

While our screen identified a novel role for IGFs and insulin to regulate early *in vitro* cardiogenesis, Activin A, BMP2, and BMP4 treatment at this stage of development inhibited cardiac differentiation. The latter ligands have been shown to play a context-dependent role in cardiac development in previous studies.[28-30] For example, a recent small-molecule screening study revealed the ability of a BMP inhibitor, dorsomorphin, to activate cardiogenic differentiation.[30] Other studies have shown both a dose- and stage-specific requirement for BMP signaling to enhance cardiac differentiation.[5] This context-dependent effect of BMP treatment has been previously reported.[31] Consistent with this, the addition of Activin A or BMPs alone to differentiating ESC cultures has been shown to preferentially induce endodermal lineage development while combined Activin A and BMP treatment was shown to induce mesodermal cardiac lineage formation.[32]

Role of IGF/insulin in early cardiomyogenesis

Some studies have revealed a negative modulatory effect of IGF and insulin expression on cardiomyocyte differentiation in human ESCs.[33-35] Similarly, a recent study found differential effects of FGF signaling pathway activation in mouse and human ESCs.[36] The apparent discrepancy between these and our findings are most likely due to differences in the developmental stage in which IGF/insulin was added to cells. In this study, we examined the effect of IGF/insulin on mesodermal cells, whereas prior human ESC studies examined the effect of IGF/insulin on cardiomyocyte differentiation from postmesodermal cells. It has been well-recognized that developmental regulators such as Wnt and Notch exert a biphasic role on cardiac lineage differentiation. We believe this is likely to be the case for IGF/insulin as well. Indeed, our preliminary data showed that treatment of mouse CPCs with IGF results in decreased cardiomyocyte differentiation. This suggests that our findings in mouse ESC here are largely consistent with those from prior human ESC studies.

Our data showed that IGF signaling results in selective expansion of Brachyury$^+$ mesodermal cells through induction of proliferation. As such, the mechanism by which IGFs and insulin stimulate CPC formation appears different from that of Wnt3a or FGF2, which showed no obvious proliferation rate changes in Brachyury$^+$ mesodermal cells (*Figure 5*). Furthermore, combining either Wnt3a or FGF2 with IGF/insulin resulted in additive increases in Nkx2.5-eGFP$^+$ cells, which are no greater than the increases when each factor was added singularly

(*Supporting Information Figure S3*). These data further support that IGF and insulin act through a different downstream signaling pathway than FGF2 or Wnt3a.

In support of our findings, Morali et al. found that IGF2$^{-/-}$ ESC lines exhibit an impaired ability to differentiate into cardiogenic and myogenic lineages when differentiated *in vitro*. This phenotype could be partially rescued when differentiating cells were treated with exogenous IGF2.[37] A more recent study describes the ability of IGF to increase ESC-derived vascular cells as well, a finding that may also be related to the upregulation of Brachyury^{+} mesodermal cells.[38] Additionally, IGF and insulin treatment showed a marked induction of endodermal markers (*Figure 3D*), suggesting this pathway could also be active during endodermal lineage formation. A recent study showed IGF treatment directed hepatocyte differentiation from definitive endoderm.[39]

Conclusion

To our knowledge, this is the first study demonstrating a direct role for IGFs and insulin to promote the *in vitro* differentiation of PSCs into mesoderm. We showed that IGF/insulin treatment of murine ESCs selectively expands the Brachyury^{+} mesodermal cell pool via activation of PI3K, Akt, and mTOR, leading to increased cell proliferation. These findings contribute to our understanding of early developmental events and provide a new strategy to enhance directed cardiac differentiation for regenerative applications.

Acknowledgements

Laura Prickett-Rice and Katherine Folz-Donahue at the Center for Regenerative Medicine Harvard Stem Cell Institute Flow Cytometry Core provided assistance with flow cytometry experiments. Andrew Olson at the Stanford Neuroscience Microscopy Service (supported by NIH NS069375) at Stanford School of Medicine provided technical assistance with confocal microscopy imaging. M.C.E. was supported by a Mosaic grant from the Netherlands Organization for Scientific Research (NWO 017.007.064). D.A.P. was supported by NWO (VENI grant 91611070) and S.M.W. was supported by NIH/Officer of the Director's New Innovator Award (OD004411), NIH/NHLBI U01 (HL099776) and K08 (HL081086) and by the Harvard Stem Cell Institute.

Author Contributions

M.C.E.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript; K.R., R.F., and A.S.: collection and/or assembly of data, data analysis and interpretation, and final approval of manuscript; U.B.N.: provision of study material, financial support, and final approval of

manuscript; M.J.S.: financial support, data analysis and interpretation, and final approval of manuscript; A.A.F.V.: data analysis and interpretation, manuscript writing, and final approval of manuscript; D.A.P.: conception and design, data analysis and interpretation, manuscript writing, and final approval of manuscript; S.M.W.: conception and design, financial support, data analysis and interpretation, manuscript writing, and final approval of manuscript.

Disclosure of potential conflicts of interest

U.B.N. is the chief executive officer of Silver Creek Pharmaceuticals, Inc.

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Supporting information

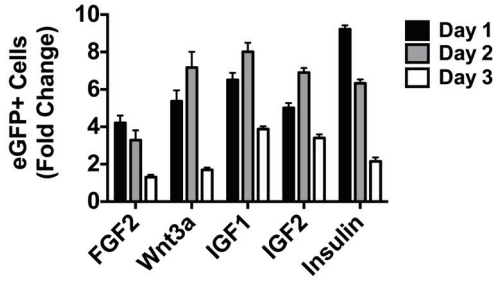

Figure S1. Flow cytometric assessment of the optimal timing of IGF/insulin stimulation-induced formation of CPCs from murine ESCs. The growth factors were added on the indicated day after LIF removal. Bars represent fold change in eGFP+ cell count by growth factor treatment. Mean ± SEM of triplicate experiments is shown.

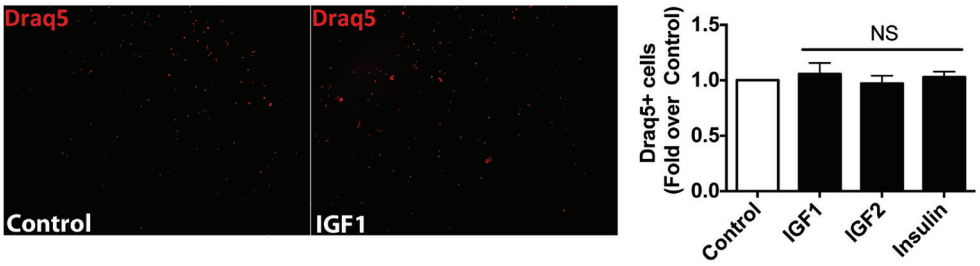

Figure S2. Representative microscopic images of Draq5-labeled untreated controls and IGF1-treated cells (day 11) after fluorescence-activated cell sorting of Nkx2.5-eGFP+ cells at day 6. Quantification of Draq5+ cells after treatment with IGF1 (125 µg/mL), IGF2 (300 µg/mL), insulin (10mg/mL) or untreated controls of sorted Nkx2.5-eGFP+ cells. NS, not significant.

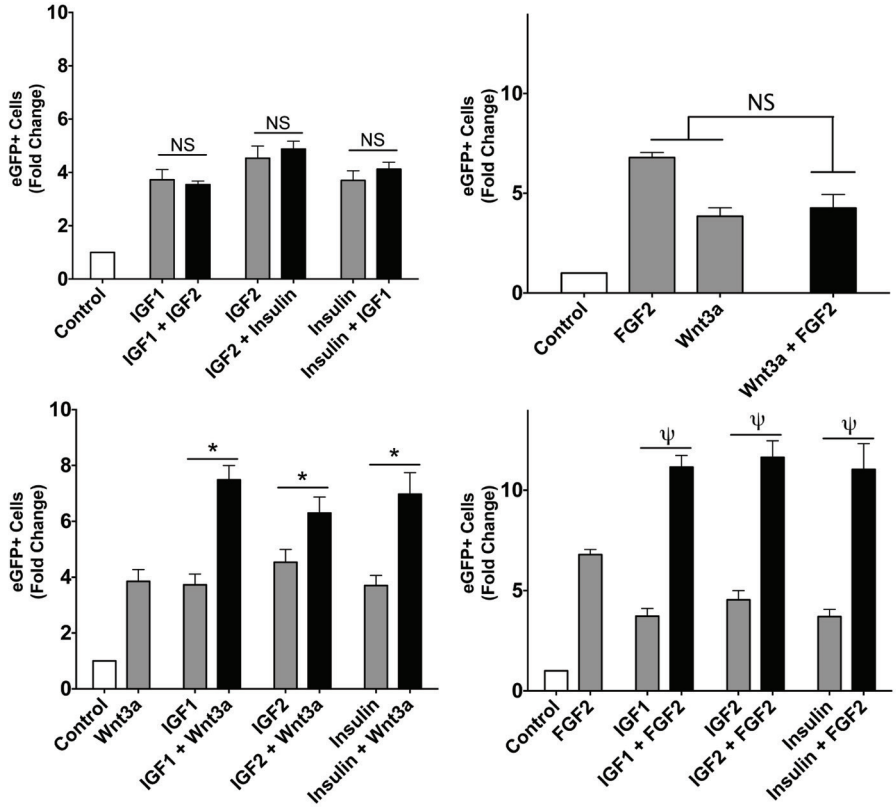

Figure S3. IGFs and insulin synergize with Wnt3a and FGF2 in stimulating the formation of CPCs from murine ESCs. Nkx2.5-eGFP ESCs were exposed to IGF1 (125 µg/mL), IGF2 (300 µg/mL), insulin (10 mg/mL), FGF2 (200 µg/mL) or Wnt3a (125 µg/mL) alone or in pairs as indicated below the graphs and the resulting number of eGFP+ CPCs was determined by flow cytometry. Bars represent mean of SEM in triplicate experiments. NS, non-significant; *, $p<0.05$; ψ, $p<0.0001$.

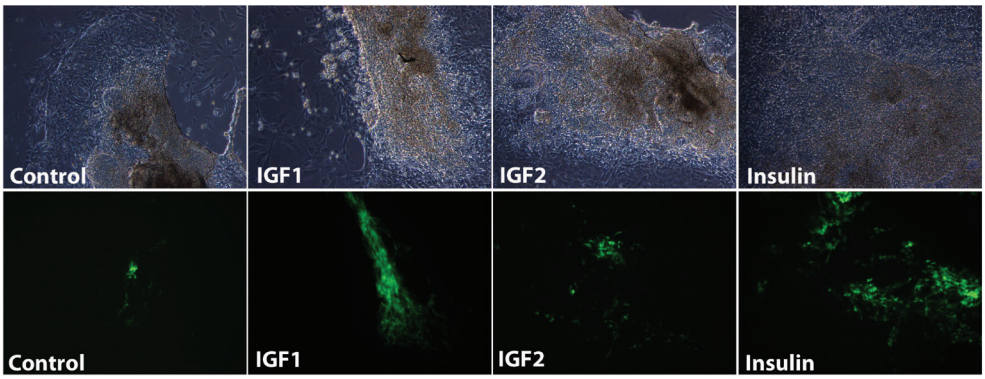

Figure S4. Representative phase contrast photomicroscopic images of differentiating murine Nkx2.5-eGFP ESCs as untreated control or following treatment with IGF1, IGF2 or insulin.

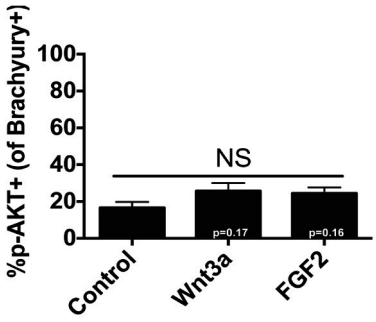

Figure S5. Treatment of Nkx2.5-eGFP ESCs with Wnt3a (125 µg/mL) or FGF2 (200 µg/mL) does not increase Akt phosphorylation in Brachyury+ cells. Flow cytometric analysis of phospho-AKT (pAkt) expression in Brachyury+ cells following treatment of Nkx2.5-eGFP ESCs with Wnt3a or FGF2. Control, untreated Nkx2.5-eGFP ESCs. Bars represent mean ± SEM of triplicate experiments. NS, non-significant.

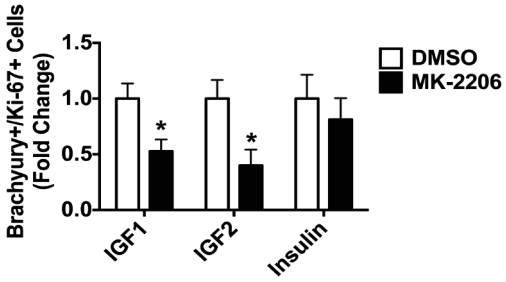

Figure S6. Selective inhibition of Akt signaling abolishes IGF/insulin-dependent proliferation of murine ESC-derived Brachyury+ cells. Flow cytometric quantification of Ki67 expression in Brachyury+ cells following treatment of 33 differentiating murine ESCs with IGF1, IGF2 or insulin in the presence of the Akt inhibitor ML-2206 or its solvent, DMSO. Mean ± SEM of triplicate experiments is shown. *, $p<0.05$.

Supplementary Table 1. Cytokines for screening assay.

Cytokine	Concentration Range (µg/mL)
Wnt3a	4-1000
IGF1	4-1000
IGF2	4-1000
Insulin	4-1000
FGF2	4-1000
LIF	4-1000
Wnt7a	4-1000
Wnt11	4-1000
SDF-1α	4-1000
TGFα	4-1000
MCP1	4-1000
G-CSF	4-1000
IL-3	4-1000
IL-6	4-1000
EPO	4-1000
TNFα	4-1000
NRG EGF domain	4-1000
TGFβ1	4-1000
TGFβ2	4-1000
Betacellulin	4-1000
Wnt1	4-1000
Wnt2	4-1000
EGF	4-1000
FGF4	4-1000
HGF	4-1000
PDGF-AA	4-1000
PDGF-AB	4-1000
PDGF-BB	4-1000
SCF	4-1000
VEGF-A 121	4-1000
VEGF-A 165	4-1000
VEGF-B	4-1000
Thymosinβ-4	4-1000
GLP-1	4-1000
Exenatide	4-1000
VEGF	4-1000
FGF10	4-1000
DKK-1	4-1000
A83	4-1000
Dorsomorphin	4-1000
Carbonoxolone	4-1000
BMP4	4-1000
Activin A	4-1000
BMP2	4-1000

Supplementary Table 2. Primers used for RT-qPCR.

Target Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')
β-actin	GAAGGAGATTACTGCTCTGGCTCCTAGCAC	TCATCGTACTCCTGCTTGCTGATCC
Mesp1	GCTCGGTCCCCGTTTAAGC	ACGATGGGTCCCACGATTCT
Isl-1	ATGATGGTGGTTTACAGGCTAAC	TCGATGCTACTTCACTGCCAG
Nkx2.5	ACAAAGCCGAGACGGATGG	CTGTCGCTTGCACTTGTAGC
GATA4	CACCCCAATCTCGATATGTTTGA	GCACAGGTAGTGTCCCGTC
MLC2a	AAGGGAAGGGTCCCATCAACTTCA	AACAGTTGCTCTACCTCAGCAGGA
Oct4	CTGAGGGCCAGGCAGGAGCACGAG	CTGTAGGGAGGGCTTCGGGCACTT
Nanog	TCTTCCTGGTCCCCACAGTTT	GCAAGAATAGTTCTCGGGATGAA
Eomes	GCAATAAGATGTACGTTCACCCA	GCAGAGACTGCAACACTATCAT
Brachyury	GCTGGATTACATGGTCCCAAG	GGCACTTCAGAAATCGGAGGG
GATA4	CACCCCAATCTCGATATGTTTGA	GCACAGGTAGTGTCCCGTC
Goosecoid	CAGATGCTGCCCTACATGAAC	TCTGGGTACTTCGTCTCCTGG
AFP	CCATCACCTTTACCCAGTTTGT	CCCATCGCCAGAGTTTTTCTT
HNF1b	AGGGAGGTGGTCGATGTCA	TCTGGACTGTCTGGTTGAACT
HNF3b	GGAGGCAAGAAGACCGCTC	CCTTTAGCTCGCTTAGGCCAC
SOX17	GATGCGGGATACGCCAGTG	CCACCTCGCCTTTCACCTTTA
Nestin	CCCTGAAGTCGAGGAGCTG	CTGCTGCACCTCTAAGCGA
GBX2	CAACTTCGACAAAGCCGAGG	ACTCGTCTTTCCCTTGCCCT
FGF5	TGTGTCTCAGGGGATTGTAGG	AGCTGTTTTCTTGGAATCTCTCC
Pax6	TACCAGTGTCTACCAGCCAAT	TGCACGAGTATGAGGAGGTCT