Abi3bp Regulates Cardiac Progenitor Cell Proliferation and Differentiation

Conrad P. Hodgkinson, Jose A. Gomez, Alan J. Payne, Lunan Zhang, Xiaowen Wang, Sophie Dal-Pra, Richard E. Pratt, Victor J. Dzau

Rationale: Cardiac progenitor cells (CPCs) are thought to differentiate into the major cell types of the heart: cardiomyocytes, smooth muscle cells, and endothelial cells. We have recently identified ABI family, member 3 (NESH) binding protein (Abi3bp) as a protein important for mesenchymal stem cell biology. Because CPCs share several characteristics with mesenchymal stem cells, we hypothesized that Abi3bp would similarly affect CPC differentiation and proliferation.

Objective: To determine whether Abi3bp regulates CPC proliferation and differentiation.

Methods and Results: In vivo, genetic ablation of the Abi3bp gene inhibited CPC differentiation, whereas CPC number and proliferative capacity were increased. This correlated with adverse recovery after myocardial infarction. In vitro, CPCs, either isolated from Abi3bp knockout mice or expressing an Abi3bp shRNA construct, displayed a higher proliferative capacity and, under differentiating conditions, reduced expression of both early and late cardiomyocyte markers. Abi3bp controlled CPC differentiation via integrin-β1, protein kinase C-ζ, and v-akt murine thymoma viral oncogene homolog.

Conclusions: We have identified Abi3bp as a protein important for CPC differentiation and proliferation.

Key Words: extracellular matrix ■ integrin-β

Cardiac progenitor cells (CPCs) are thought to give rise to the major cell types of the heart; these being cardiomyocytes, smooth muscle cells, and endothelial cells. V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog (c-Kit+)/Sca-1+ CPCs have been the most heavily studied; however, other proteins such as homeobox protein Nkx-2.5, Wilms tumor 1, Islet-1, T-box 5, and Heart and neural crest derivatives expressed have also been used to define these cells. Endogenous CPCs are present in too low a number to regenerate a damaged heart after injury completely. However, recent reports have highlighted the potential therapeutic benefits of injected CPCs in the human heart after myocardial infarction. Enhancing these beneficial effects requires characterization of the mechanisms by which CPCs differentiate and proliferate.

We have recently discovered that ABI family, member 3 (NESH) binding protein (Abi3bp) promotes mesenchymal stem cell (MSC) differentiation, while simultaneously inhibiting proliferation. MSCs prepared from Abi3bp knockout mice were unable to differentiate into osteocytes and adipocytes. Significant impairment of chondrogenic and smooth muscle differentiation was also observed. Conversely, knockout of Abi3bp increased MSC proliferation, with integrin-β1 and ERK being found to be necessary for the effect. Abi3bp is a protein of relatively unknown function; with roles in the olfactory system and tumorigenesis being ascribed to the protein. Abi3bp was shown in vitro to reduce mitral cell dendritic complexity. This process is important in the developing brain because functional circuits are established by pruning immature connections. Reduced Abi3bp expression has been observed in thyroid tumors. Re-expression of Abi3bp in thyroid cancer cells prevented tumor formation when the cells were injected into nude mice.

C-Kit+ CPCs have been shown to possess mesenchymal markers, suggesting the possibility that Abi3bp may also similarly affect CPC differentiation and proliferation. Indeed, in this study, we demonstrate both in vivo and in vitro that Abi3bp is important for the control of CPC proliferation and differentiation.

Methods

Abi3bp Knockout Mice

Abi3bp mice, harboring a neomycin resistance gene replacement of the first exon, were originally purchased from Taconic. All experiments were performed with wild-type (WT; Abi3bp+/+) and...
Abi3bp knockout (Abi3bp−/−) littermates in accordance with institutional guidelines (Division of Laboratory Animal Resources and Institutional Animal Care & Use Committee).

CPC Isolation

Enzymatic Digestion
c-KIt+ CPCs were isolated from 8-week-old male WT and Abi3bp knockout littermates. Minced ventricular tissue was digested in 100 U of collagenase in Hank Buffered Saline solution at 37°C for 15 minutes. Single cells were then selected by magnetic bead isolation (Miltenyi Biotech, Boston, MA) and further cultured in CPC-maintenance media. Cells were differentiated at passage 3. At this passage, the cells were positive for c-Kit and CD29 (Online Figure IIA). Necrosis was not significantly different between c-Kit+ CPCs derived from WT and Abi3bp knockout mice (Online Figure IB). In CPC-maintenance media, Abi3bp knockout c-KIt+ CPCs expressed significantly lower levels of Abi3bp, myocyte enhancer factor 2C (Me2C), and cardiac troponin-I (cTroponin-I) when compared with WT c-KIt+ CPCs; however, the expression of Gata4 and Gata6 was not significantly different between WT and Abi3bp knockout c-KIt+ CPCs (Online Figure IIB). These data suggested that Abi3bp may be important for the differentiation of CPCs and the maintenance of the differentiated phenotype in cardiomyocytes. To test this hypothesis, we performed differentiation

CPC Differentiation

CPCs were seeded at 25000 cells/cm² in CPC-maintenance media. Twenty-four hours later the media was replaced with CPC-differentiation media (Advanced DMEM/F12, 0.2% w/vol BSA, 2 mmol/L t-glutamine, 1x insulin-transferrin-selenium, 250 μmol/L ascorbic acid). Media was changed every 2 days.

Stable Abi3bp Knockdown and Re-expression

Full Methods is provided in the Online Data Supplement. Significant knockdown of Abi3bp was observed (Online Figure IIIA). Scrambled shRNA had no effect on the basal expression of Abi3bp, Me2C, and Gata4 (Online Figure IIIB). Abi3bp expression levels in WT, Abi3bp knockout expressing cmyc, and Abi3bp knockout CPCs expressing cmyc-Abi3bp are shown in Online Figure IIIC.

Myocardial Infarction (Acute Left Anterior Descending Coronary Artery Ligation)

Anesthetized (ketamine [100 mg/kg] and xylazine [5 mg/kg] by IP injection) 10- to 12-week-old WT or Abi3bp knockout mice were intubated before left thoracotomy. Mice were ventilated at a tidal volume 0.7 to 1 mL, respiratory rate 120 breaths per minute. Chest cavity was opened and a 7-0 nylon suture placed through the myocardium into the anterolateral LV wall, corresponding to the course of the left anterior descending artery. The suture was tied off (myocardial infarction), and apex of the LV observed for evidence of myocardial blanching indicating interruption in coronary flow. The wound was closed and after the resumption of spontaneous respiration the animal was allowed to recover. Postoperative analgesia was used for 5 days. Echocardiographic analysis was performed under minimal iso-flurane anesthesia. The full Methods and procedure for staining is described in the Online Data Supplement.

Cardiac Fibroblast and Cardiomyocyte Isolation

Cardiac fibroblasts and cardiomyocytes were isolated as previously described.16

Images

Figures were prepared using CorelDraw. Microscopy images were exported from Axiovision Rel4.8 software.

Statistics

Statistical analysis was performed with GraphPad or R. Experiments containing 2 conditions a t test was performed. ANOVA was used for experiments with ≥3 conditions followed by Bonferroni post hoc tests for comparisons between individual groups. Mann–Whitney U tests, a nonparametric test that does not rely on assumptions of normality, was used for n=3 data.

Results

To test the hypothesis that Abi3bp is important for CPC biology, we isolated WT and Abi3bp knockout c-KIt+ CPCs from the noncardiomyocyte fraction of the adult mouse heart. The characteristics of these isolated c-KIt+ CPCs, as described in the Methods section of this article, were consistent with resident CPCs reported in the literature.5,17 These cells also expressed integrin-β1/CD29 (Online Figure IA).

The heart contains many cell types, the majority being cardiomyocytes and cardiac fibroblasts. Abi3bp expression was easily detected in cardiomyocytes but was not detected in cardiac fibroblasts and undifferentiated CPCs (Figure 1A). However, after 14-day culture in CPC-differentiation media, expression levels of Abi3bp in differentiated CPCs were comparable with cardiomyocytes (Figure 1A). These data suggested that Abi3bp may be important for the differentiation of CPCs and the maintenance of the differentiated phenotype in cardiomyocytes. To test this hypothesis, we performed differentiation
time-course experiments with WT and Abi3bp knockout CPCs. The expression of cardiac genes was assessed at 0, 3, 8, and 14 days after the addition of CPC-differentiation medium. In WT CPCs, Abi3bp mRNA increased dramatically during differentiation, reaching ≈120-fold by day 14 (Figure 1B). As expected, no Abi3bp expression was observed in c-Kit+ CPCs prepared from Abi3bp knockout mice (Figure 1B). At the protein level, Abi3bp expression increased ≈3-fold after 14 days of culture in differentiation media (Figure 1B).

Markers of lineage commitment were then assessed in differentiating WT and Abi3bp KO CPCs. mRNAs encoding Gata4, Mef2C, homeobox protein Nkx-2.5, Gata6, and v-ets avian erythroblastosis virus E26 oncogene homolog 1 are present in the early stages of CPC differentiation, whereas cardiac troponins and α-myosin heavy chain (αMHC) mRNA are observed in the later stages of CPC differentiation toward a cardiomyocyte fate. In WT c-Kit+ CPCs, mRNA encoding the early markers Gata4, Mef2C, and Gata6 increased during...
transiently transfected with a vector encoding Abi3bp. WT c-Kit+ CPCs were used as a positive control. Gata4, Gata6, Mef2C, and Tnni3 mRNA levels were significantly increased after transient re-expression of Abi3bp in Abi3bp knockout cells (Figure 2C). Interestingly, re-expression of Abi3bp in Abi3bp knockout c-Kit+ CPCs was sufficient to recover the phenotype. Gata4, Gata6, Mef2C, and Tnni3 expressions were identical in WT and Abi3bp knockout c-Kit+ CPCs transiently transfected with the Abi3bp vector (Figure 2C). Similarly, re-expression of Abi3bp in knockout CPCs increased Gata4, cardiac troponin-I, and αMHC protein levels at both baseline and after differentiation (Figure 2D).

To investigate whether Abi3bp affected CPC differentiation in vivo, we subjected WT and Abi3bp knockout mice to myocardial injury (MI). Abi3bp was found to be expressed in the normal and injured heart at similar levels (Figure 3A). Masson Trichrome staining was performed to assess fibrosis. One week after MI, fibrosis levels were similar between WT and Abi3bp knockout (Figure 3B). However 1 month after MI, fibrosis levels were significantly higher in the Abi3bp knockout animals (Figure 3C). Interestingly, at 1 month after MI, cardiac tissue from injured Abi3bp knockout mice showed more fragile than that from the WT mice. After embedding into paraffin, ≈90% of the sections from Abi3bp knockout hearts showed breakage in the injured area as opposed to only ≈10% in sections from WT hearts (Figure 3C).

We next examined cardiac function by echocardiography. Knockout of the Abi3bp gene was associated with lower ejection fraction and fractional shortening 1-month after MI (Figure 3D; Online Table I for preinjury, sham, and MI echocardiographic data).

We used immunostaining to assess c-Kit+ CPC number and differentiation in the sham and MI hearts 7 days after injury. Sequential sections through the entire infarct region of the heart were assayed for c-Kit and Gata4 expression. This allowed an assessment of the total number of c-Kit+ and Gata4+ cells in the peri-infarct region of each animal (Figure 4A; larger sections can be found in Online Figure V). Cardiac injury increased the number of c-Kit+ cells in both WT and Abi3bp knockout mice (Figure 4B). The number of c-Kit+ cells were significantly higher in Abi3bp knockout mice in both sham and MI animals (Figure 4B). Cardiac injury was associated with an increase in the percentage of double-positive (c-Kit+/Gata4+) cells in WT mice (Figure 4B). In contrast, MI had no effect on the percentage of c-Kit+/Gata4+ cells in Abi3bp knockout mice (Figure 4B), suggesting that despite the increase in the numbers of c-Kit+ cells in these animals lineage commitment was being inhibited. The immunostaining results were verified by flow cytometry. The numbers of c-Kit+ cells was found to increase with injury in both WT and Abi3bp knockout mice; with significantly higher numbers observed in Abi3bp knockout animals (Figure 4C). Similarly, an increase in the percentage of double-positive (c-Kit+/Gata4+) cells after MI was only observed in WT animals (Figure 4C). Cardiomyocyte proliferation in the border zone, 1 week after injury, was not significantly different between WT and Abi3bp knockout mice (Figure 4D).
Taken together, the above data indicated that Abi3bp is important for CPC cardiac differentiation.

We have previously shown that integrin-β1 functions as the Abi3bp receptor.11 Moreover, c-Kit+ CPCs prepared by enzymatic dissociation and those prepared by explant, were found to express integrin-β1. Furthermore, c-Kit+ cells in vivo were found to express integrin-β1 (Online Figure VI). To examine the potential role of integrin-β1, isotype-control, or integrin-β1 blocking antibodies were added to WT c-Kit+ CPCs cultured under differentiation conditions. Gata4 and Gata6 expression were evaluated by quantitative polymerase chain reaction and compared with WT c-Kit+ CPCs cultured in CPC-differentiation media in the absence of antibody. The isotype control antibody had no effect on CPC differentiation; Gata4 and Gata6 expression levels were comparable with cells differentiated in the absence of antibody (Figure 5A). In contrast, addition of the integrin-β1 blocking antibody markedly inhibited Gata4 and Gata6 expression (Figure 5A), indicating that the effects on CPC differentiation were mediated by integrin-β1. This experiment was also performed with Abi3bp knockout c-Kit+ CPCs transiently overexpressing either a control or Abi3bp expression plasmid. Isotype or integrin-β1 blocking antibodies were added to CPCs cultured under differentiation conditions and Gata4 expression evaluated by quantitative polymerase chain reaction. Integrin-β1 blocking antibodies completely abrogated the positive effects of re-expression of Abi3bp (Figure 5B). Gata4 expression was not significantly different to cells expressing the control plasmid (Figure 5B). Phosphorylation of focal adhesion kinase (FAK) is a key event after integrin activation. As expected, phosphorylation of FAK levels were markedly lower in Abi3bp knockout c-Kit+ cells (Figure 5C). Furthermore, re-expression of Abi3bp in Abi3bp knockout c-Kit+ CPCs...
increased FAK phosphorylation (Figure 5D). No significant differences in total FAK were observed.

Having ascertained that integrin-β1 was important for the effects of Abi3bp on CPC differentiation, we identified the signaling proteins involved. WT and Abi3bp knockout c-KiT+ CPCs were cultured for 14 days in CPC-differentiation media. Protein extracts from these cells were subsequently immunoblotted for protein kinase C (PKC) -ζ and v-akt murine thymoma viral oncogene homolog (Akt). Abi3bp knockout had no effect on the levels of total PKC -ζ and Akt in c-Kit+ CPCs (Figure 5E, with quantification shown in Online Figure VIIB). In contrast to WT c-KiT+ CPCs, expressing the Abi3bp shRNA construct were made to express either a scrambled control or Abi3bp shRNA stably. The concentrations used for the pharmacological inhibitors were effective at inhibiting their respective kinases (Online Figure VIIIC). Pharmacological inhibition of Akt significantly attenuated the increase in Mef2C, Gata4, and Gata6 mRNA levels and cardiac troponin-T protein expression observed during WT CPC differentiation (Figure 5G, FACS traces Online Figure VIID). The vehicle had no effect on cardiac gene expression (Figure 5G). The pharmacological inhibition of PKCζ was similar to that of Akt; increases in Mef2C, Gata6, and cardiac troponin-T expression that occur during WT CPC differentiation were significantly reduced by the inhibitor (Figure 5H, FACS traces Online Figure VIID). However, no effect was observed with Gata4 (Figure 5H), suggesting that the 2 kinases control different pathways.

We hypothesized, based on our previous studies, that Abi3bp would affect CPC proliferation. WT and Abi3bp knockout c-KiT+ CPCs were seeded at the same density, cultured for 3 days, and manually counted on a daily basis. Re-expression of Abi3bp in Abi3bp knockout (KO) is associated with lower cardiac function and increased fibrosis after myocardial injury (MI). A, Protein extracts (50 μg) from sham and infarct hearts were immunoblotted with antibodies to the indicated proteins. β-tubulin was used as a loading control. Intensities were normalized to the loading control; normalized intensity of sham animals was taken to be 1. n=3. Serial sections from sham and MI mice were stained with Masson trichrome. Fibrosis area is represented as a percentage of the left ventricle. B, One week after infarct and (C) 1 month after infarct. n=4 to 6 per group. Significance between MI groups is shown. **P<0.01. D, One month after injury echocardiography was performed. Left and center, Ejection fraction and fractional shortening shown for wild-type (WT) and Abi3bp KO mice subjected to MI. P values indicated. n=11 for WT, n=6 for Abi3bp KO. Right, Sham ejection fraction and fractional shortening values (n=5 per group). Other parameters for preoperative, MI, and sham animals are shown in Online Table I.
of bromodeoxyuridine-positive CPCs indicating a greater proportion of cells in S-phase (Figure 6D). Knockdown and knockdown of Abi3bp removed contact inhibition; the exponential growth curves of Abi3bp knockout and knockdown CPCs continued beyond the time-frame of the experiment (data not shown).

Additional experiments were performed with c-Kit+ CPCs prepared from cardiac biopsies. In culture, Abi3bp knockout c-Kit+ CPC number was observed to be significantly higher 1, 2, 3, and 4 days after seeding when compared with WT c-Kit+ CPCs (Online Figure VIIIIA) with a doubling time of 0.95 versus 1.3 days for the WT cells. Similarly, knockout of the Abi3bp gene increased bromodeoxyuridine incorporation in c-Kit+ CPCs (Online Figure VIIIB).

We then assessed the c-Kit+ CPC proliferation in WT and Abi3bp knockout mice. Consequently, we measured bromodeoxyuridine uptake in WT and Abi3bp knockout mice. Abi3bp knockout increased the proliferation of both c-Kit+ positive hematopoietic lineage-negative CPCs, as shown by the a10-fold increase in bromodeoxyuridine incorporation (Figure 6E).

**Discussion**

In this study, we show that Abi3bp regulates critical aspects of CPC biology, such as differentiation and proliferation.

After injury to the heart, the extracellular matrix (ECM) undergoes several changes that affect cardiac function. The ECM has been shown to affect the behavior of certain types of stem cells, such as MSCs. ECM stiffness and composition have a strong effect on MSC differentiation. ECM cross-linking proteoglycans of the heparan sulfate and chondroitin sulfate families are likely to be important in the lineage specification of MSCs, especially as they help to control matrix stiffness. In contrast, less is known about how the ECM regulates CPC behavior. ECM components, such as fibronectin, laminin, and vitronectin have been shown to augment Sca-1+ and Flk-1+ CPC proliferation. In our study, we show that Abi3bp, an ECM protein, is important for promoting CPC differentiation. Our study also suggests that like MSCs, ECM stiffness may have a role to play in CPC proliferation and differentiation. Abi3bp is itself a proteoglycan, and we previously found that the loss of the protein increased tensile stresses on MSCs.

We found that Abi3bp promotes CPC differentiation via integrin-β1, Akt, and PKCζ. To our knowledge, this is the first report linking these proteins to CPC differentiation. Akt helps to mediate IGF protein stimulation of embryonic stem cell differentiation to homeobox protein Nkx-2.5+ CPCs, which agrees with our finding that Akt is important for CPC differentiation. The mechanisms involved in CPC proliferation have been more heavily characterized than those governing CPC differentiation. Activated β2-adrenergic receptors promote CPC proliferation, in part, through Akt. Our results suggest that other pathways exist that are important for CPC proliferation because in our model higher Akt phosphorylation levels were found to correlate with reduced CPC proliferation. Indeed, fibronectin has been shown to increase CPC proliferation through an Akt-independent pathway, involving integrin-β1, FAK, signal transducer and activator of transcription 3, and Pim-1 oncogene. In our previous MSC
Figure 5. ABI family, member 3 (NESH) binding protein (Abi3bp) controls cardiac progenitor cells (CPC) differentiation through integrin-β1, protein kinase C (PKC)-ζ, and v-akt murine thymoma viral oncogene homolog (Akt). A, Wild-type (WT) c-Kit+ CPCs were cultured for 14 days in CPC-differentiation media. Where necessary isotype control or integrin-β1 blocking antibodies (10 μg/mL) were added to the media for the duration of the experiment. Gata4 and Gata6 expressions were determined by quantitative polymerase chain reaction (qPCR). Data are shown as a fold-change where expression values in control WT c-Kit+ CPCs were taken to be 1. Control WT c-Kit+ CPCs were differentiated for 14 days in the absence of antibody. n=3. Comparisons made between isotype and integrin-β1 blocking antibody-treated cells, *P≤0.05, **P≤0.01. B, Abi3bp KO c-Kit+ CPCs were transiently transfected with either a myc or mycAbi3bp plasmid. Cells were cultured for 14 days in CPC-differentiation media with isotype control or integrin-β1 blocking antibodies (10 μg/mL). Gata4 gene expression was determined by qPCR. Data are shown as a fold-change where expression values in day 0 cells were taken to be 1. n=3. No significant difference was observed between the integrin-β1 treated groups. C, WT and Abi3bp KO c-Kit+ CPCs were cultured for 14 days with CPC-differentiation media. Protein extracts (5 μg), taken at the indicated times, were immunoblotted for phosphorylation of focal adhesion kinase (p-FAK), FAK, and actin. n=3. *P≤0.05, ns not significant, comparisons made to WT day 0 cells. D, Abi3bp KO c-Kit+ CPCs were transiently transfected with either a myc or mycAbi3bp plasmid and cultured in growth media after transfection. Protein extracts (7.5 μg) were immunoblotted for p-FAK, FAK, and actin. n=3. *P≤0.05, ns not significant, comparisons made to the myc-expressing cells. E, WT and Abi3bp KO c-Kit+ CPCs were cultured for 14 days with CPC-differentiation media. Protein extracts (5 μg), taken at the indicated times, were immunoblotted for p-PKCζ, p-Akt, PKCζ, Akt, and actin. n=3. Quantification supplied in Online Figure VIIA. F, Abi3bp KO c-Kit+ CPCs were transiently transfected with either a myc or mycAbi3bp plasmid and cultured in growth media after transfection. Protein extracts (7.5 μg) were immunoblotted for p-PKCζ, p-Akt, PKCζ, Akt, and actin. n=3. Quantification supplied in Online Figure VIIB. G, WT c-Kit+ CPCs were cultured for 14 days with CPC-differentiation media. Where appropriate c-Kit+ CPCs were treated with either vehicle (dimethyl sulfoxide), or Akt inhibitor (DMSO soluble). Myocyte enhancer factor 2C (Mef2C), Gata4, and Gata6 expression was determined by qPCR. Expression at day 0 was taken to be 1. n=3. Comparisons made with day 14 CPCs exposed to neither vehicle nor inhibitor, *P≤0.05, **P≤0.01, ***P≤0.001. Flow cytometry was used to determine the number of cardiac troponin-T–positive cells. n=3. Comparisons made with day 14 CPCs exposed to neither vehicle nor inhibitor, *P≤0.05, **P≤0.01, ***P≤0.001. Flow cytometry was used to determine the number of cardiac troponin-T–positive cells. n=3. Comparisons made with day 14 CPCs exposed to neither vehicle nor inhibitor, *P≤0.01.
study, we found that Abi3bp inhibited proliferation by ERK sequestration at the plasma membrane by Src, an event mediated by integrin-β1 activation and paxillin phosphorylation.11 Considering the similarities between MSCs and CPCs Abi3bp may inhibit CPC proliferation through the same pathway.

Our data have implications both for the therapeutic use of CPCs and the controversy surrounding these cells. c-Kit+ CPCs have been shown to be beneficial therapeutically.7 Controversy exists on the differentiation potential of c-Kit+ CPCs. Using different models, 2 lineage tracing experiments arrived at a polar conclusion. One study found that c-Kit+ CPCs robustly differentiated to cardiomyocytes after injury28; the other study found that c-Kit+ cells only minimally contributed to the formation of cardiomyocytes.29 In our study, c-Kit+ CPC numbers were elevated in the Abi3bp knockout mouse, similar results were obtained with Sca-1+ CPCs (data not shown). However, ablation of the Abi3bp gene inhibited CPC differentiation and adversely affected recovery after MI. Considering that cardiomyocyte proliferation was unaffected by Abi3bp knockdown, this suggests that the critical determinant of the therapeutic potential of CPCs may not be cell number but rather their differentiation ability. It is worth noting that Abi3bp may have other effects on heart biology, independent of driving CPC differentiation. Lineage tracing experiments will be necessary to address this question. However, our model offers the potential to provide insight into the relative roles of cardiomyocyte proliferation versus CPC differentiation in the formation of new cardiomyocytes.

In conclusion, we have identified Abi3bp as an ECM protein important for promoting CPC differentiation via integrin-β1, Akt, and PKCζ. The findings of this study are potentially important for the therapeutic uses of CPCs.

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Disclosures

None.

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Removal of the ABI family, member 3 (NESH) binding protein (Abi3bp)

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Supplemental Material

Detailed Materials and Methods

Pharmacological inhibitors
Pharmacological inhibitors were purchased from Calbiochem. The company name for the Akt inhibitor is Akt inhibitor, Cat no 124005. The company name for the PKCζ inhibitor is PKCζ Pseudosubstrate Inhibitor, Myristoylated, Cat no 539624. The PKCζ inhibitor is a myristoylated pseudosubstrate peptide. The myristoylation enables the inhibitor to efficiently cross the plasma membrane. The peptide then binds to the pseudosubstrate domain within PKCζ which prevents the kinase from phosphorylating, and thus activating, downstream targets. The Akt inhibitor prevents ATP from binding to the active site.

Antibodies

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Blocking
Name | Company | Cat no
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CD29 | Abcam | ab24693

Primers
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Actn2 | Invitrogen | Mm00473657_m1
Cardiac troponin-I | Invitrogen | Mm00437164_m1
GAPDH | Invitrogen | Mm99999915_m1
Gata4 | Invitrogen | Mm00437164_m1
Gata6 | Invitrogen | Mm00802636_m1
Mef2C | Invitrogen | Mm01340482_m1

Abi3bp knockout mice
Abi3bp knockout mice are viable, however, non-Mendelian effects were observed with progeny from the heterozygous breeding pairs. The relative percentages of progeny were 27.5%:57.5%:15% (wild-type:heterozygous KO:homozygous KO).

Cardiac Progenitor Cell Isolation: Explant from cardiac biopsies
c-Kit+ CPCs were isolated from the cardiac biopsies of 4 week old male wild-type and Abi3bp knockout litter-mates according to the method of Hatzistergos et al with minor modifications due to differences in organisms used in the two studies. Cardiac biopsies were harvested, minced to ~1mm³ cubes and digested for 10 minutes at 37°C in a solution containing 200units/ml collagenase-II (Worthington), DMEM/F12 (Invitrogen), and 1xpenicillin-streptomycin (Invitrogen). Fetal bovine serum (Invitrogen) was added to a final concentration of 10% v/v to inhibit the reaction. The mixture was centrifuged at 500g for 5 minutes, supernatant was removed and pellet resuspended in DMEM containing 5xpenicillin-streptomycin (Invitrogen). The mixture was again centrifuged at 500g for 5 minutes and the pellet washed again. The pellet was resuspended in DMEM/F12, 20% FBS, 1xpenicillin-streptomycin and plated onto a well of a 6-well plate containing 50,000 bone marrow derived wild-type MSCs. After 7 days, samples were collected with enzyme free cell dissociation buffer (Invitrogen) to prevent cleavage of c-Kit at the plasma membrane.
c-Kit+ cells were isolated by repeated immunopanning as described. Immunopanning required that plastic petri-dishes were first incubated with 10μg/ml anti-rat IgG (eBioscience) overnight in 50mM Tris pH9.5 at 4°C. Following 2 washes with PBS the dishes were blocked with 1xPBS 5%BSA 2mM EDTA for 30 minutes at room temperature. The dishes were then incubated with c-Kit antibody (eBioscience, raised in rat, 2μg/ml, 1xPBS 5%BSA 2mM EDTA) for two hours at room temperature. Following two washes with PBS, cells, resuspended in 1xPBS 5%BSA 2mM EDTA, were added to the dishes. After 1 hour at room temperature, the dishes were washed twice with PBS, and attached cells removed with 0.05% trypsin. Fetal bovine serum was added to a final concentration of 10% v/v to stop the reaction. Following a 500g centrifugation for 5 minutes the cell pellet was resuspended and plated in F12K (Invitrogen), 5% FBS, 10ng/ml bFGF (R&D Systems), 20ng/ml LIF (Sigma), 1xpenicillin-streptomycin. After 7 days the c-Kit+ CPCs, which were found to be weakly adherent, were cultured in CPC-maintenance media. Following expansion the c-Kit+ CPCs were used at passage 1. At this passage the cells were positive for c-Kit and CD29 (Online Figure IIA). Necrosis was not significantly different between c-Kit+ CPCs derived from wild-type and Abi3bp knockout mice (Online Figure IIB), though a slight elevation in apoptosis was noted in the Abi3bp knockout cells (Online Figure IIB). In CPC-
maintenance media, Abi3bp knockout c-Kit+ CPCs expressed significantly lower levels of Abi3bp, Mef2C, and cardiac troponin-I (cTroponin-I) when compared to wild-type c-Kit+ CPCs, however, expression of Gata4 and Gata6 was not significantly different between wild-type and Abi3p knockout c-Kit+ CPCs (Online Figure IIC).

Stable Abi3bp knockdown in c-Kit+ CPCs
The shRNA plasmids were supplied by Origene. One plasmid contained a scrambled shRNA which was used as a control. The sequence of the Abi3bp shRNAs is FI510383 (shRNA 83): 5'CACCAGCGACTCCATCCTCTTGAAGTTCC3'.
c-Kit+ CPCs at passage 2 (5x10^4) were seeded into a well of a 6-well plate in 2ml CPC-maintenance medium one day prior to transfection. On the day of transfection 1μg of vector DNA (10μl) was diluted in optimem-serum-free media (90μl, Optimem-SF, Invitrogen). In a separate tube lipofectamine 2000 (2.5μl) was added to Optimem-SF (47.5μl). Solutions were left to incubate for 5' at room temperature then combined. After 20’ at room temperature the transfection complex was added to the cell layer with 2ml of growth media. One day after transfection cells were removed by incubation with 0.05%w/v trypsin and seeded into a 75cm^2 flask. Selection was performed at 3μg/ml puromycin for 7 days. Stable cell-lines were made at passage 3. Significant knockdown of Abi3bp was observed (Online Figure IIIA). The scrambled shRNA had no effect on the basal expression of Abi3bp, Mef2C and Gata4 when compared to unmodified wild-type c-Kit+ CPCs (Online Figure IIIB).

Re-expression of Abi3bp in Abi3bp knockout c-Kit+ CPCs.
Control- and C-terminal myc-tagged mouse Abi3bp (Accession number NM_178790) vectors were purchased from Genecopoeia. Transfection grade DNA was isolated by EndoFree Plasmid Maxi kit (Qiagen). Abi3bp knockout c-Kit+ CPCs (passage 2) were seeded at 5x10^4 cells per cm^2 one day prior to transfection in a 6-well plate. Transfection was performed with lipofectamine 2000 according to manufacturer guidelines for a 6-well plate format (Invitrogen). Cells were used three days after transfection.

Mesenchymal Stem Cell isolation
Mesenchymal stem cells (MSCs) were isolated as described previously from the bone marrow of 8-week old male Abi3bp wild-type^2. MSCs were used at passage 5.

Immunofluorescence
Cells were fixed with 2%v/v paraformaldehyde (EMS) as described previously^2. Fixed cells were incubated with primary antibodies, at the manufacturer’s recommended concentration, overnight at 4°C in antibody buffer (5%w/v BSA, 0.3%v/v Triton X-100, in PBS). Nuclei were stained by DAPI at 1μg/ml for 5 minutes at room temperature in PBS. Alexa-Fluor conjugated secondary antibodies (Invitrogen) were used at 1:500 dilution in antibody buffer for 1hr at room temperature. FITC conjugated Cardiac troponin-T, alpha-myosin heavy chain (αMHC), Mef2C antibodies were purchased from Abcam.

Cell growth curves
MTS assay:CPC growth curves were determined by Promega CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (MTS) used in accordance with the manufacturer’s
guidelines. Incubations were all 90 minutes at 37°C. CPCs were all seeded at 5000 cells per cm² and a 48-well format was used.

**Manual cell counting:** c-Kit+ CPCs were plated at 5000 cells per cm². Each day the cells were removed with 0.05% v/v trypsin and counted by hemocytometer. Each well was assayed in triplicate by a blinded investigator.

**Flow cytometry**

**Cell surface characterization:** Cell surface marker characterization was performed as described previously.

c-Kit and Gata4 analysis following collagenase digestion of the heart: hearts were excised and digested (HBSS, 100Units per ml collagenase-II) at 37°C for 15 minutes. The mixture was passed through a 100 μm sieve, and cells pelleted by centrifugation (500g, 5 minutes). Cells were then incubated with PE conjugated c-Kit (BD Biosciences, eBioscience) antibody (0.4μg/10⁶ cells) for 1hr at 4°C in FACS buffer (1xPBS, 0.5% BSA, 2mM EDTA). Cells were washed copiously with FACS buffer, and then fixed in 2% PFA for 15 minutes at 4°C. Following washing cells were incubated with APC conjugated Gata4 antibody (BD Biosciences, 0.4μg/10⁶ cells) in the presence of FACS buffer plus 0.2% w/v saponin for 1hr at 4°C. Following copious washing cells were analyzed by FACS on a FACSCantoll (BD Biosciences). FlowJo version 10 was used to compensate and analyze the data. Isotype control antibodies (BD Biosciences & eBioscience) were used throughout the experiment.

**In vitro cell cycle analysis with BrdU and 7-AAD:** this was performed according to the method outlined in Hodgkinson et al².

**In vitro analysis of apoptosis and necrosis:** this was performed with the Annexin V Apoptosis Detection eFluor®450 kit (eBioscience) according to the manufacturer’s instructions. Annexin V detects apoptosis, 7-AAD necrosis.

**qPCR**

Total RNA was extracted using Rn ease Plus Micro Kits according to the manufacturer’s instructions (Qiagen). Total RNA (500ng) was converted to cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems). cDNA was used in a standard qPCR reaction involving FAM conjugated gene specific primers and TaqMan Gene Expression Master Mix (Applied Biosystems).

**Protein extraction and Immunoblotting**

For cultured cells the cell layer was washed once with PBS and proteins lysed with 150μl lysis buffer (62.5mM Tris pH8, 1%v/v SDS, 1%v/v mammalian protease inhibitor cocktail [Sigma], 10%v/v phosphatase inhibitor cocktail [Roche, stock 1 tablet in 1ml water]) per well of a 6-well plate on ice. For trypsinized cells, centrifugation at 500g for 5’ was used to pellet the cells, trypsin removed, 5ml PBS added and cells re-centrifuged at the same settings. Once PBS was removed the cell pellet was lysed in 150μl lysis buffer per 5x10⁴ cells. Proteins from cultured cells/conditioned media were separated by SDS-PAGE (Invitrogen) and transferred to nitrocellulose (Bio-Rad). Primary and secondary antibodies were used according to manufacturer’s instructions. Proteins were visualized by chemiluminescence using ECL-Plus (GE Healthcare). Band intensities were determined by Image J or Syngene software.
**BrdU Flow cytometry in vivo:** For BrdU experiments in vivo mice were injected i.p. with 2mg of BrdU in a total volume of 200µl in PBS (BD Biosciences). Animals were sacrificed 24 hours later and hearts digested with collagenase as described in the Methods section. Cells were re-suspended in red blood cell lysis buffer (Sigma) and incubated for 5 minutes at room temperature. Reaction was stopped by the addition of 40ml PBS. Cells were pelleted at 500g for 5 minutes. Cells were fixed with 4%v/v paraformaldehyde for 15' at 4°C. After fixation cells were washed with antibody dilution buffer (1xPBS, 0.5%w/v BSA, 2mM EDTA). Cells were incubated with 0.4µg antibody for 1hr at 4°C in antibody dilution buffer. After washing with antibody dilution buffer, cells were re-fixed with 1%v/v paraformaldehyde and incubated with 7-AAD in antibody dilution buffer containing 0.2% saponin to allow 7-AAD entry into the cell.

**Myocardial infarction (acute left anterior descending (LAD) coronary artery ligation)**

Anesthetized 10-12 week old wild-type or Abi3bp knockout mice were intubated prior to left thoracotomy and exposure of the left ventricle of the heart. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg) by i.p. injection. Ophthalmic ointment was applied to both eyes to prevent corneal desiccation during the procedure. Following washing of the skin with iodophore/alcohol an endotracheal intubation was performed and the mouse connected to a ventilator (model 683, Harvard Apparatus, tidal volume 0.7-1ml, respiratory rate 120 breaths per minute) via an LV catheter (20 GA 1 IN) as the cannula under direct laryngoscopy. The chest cavity was opened between the fourth and the fifth rib in the intercostals muscle, the heart externalized and a 7-0 nylon suture be placed through the myocardium into the anterolateral LV wall, corresponding to the course of the left anterior descending artery. The suture was positioned approximately midway between the apex and base and a ligature made. Before ligation, left coronary artery entrapment was confirmed by upward traction. The suture was completely tied off (myocardial infarction), and the apex of the LV be observed for evidence of myocardial blanching indicating interruption in coronary flow. The wound was then closed using 7-0 nylon, and the animal allowed to recover. The chest cavity was subsequently closed in layers with 5-0 monofilament suture, negative pressure re-established at closure, and the animal gradually removed from the respirator. Following the resumption of spontaneous respiration, the endotracheal tube was removed and the animal placed on a deltaphase isothermal pad at 37°C. The animal remained in a supervised setting until fully conscious and then returned to their cages and given standard chow and water. Post-operative analgesia was used: bupivicaine locally + buprenorphine SQ at BID for 5 days. All skin sutures were removed by 7 days post-operation, if the sutures remained.

For staining, the hearts of anesthetized (isoflurane) mice were arrested via cold cardioplegic solution (35 mmol/L KCl, 68.6 mmol/L mannitol, 5% dextrose, 1.6 mmol/L bicarbonate, 1000 units heparin in normal saline). Hearts were excised and fixed overnight at 4°C in 10%w/v neutral buffered formalin, followed by incubation in 30%w/v sucrose for a further 24hrs at 4°C. The entire heart into 0.5mm sections using a Zivic Stainless Steel Mouse Heart Matrix (HSHS005-1). These sections were paraffin embedded and 2 10-micron slices cut for every 0.5mm section. One section was stained by Masson's trichrome to assess fibrosis. The other section was immunostained with c-Kit and Gata4 antibodies by a blinded investigator. The entire peri-infarct region in each tissue slice was visualized and counted the number of c-Kit+ and double positive c-Kit+/ Gata4+ cells. Primary (c-Kit R&D Systems #BAF1356 1:100, Gata4 Santa Cruz #sc9053 1:100) and secondary antibodies (anti-rabbit Alexa-Fluor 546 Invitrogen 1:500, goat IgG-biotin Santa Cruz 1:800, streptavidin Alexa-Fluor 488 Invitrogen 1:500) were used according to standard techniques.
**Echocardiographic analysis:** Animals will be anesthetized with isofluorane (via nose cone) and placed on a heating pad (Deltaphase pads will be used for this purpose) for the entire duration of procedure. To assess left ventricular mass, geometry, and function, echocardiographic analysis will be performed with spontaneous respiration under light anesthesia using isofluorane to maintain a heart rate < 400 bpm. Short-axis two-dimensional images using an 8-12 MHz transducer placed at the midpapillary levels of the left ventricle will be stored as digital loops.

**References for Detailed Materials and Methods**
2. Hodgkinson CP, Naidoo V, Patti KG, Gomez JA, Schmeckpeper J, Zhang Z, Davis B, Pratt RE, Mirotou M, Dzau VJ. Abi3bp is a multifunctional autocrine/paracrine factor that regulates mesenchymal stem cell biology. *Stem Cells.* 2013;31:1669-1682

**Online Figures**

**Online Figure I. C-Kit+ CPCs express CD29 (integrin-β1).**
(A) c-Kit and CD29 expression in passage 3 CPCs was determined by flow cytometry.
(B) Apoptosis and necrosis was determined by Annexin-V and 7-AAD staining respectively in cells two days post-seeding. N=3. No significance was observed between groups.
(C) Expression of the indicated genes in c-Kit+ CPCs cultured in growth media at passage 3 was determined by qPCR and expressed relative to GAPDH. N=3. ***P≤0.001

**Online Figure II. C-Kit+ CPCs derived from cardiac biopsies express integrin-β1.**
(A) c-Kit and CD29 expression in passage 1 CPCs was determined by flow cytometry.
(B) Apoptosis and necrosis was determined by Annexin-V and 7-AAD staining respectively in cells two days post-seeding. N=3. * P≤0.05.
(C) Expression of the indicated genes in c-Kit+ CPCs cultured in growth media at passage 3 was determined by qPCR and expressed relative to GAPDH. N=3. ***P≤0.001

**Online Figure III. Relative gene expression in knockdown and over-expression systems.**
(A) Expression of Abi3bp mRNA in wild-type c-Kit+ CPCs expressing either a scrambled or Abi3bp shRNA and cultured in either growth media or differentiation media for 14 days. Values are relative to GAPDH. N=3. ***P≤0.001.

(B) Expression of the indicated genes in either unmodified CPCs or CPCs expressing a scrambled shRNA was determined by qPCR and compared to GAPDH. N=3. No significant differences were observed between groups.

(C) Expression of Abi3bp mRNA in wild-type or Abi3bp knockout c-Kit+ CPCs expressing either a myc or mycAbi3bp plasmid. Values are relative to GAPDH. N=3. ***P≤0.001.
Online Figure IV. Abi3bp knockout inhibits differentiation of c-Kit+ CPCs derived from cardiac biopsies. c-Kit+ CPCs prepared from wild-type and Abi3bp knockout heart biopsies were assayed for:

(A) Wild-type and Abi3bp knockout c-Kit+ CPCs were immunostained with Mef2C, cardiac troponin-T, or αMHC antibodies to measure basal expression of these proteins. DAPI was used to stain nuclei. N=3. Scale bar 100 microns. Representative images shown. ***P≤0.001.

(B) Protein extracts (7.5µg) from wild-type and Abi3bp knockout c-Kit+ CPCs and cultured in CPC-differentiation media for either 0 or 14 days were probed for the indicated proteins (0 days represents basal expression). Actin was used as a loading control. Intensities were normalized to the loading control; normalized intensity of wild-type cells at day 0 was taken to be 1. N=3. ***P≤0.001, *P≤0.05.

(C) Flow cytometry was performed with wild-type and Abi3bp knockout c-Kit+ CPCs following incubation with a Mef2C antibody to measure basal expression of this protein. N=3. **P≤0.01.

(D) Wild-type and Abi3bp c-Kit+ CPCs were cultured for 14 days in CPC-differentiation media. Expression of Gata4, Gata6, and Mef2C was determined by qPCR. Expression in day 0 wild-type CPCs was taken to be 1. N=3. ***P≤0.001.

Online Figure V. C-Kit and Gata4 expression post-MI. Seven days following injury CPC differentiation was assessed by immunostaining. Peri-infarct regions were stained with c-Kit, Gata4, and DAPI. Scale bar 100 microns.

Online Figure VI. C-Kit cells express integrin-β1 (CD29) in vivo. Heart sections were stained with c-Kit, integrin-β1 (CD29) and DAPI. Scale bar 50 microns.

Online Figure VII. Abi3bp controls CPC differentiation through integrin-β1.

(A) Wild-type and Abi3bp knockout c-Kit+ CPCs were cultured for 0-14 days with CPC-differentiation media. Protein extracts [5µg], taken at the indicated times, were immunoblotted for p-PKCζ, p-Akt, PKCζ, Akt and actin. N=3. Intensities were normalized to the actin loading control and the normalized intensity of wild-type c-Kit+ CPCs at day 0 was taken to be 1. N=3. *P≤0.05, **P≤0.01, ***P≤0.001.

(B) Abi3bp knockout CPCs were transiently transfected with either a myc or mycAbi3bp plasmid. Protein extracts [7.5µg] were immunoblotted for p-PKCζ, p-Akt, PKCζ, Akt and actin. N=3. Intensities were normalized to the actin loading control and the normalized intensity of wild-type CPCs at day 0 was taken to be 1. N=3. *P≤0.05, **P≤0.01, ***P≤0.001.

(C) Wild-type c-Kit+ CPCs were treated with vehicle or the indicated pharmacological inhibitors. Intensities were normalized to the actin loading control and the normalized intensity of untreated CPCs was taken to be 1. N=3. *P≤0.05, **P≤0.01, ***P≤0.001.

(D) FACS traces for the pharmacological inhibitors.
Online Figure VIII. Abi3bp knockout c-Kit+ CPCs derived from cardiac biopsies have augmented proliferative capacity.

(A) Wild-type and Abi3bp knockout c-Kit+ CPCs derived from cardiac biopsies were seeded at the same density and manually counted for up to four days post-seeding. N=3. Comparisons between groups at the same time point **P≤0.01, ***P≤0.001.

(B) Wild-type and Abi3bp knockout c-Kit+ CPCs were incubated with BrdU for 6 hours and analyzed by flow cytometry. N=3. ***P≤0.001.
A c-Kit and CD29 expression in CPCs

B Apoptosis and necrosis in c-Kit+ CPCs

C mRNA expression of indicated genes relative to GAPDH

Online Figure I
A  c-Kit and CD29 expression in CPCs derived from cardiac biopsies

B  Apoptosis and necrosis in c-Kit+ CPCs derived from cardiac biopsies

C  mRNA expression of the indicated genes relative to GAPDH

Online Figure II
A shRNA mediated knockdown of Abi3bp gene expression

Growth media vs Differentiation media (14 days)

Expression relative to GAPDH (x10^-5)

B control shRNA has no effect on Abi3bp, Mef2C and Gata4 gene expression

Expression relative to GAPDH (Abi3bp x10^-17, Mef2C x10^-13, Gata4 x10^-17)

C Abi3bp gene re-expression in Abi3bp knockout c-Kit+ CPCs

Expression of Abi3bp relative to GAPDH (x10^-3)

Online Figure III
Online Figure IV
Wild-type

Knockout

Online Figure V
Online Figure VIII
**Online Table I.** Echocardiographic data for wild-type (N=11) and Abi3bp knockout (N=6) mice pre-injury and one month following sham or MI operation. Comparisons made between wild-type and Abi3bp knockout mice in each group. Significance <0.05 is marked in bold.

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