Abi3bp Regulates Cardiac Progenitor Cell Proliferation and Differentiation


Mandel Center for Hypertension Research and Division of Cardiovascular Medicine, Department of Medicine, Duke University Medical Center, Durham, NC 27710, USA.

Running title: Abi3bp Regulates Cardiac Progenitor Cells

Subject codes:
[6] Cardiac development
[108] Other myocardial biology

Address correspondence to:
Dr. Victor J Dzau
Mandel Center for Hypertension and Atherosclerosis
Division of Cardiovascular Medicine
Department of Medicine
Duke University Medical Center
Durham, NC 27710, USA.
Tel: 919 681 3828
Fax: 919 681 8371
victor.dzau@duke.edu

In September, 2014, the average time from submission to first decision for all original research papers submitted to Circulation Research was 14.29 days.
ABSTRACT

Rationale: Cardiac progenitor cells (CPCs) are believed to differentiate into the major cell types of the heart; cardiomyocytes, smooth muscle cells, and endothelial cells. We have recently identified Abi3bp as a protein important for mesenchymal stem cell (MSC) biology. Since CPCs share several characteristics with MSCs 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 was increased. This correlated with adverse recovery following 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, PKCζ, and Akt.

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

Keywords: Cardiac progenitor cells, extracellular matrix, integrin, cell signaling.
Nonstandard Abbreviations and Acronyms:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>αMHC</td>
<td>Alpha myosin heavy chain</td>
</tr>
<tr>
<td>Abi3bp</td>
<td>ABI family, member 3 (NESH) binding protein</td>
</tr>
<tr>
<td>Akt</td>
<td>v-akt murine thymoma viral oncogene homolog</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>c-Kit</td>
<td>V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog</td>
</tr>
<tr>
<td>CPC</td>
<td>Cardiac progenitor cell</td>
</tr>
<tr>
<td>DHVI</td>
<td>Duke Human Vaccine Institute</td>
</tr>
<tr>
<td>DLAR</td>
<td>Division of Laboratory Animal Resources</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>eHAND</td>
<td>Heart and neural crest derivatives expressed 1</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>Ets1</td>
<td>v-ets avian erythroblastosis virus E26 oncogene homolog 1</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GATA</td>
<td>Gata binding protein</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care &amp; Use Committee</td>
</tr>
<tr>
<td>Isl1</td>
<td>Islet-1</td>
</tr>
<tr>
<td>ITS</td>
<td>Insulin-transferrin-selenium</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia Inhibitory Factor</td>
</tr>
<tr>
<td>Mef2C</td>
<td>Myocyte enhancer factor 2C</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>NeoR</td>
<td>Neomycin resistance gene</td>
</tr>
<tr>
<td>Nkx2.5</td>
<td>Homeobox Protein Nkx-2.5</td>
</tr>
<tr>
<td>PIM1</td>
<td>Pim-1 oncogene</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>Stat3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>Tbx5</td>
<td>T-box 5</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>Wt1</td>
<td>Wilms tumor 1</td>
</tr>
</tbody>
</table>
INTRODUCTION

Cardiac progenitor cells (CPCs) are believed to give rise to the major cell types of the heart; these being cardiomyocytes, smooth muscle cells, and endothelial cells. c-Kit+/Sca-1+ CPCs have been the most heavily studied, however other proteins such as Nkx2.5, Wt1, Isl1, Tbx5 and eHand have also been used to define these cells. Endogenous CPCs are present in too low a number to completely regenerate a damaged heart following injury. However, recent reports have highlighted the potential therapeutic benefits of injected CPCs in the human heart following myocardial infarction. Enhancing these beneficial effects requires characterization of the mechanisms by which CPCs differentiate and proliferate.

We have recently discovered that Abi3bp promotes mesenchymal stem cell (MSC) differentiation whilst 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 as 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 neoR replacement of the first exon, were originally purchased from Taconic. All experiments were performed with wild-type (Abi3bp+/+) and Abi3bp knockout (Abi3bp-/-) littermates in accordance with institutional guidelines (DLAR and IACUC).

Cardiac progenitor cell isolation.

Enzymatic digestion: c-Kit+ CPCs were isolated from 8 week old male wild-type and Abi3bp knockout litter-mates. Minced ventricular tissue was digested in 100U of collagenase in Hank’s Buffered Saline solution at 37° C for 15 minutes. Single cells were passed through a 100 μm sieve and low density cells were separated on a discontinuous Percoll gradient. Primary cells were cultured for 3 days in CPC-maintenance media (DMEM/F12-K 1:1, 20% ES cell qualified FBS, 10 ng/mL bFGF, 20 ng/mL EGF, 100U LIF, and 1x ITS (insulin-transferrin-selenium)). c-Kit+ 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 IA). Apoptosis and necrosis was not significantly different between c-Kit+ CPCs derived from wild-type and Abi3bp knockout mice (Online Figure IB). In CPC-maintenance media, Abi3bp knockout c-Kit+ CPCs expressed significantly lower levels of Abi3bp, Mef2C, and cardiac troponin-I when compared to wild-type c-Kit+ CPCs, however, expression of Gata4 and Gata6 was not significantly different between wild-type and Abi3bp knockout c-Kit+ CPCs (Online Figure IC). CPCs were not observed to beat during the experiments.
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\(^\text{15}\) with minor modifications due to differences in organisms used in the two studies. A full method is provided in the Supplementary Methods. c-Kit+ CPCs isolated by this method were found to be weakly adherent\(^\text{15}\). 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). CPCs were not observed to beat during the experiments.

CPC differentiation.
CPCs were seeded at 25000 cells/cm\(^2\) in CPC-maintenance media. Twenty-four hours later the media was replaced with CPC-differentiation media (Advanced DMEM/F12, 0.2% w/v BSA, 2 mM L-glutamine, 1x ITS, 250 \(\mu\)mol/L ascorbic acid). Media was changed every two days.

Stable Abi3bp knockdown and re-expression.
Full method described in Online Supplement. Significant knockdown of Abi3bp was observed (Online Figure IIIA). Scrambled shRNA had no effect on the basal expression of Abi3bp, Mef2C and Gata4 (Online Figure IIIB). Abi3bp expression levels in wild-type, Abi3bp knockout expressing cmyc, and Abi3bp knockout CPCs expressing cmyc-Abi3bp are shown in Online Figure IIIC.

Myocardial infarction (acute left anterior descending (LAD) coronary artery ligation).
Anesthetized (ketamine (100 mg/kg) and xylazine (5 mg/kg) by i.p. injection) 10-12 week old wild-type or Abi3bp knockout mice were intubated prior to left thoracotomy. Mice were ventilated at a tidal volume 0.7-1ml, 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 following the resumption of spontaneous respiration the animal was allowed to recover. Post-operative analgesia was used for 5 days. Echocardiographic analysis was performed under minimal isofluorane anesthesia. The full method and procedure for staining is described in the Online Supplement.

Cardiac fibroblast and cardiomyocyte isolation.
Cardiac fibroblasts and cardiomyocytes were isolated as previously described \(^\text{16}\).

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 two conditions a t-test was performed. ANOVA was used for experiments with three or more conditions followed by Bonferroni post-hoc tests for comparisons between individual groups. Mann-Whitney U tests, a non-parametric test which 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 wild-type and Abi3bp knockout c-Kit+ CPCs from the non-cardiomyocyte fraction of the adult mouse heart. The characteristics of these isolated c-Kit+ CPCs, as described in the Methods Section, were consistent with resident CPCs reported in the literature. 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 ~100-fold lower in cardiac fibroblasts and un-differentiated CPCs (Figure 1A). However, following 14 days culture in CPC-differentiation media, expression levels of Abi3bp in differentiated CPCs were comparable to cardiomyocytes (Figure 1A). This 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 wild-type and Abi3bp knockout CPCs. Expression of cardiac genes was assessed at 0, 3, 8 and 14 days following the addition of CPC-differentiation medium. In wild-type 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 following 14 days of culture in differentiation media (Figure 1B).

Markers of lineage commitment were then assessed in differentiating wild-type and Abi3bp knockout c-Kit+ CPCs. mRNA encoding Gata4, Mef2C, Nkx2-5, Gata6, and Ets1 are present in the early stages of CPC differentiation whilst cardiac troponins and α-myosin heavy chain mRNA is observed in the later stages of CPC differentiation towards a cardiomyocyte fate. In wild-type c-Kit+ CPCs, mRNA encoding the early markers Gata4, Mef2C, and Gata6 increased during differentiation (Figure 1C). However, Abi3bp knockout markedly inhibited the expression of these early markers at all the time-points tested (Figure 1C). Identical results were obtained with the late marker cardiac troponin-I. Wild-type c-Kit+ CPCs expressed cardiac troponin-I mRNA at increasing amounts when cultured in CPC-differentiation media. However, Abi3bp knockout c-Kit+ CPCs expressed cardiac troponin-I at a markedly lower level throughout the time-course of the experiment (Figure 1D).

To validate the qPCR experiments, c-Kit+ CPCs were cultured for 14 days with CPC-differentiation media and then stained for various cardiac markers. Differentiated wild-type CPCs showed robust staining for Mef2C, cardiac troponin-T and α-myosin heavy chain (αMHC) (Figure 1E). However, the number of Mef2C, cardiac troponin-T, and αMHC positive cells were severely reduced in differentiated Abi3bp knockout CPCs (Figure 1E). Immunoblotting for several cardiac markers was used to verify the immunostaining results. Wild-type c-Kit+ CPCs were found to express Gata4, cardiac troponin-I, and αMHC protein (Figure 1F). Expression increased in CPC differentiation media (Figure 1F). In contrast, Abi3bp knockout c-Kit+ CPCs expressed significantly lower amounts of Gata4, cardiac troponin-I, and αMHC both at baseline and during differentiation (Figure 1F).

To ensure that the characteristics of the c-kit+ CPCs isolated from wild-type and Abi3bp knockout mice were not an artifact of the isolation procedure, we tested explanted c-Kit+ CPCs isolated from cardiac biopsies (see Methods). These cells yielded results identical to those from enzymatically dispersed cells. Compared to wild-type c-Kit+ CPCs, Abi3bp knockout c-Kit+ CPCs isolated by this method expressed lower basal levels of the Mef2C, Gata4, cardiac troponin-I, cardiac troponin-T, and αMHC protein (Online Figure IVA-C) and mRNA (Online Figure IVD) at baseline and following differentiation.

To verify that these findings were not due to a clonal effect, Abi3bp was stably knocked down in wild-type c-Kit+ CPCs by shRNA. Wild-type c-Kit+ CPCs, expressing either a scrambled control or Abi3bp
shRNA, were cultured for 14 days in CPC-differentiation media. Abi3bp expression increased ~170-fold in wild-type c-Kit+ CPCs expressing the scrambled control shRNA (data not shown). In contrast, as described in the Methods Section, no Abi3bp expression was observed in wild-type CPCs expressing the Abi3bp targeting shRNA. In c-Kit+ CPCs expressing the scrambled control shRNA, exposure to CPC differentiation media for 14 days increased expression of both Gata4 and Mef2C (Figure 2A). However, knockdown of Abi3bp had a significant inhibitory effect on the expression of both genes (Figure 2A). Following 14 days of culture in CPC-differentiation media the number c-Kit+ CPCs positive for either Mef2C or cardiac troponin-I was assessed by flow cytometry. Approximately 80% of wild-type c-Kit+ CPCs expressing the scrambled control shRNA were positive for either Mef2C or cardiac troponin-I following differentiation (Figure 2B). In contrast, Mef2C or cardiac troponin-I positive c-Kit+ CPCs were non-existent in Abi3bp-knockdown wild-type c-Kit+ CPCs (Figure 2B).

Finally we verified our findings by re-expression of Abi3bp in knockout c-Kit+ CPCs. Abi3bp knockout c-Kit+ CPCs were transiently transfected with a vector encoding Abi3bp. Wild-type c-Kit+ CPCs were used as a positive control. Gata4, Gata6, Mef2C, and Tnni3 mRNA levels were significantly increased following 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 expression was identical in wild-type 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 following differentiation (Figure 2D).

To investigate whether Abi3bp affected CPC differentiation in vivo, we subjected wild-type 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’s Trichrome staining was performed to assess fibrosis. One week post MI fibrosis levels were similar between wild-type and Abi3bp knockout (Figure 3B). However, one month post-MI fibrosis was significantly higher in the Abi3bp knockout animals (Figure 3C). Interestingly, at one month post MI, cardiac tissue from injured Abi3bp knockout mice appeared more fragile than that from the wildtype mice. Following embedding into paraffin, ~90% of the sections from Abi3bp knockout hearts showed breakage in the injured area as opposed to only about 10% in sections from wildtype hearts (Figure 3C).

We next examined cardiac function by echocardiography. Knockout of the Abi3bp gene was associated with lower ejection fraction and fractional shortening one-month post MI (Figure 3D, see Online Table I for pre-injury, sham, and MI echocardiographic data).
border zone, one week following injury, was not significantly different between wild-type 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. Moreover, c-Kit+ CPCs prepared by enzymatic dissociation as well as 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 wild-type c-Kit+ CPCs cultured under differentiation conditions. Gata4 and Gata6 expression were evaluated by qPCR and compared to wild-type c-Kit+ CPCs cultured in CPC-differentiation media in the absence of either antibody. The isotype control antibody had no effect on CPC differentiation, Gata4 and Gata6 expression levels were comparable to 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 over-expressing 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 qPCR. 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 FAK is a key event following integrin activation. As expected, p-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 upon CPC differentiation we wished to identify the signaling proteins involved. Wild-type and Abi3bp knockout c-Kit+ CPCs were cultured for up to 14 days in CPC-differentiation media. Protein extracts from these cells were subsequently immunoblotted for PKCζ and Akt. Abi3bp knockout had no effect on the levels of total PKCζ and Akt in c-Kit+ CPCs (Figure 5E, with quantification provided in Online Figure VIIA). Both phospho-PKCζ (Thr410) and phospho-Akt (S473) were observed in wild-type c-Kit+ CPCs (Figure 5E). Phosphorylation levels of both kinases showed a modest, but significant increase, during differentiation (Figure 5E, with quantification shown in Online Figure VIIA). In contrast to wild-type c-Kit+ CPCs, Abi3bp knockout CPCs displayed markedly lower levels of both p-PKCζ and p-Akt at all time-points tested (Figure 5E, with quantification shown in Online Figure VIIA). Phosphorylation levels of PKCζ and Akt did not change when Abi3bp knockout CPCs were cultured in CPC-differentiation media (Figure 5E, with quantification shown in Online Figure VIIA). Re-expression of Abi3bp in Abi3bp knockout c-Kit+ CPCs increased phosphorylation of PKCζ and Akt (Figure 5F, with quantification shown in Online Figure VIIIB).

The differences between wild-type and Abi3bp knockout c-Kit+ CPCs in the levels of p-PKCζ and p-Akt suggested that Abi3bp promotes CPC differentiation through these kinases. Pharmacological inhibition was employed to test this hypothesis. Wild-type c-Kit+ CPCs were cultured for 14 days in CPC differentiation media supplemented with either vehicle or pharmacological inhibitors. The concentrations employed 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 wild-type CPC differentiation (Figure 5G, FACS traces Online Figure VIIID). 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 wild-type CPC differentiation were significantly reduced by the inhibitor (Figure 5H, FACS traces Online Figure VIIID). However, no effect was observed with Gata4 (Figure 5H) suggesting that the two kinases control different pathways.
We hypothesized, based on our previous studies, that Abi3bp would affect CPC proliferation. Wild-type and Abi3bp knockout c-Kit+ CPCs were seeded at the same density, cultured for three days, and manually counted on a daily basis. Abi3bp knockout c-Kit+ CPC number was significantly higher than their wild-type counterparts 1, 2, and 3 days post-seeding (Figure 6A) with a doubling time of 1.3 days versus 1.7 days. Re-expression of Abi3bp in Abi3bp knockout cells decreased cell proliferation as determined by cell counting (Figure 6B). Re-expression of Abi3bp in Abi3bp knockout CPCs increased the doubling time to 2.3 days. Wild-type c-Kit+ CPCs were made to stably express either a scrambled-control or Abi3bp shRNA. C-Kit+ CPCs expressing the Abi3bp shRNA construct were found to have a higher growth rate when compared to control cells expressing a control scrambled shRNA (Figure 6C). Wild-type CPCs, expressing either the scrambled or Abi3bp shRNA construct, were incubated with the thymidine analogue BrdU to determine whether the changes in cell number arose from altered cell cycle kinetics. Flow cytometry was used to determine the number of BrdU-positive cells. Knockdown of Abi3bp increased the number of BrdU-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 post-seeding when compared to wild-type c-Kit+ CPCs (Online Figure VIIIA) with a doubling time of 0.95 days versus 1.3 days for the wild-type cells. Similarly, knockout of the Abi3bp gene increased BrdU incorporation in c-Kit+ CPCs (Online Figure VIIIB).

We then assessed the c-Kit+ CPC proliferation in wild-type and Abi3bp knockout mice. Consequently, we measured BrdU uptake in wild-type and Abi3bp knockout mice. Abi3bp knockout increased the proliferation of both c-Kit+ positive hematopoietic lineage-negative CPCs, as shown by the ~10-fold increase in BrdU incorporation (Figure 6E).

**DISCUSSION**

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

Following injury to the heart the extracellular matrix undergoes a number of changes which affect cardiac function. The extracellular matrix (ECM) has been shown to affect the behavior of certain types of stem cells, such as mesenchymal stem cells (MSCs). ECM stiffness and composition has 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 found previously that 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 Nkx2-5+ 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; as 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 a Akt independent pathway involving integrin-β1, focal adhesion kinase (FAK), Stat3, and PIM1. In our previous MSC 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. Considering the similarities between MSCs and CPCs Abi3bp may inhibit CPC proliferation through the same pathway.

Our data has implications both for the therapeutic use of CPCs and the controversy surrounding these cells. c-Kit+ CPCs have been shown to be therapeutically beneficial. Controversy exists regarding the differentiation potential of c-Kit+ CPCs. Using different models, two lineage tracing experiments arrived at a polar conclusion. One study found that c-Kit+ CPCs robustly differentiated to cardiomyocytes following injury, the other study found that c-Kit+ cells only minimally contributed to the formation of cardiomyocytes. 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 following 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.

ACKNOWLEDGEMENTS
We would like to thank John Wong of the DHVI (Duke Human Vaccine Institute) for the use of flow cytometers. This work was supported by the NIH.

SOURCES OF FUNDING
Research conducted in these studies was supported by National Heart, Lung, and Blood Institute grants RO1 HL35610, HL81744, HL72010, and HL73219 and the Edna and Fred L. Mandel, Jr. Foundation

DISCLOSURES
None

CONTRIBUTIONS
Conrad P Hodgkinson: conception and design, collection of data, data analysis and interpretation, manuscript writing, final approval of manuscript. Jose Gomez, Alan Payne, Lunan Zhang, Xiaowen Wang. Sophie Dal-Pra: collection and/or assembly of data. Richard Pratt: data analysis and interpretation. Victor J Dzau: conception and design, manuscript writing, data analysis and interpretation, final approval of manuscript.
REFERENCES


4. Bollini S, Smart N, Riley PR. Resident cardiac progenitor cells: At the heart of regeneration. *J Mol Cell Cardiol*. 2011;50:296-303


11. 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


DOI: 10.1161/CIRCRESAHA.115.304216
FIGURE LEGENDS

**Figure 1.** Abi3bp knockout inhibits CPC differentiation. (A) Abi3bp expression in cardiomyocytes [Cm], cardiac fibroblasts [Cf], and CPCs grown in either growth media [CPC GM] or differentiation media [CPC diff] was determined by qPCR. N=3. Data is shown as a fold expression with c-Kit+ CPCs grown in CPC-maintenance media taken to be 1. (B) Wild-type and Abi3bp knockout CPCs were cultured in CPC-differentiation media for up to 14 days. Expression of Abi3bp was determined by qPCR and immunoblotting. Expression in day 0 wild-type CPCs was taken to be 1. N=3. ***P≤0.001. (C-D) Wild-type and Abi3bp knockout CPCs were cultured for 14 days in CPC-differentiation media. The cells were subsequently stained with Mef2C, cardiac troponin-T, or αMHC antibodies. DAPI was used to stain nuclei. Scale bar 100 microns. N=4. (F) Protein extracts (7.5μg) from wild-type and Abi3bp knockout CPCs cultured in CPC-differentiation media for 0, 7 and 14 days were probed for the indicated proteins. 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≤0.05.

**Figure 2.** Re-expression of the Abi3bp in Abi3bp knockout CPCs recapitulates the wild-type phenotype. (A) Wild-type CPCs, expressing either a scrambled control or an Abi3bp targeting shRNA, were cultured for 14 days in CPC-differentiation media. Gata4 and Mef2C expression was determined by qPCR. Expression of cells at day 0 was taken to be 1. N=3. Comparisons made between scrambled and Abi3bp shRNA expressing cells **P≤0.01, ***P≤0.001. (B) Following culturing in CPC-differentiation media for 14 days the percentage of CPCs positive for Mef2C or cardiac troponin-I was determined by flow cytometry, N=3. Comparisons made between scrambled and Abi3bp shRNA expressing cells, **P≤0.01, ***P≤0.001. (C) Wild-type and Abi3bp knockout CPCs, transiently transfected with either a myc or mycAbi3bp plasmid, were cultured for 7 days in CPC-differentiation media. Expression of Gata4, Gata6, Mef2C and cardiac troponin-T was determined by qPCR. Gene expression data is shown relative to GAPDH. N=3, ***P≤0.001, **P≤0.01, *P≤0.05. (D) Protein extracts (7.5μg) from Abi3bp knockout c-Kit+ CPC, expressing either the myc or mycAbi3bp plasmid, and cultured in CPC-differentiation media for either 0 or 14 days, were probed for the indicated proteins. 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≤0.05.

**Figure 3.** Abi3bp knockout is associated with lower cardiac function and increased fibrosis following MI. (A) Protein extracts (50μg) 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’s trichrome. Fibrosis area is represented as a percentage of the left ventricle. (B) One-week post-infarct and (C) One month post-infarct. N=4-6 per group. Significance between MI groups is shown. ** P≤0.01. (D) One month following injury echocardiography was performed. Left and center panels: Ejection fraction and fractional shortening shown for wild-type and Abi3bp knockout mice subjected to MI. P values indicated. N=11 for wild-type, N=6 for Abi3bp knockout. Right panel: Sham ejection fraction and fractional shortening values (N=5 per group). Other parameters for pre-operative, MI and sham animals are shown in Supplementary Table I.

**Figure 4.** Abi3bp knockout prevents CPC differentiation/commitment following cardiac injury. Wild-type and Abi3bp knockout mice were subjected to either a sham operation or myocardial infarction [MI]. (A) Seven days following injury CPC differentiation was assessed by immunostaining. Peri-infarct regions were stained with c-Kit, Gata4, and DAPI. Scale bar 50 microns. Larger regions which contain these...
sections can be found in Online Figure V. (B) The entire peri-infarct region was visualized by serial sectioning through the heart tissue. Total numbers of c-Kit+ and Gata4+ cells were determined. The numbers of c-Kit+ cells are expressed per mm² of the peri-infarct region. *P≤0.05 comparing MI to sham, †P≤0.05 comparing wild-type MI to Abi3bp knockout MI, ‡P<0.01 comparing wild-type sham to Abi3bp knockout sham. No other comparisons are significant. The numbers of double positive (c-Kit+ Gata4+) cells are expressed as a percentage of the total c-Kit+ population in the peri-infarct region. **P≤0.01 comparing wild-type MI to wild-type sham, ††P≤0.001 comparing wild-type MI to Abi3bp knockout MI. No other comparisons are significant. N=4-6 per group.

(C) Hearts were collagenase digested and differentiating CPCs counted by flow cytometry. Differentiating CPCs were defined by the presence of c-Kit, expression of Gata4, and the absence of hematopoietic lineage markers. N=4-6 per group. Data is expressed as the percentage of c-Kit positive Gata4 positive cells in the total hematopoietic negative population. P-values indicated. (D) Serial sections of one-week post-MI tissue were stained for cardiac troponin-T (Trop) and the proliferation marker PCNA. Scale bar 50 microns. N=4-6 per group. No significance was observed between groups.

**Figure 5. Abi3bp controls CPC differentiation through integrin-β1, PKCζ and Akt.** (A) Wild-type c-Kit+ CPCs were cultured for 14 days in CPC-differentiation media. Where necessary isotype control or integrin-β1 blocking antibodies (10μg/ml) were added to the media for the duration of the experiment. Gata4 and Gata6 expression was determined by qPCR. Data is shown as a fold-change where expression values in control wild-type c-Kit+ CPCs were taken to be 1. Control wild-type 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 knockout 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 (10μg/ml). Gata4 gene expression was determined by qPCR. Data is 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) Wild-type and Abi3bp knockout c-Kit+ CPCs were cultured for 14 days with CPC-differentiation media. Protein extracts [5μg], taken at the indicated times, were immunoblotted for p-FAK, FAK and actin. N=3. *P<0.05, ns not significant, comparisons made to WT day 0 cells. (D) Abi3bp knockout c-Kit+ CPCs were transiently transfected with either a myc or mycAbi3bp plasmid and cultured in growth media following transfection. Protein extracts [7.5μg] were immunoblotted for p-FAK, FAK and actin. N=3. *P<0.05, ns not significant, comparisons made to the myc-expressing cells. (E) Wild-type and Abi3bp knockout c-Kit+ CPCs were cultured for 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. Quantity supplied in Online Figure VIIA. (F) Abi3bp knockout c-Kit+ CPCs were transiently transfected with either a myc or mycAbi3bp plasmid and cultured in growth media following transfection. Protein extracts [7.5μg] were immunoblotted for p-PKCζ, p-Akt, PKCζ, Akt and actin. N=3. Quantity supplied in Online Figure VIIIB. (G) Wild-type c-Kit+ CPCs were cultured for 14 days with CPC-differentiation media. Where appropriate c-Kit+ CPCs were treated with either vehicle [DMSO], or Akt inhibitor [DMSO soluble]. 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 either vehicle or 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 either vehicle or inhibitor, **P≤0.01. (H) Wild-type c-Kit+ CPCs were cultured for 14 days with CPC-differentiation media. Where appropriate c-Kit+ CPCs were treated with a PKCζ inhibitor [media soluble]. 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 either vehicle or 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 either vehicle or inhibitor, **P≤0.01.
Figure 6. Abi3bp inhibits CPC proliferation in vitro and in vivo. (A) Wild-type and Abi3bp knockout c-Kit+ CPCs were seeded at the same density in growth media and manually counted for up to three days post-seeding. N=3. Comparisons between groups at the same time point *** $P \leq 0.001$. (B) Abi3bp knockout c-Kit+ CPCs, over-expressing either a control or Abi3bp plasmid, were seeded at the same density in growth media and manually counted for up to three days post-seeding. N=3. Comparisons between groups at the same time point ** $P \leq 0.01$. (C) MTS assay growth curves in growth media for wild-type CPCs expressing either scrambled or Abi3bp shRNA. N=6. Comparisons between groups at the same time point, *** $P \leq 0.001$. (D) Wild-type CPCs expressing scrambled or Abi3bp shRNA were incubated with in growth media supplemented with BrdU for 6 hours and analyzed by flow cytometry using 7-AAD to stain DNA. BrdU positive cells are in S-phase. N=3. *** $P \leq 0.001$. (E) Wild-type and Abi3bp knockout mice were injected with BrdU for four days, cells were collected by collagenase digestion and analyzed by FACS. Left panel, BrdU+/c-Kit+/lin- CPCs were counted and expressed as a percentage of the total c-Kit+/lin- CPC population. N=3. * $P \leq 0.05$. 

Downloaded from http://circres.ahajournals.org/ by guest on October 27, 2017
Novelty and Significance

What Is Known?

- Cardiac progenitor cells (CPCs) are believed to differentiate into cardiomyocytes, smooth muscle cells and endothelial cells.
- CPCs have been shown to be therapeutically beneficial for the treatment of heart disease.
- The mechanisms controlling CPC differentiation and proliferation are unclear.

What New Information Does This Article Contribute?

- Removal of the Abi3bp gene inhibited CPC differentiation in vivo, whereas CPC proliferation and number were increased.
- This correlated with adverse recovery following myocardial infarction.
- Abi3bp promoted CPC differentiation via integrin-β1, PKCζ, and Akt activation.

CPCs are thought to differentiate into the major cell types of the heart and injection of CPCs into damaged heart tissue following injury promotes recovery. Despite their clinical relevance mechanisms controlling important aspects of CPC biology, such as differentiation and proliferation, are unclear. We found in vivo that removal of the Abi3bp gene inhibited CPC differentiation whereas CPC proliferation and number were increased. This correlated with adverse recovery following myocardial infarction. We also found that Abi3bp promoted CPC differentiation through the activation of an integrin-β1, PKCζ and Akt signaling pathway. These data show that Abi3bp is important for CPC differentiation and proliferation.
Figure 2

Abi3bp over-expression in Abi3bp Knockout c-Kit CPCs

A

B

Gata4 mRNA

Mef2C mRNA

Fold change over Od

Fold change over Od

scrambled shRNA

Abi3bp shRNA

scrambled shRNA

Abi3bp shRNA

scrambled shRNA

Abi3bp shRNA

scrambled shRNA

Abi3bp shRNA

(cTroponin-I protein

(Mef2C protein

(C-K+ + c-K+ [\%]

(C-K+ [\%]

(C-K+ [\%]

(C-K+ [\%]

(C-K+ [\%]

Figure 2
Figure 6

(A-C) Graphs showing the number of cells over time in culture for different conditions:

- **A:** Comparison of WT (squares) and KO (diamonds) cell numbers. **B:** Comparison of KO + mycAbi3bp (squares) and KO + myc (diamonds) cell numbers. **C:** Absorbance measurements showing a significant difference between Abi3bp shRNA (squares) and scrambled shRNA (diamonds).

(D-E) Bar graphs showing the percent of cells in S-phase for different conditions:

- **D:** Comparison of scrambled shRNA and Abi3bp shRNA. **E:** Ratio of (BrdU+ c-Kit+/Lin-) cells for WT and KO conditions.
Abi3bp Regulates Cardiac Progenitor Cell Proliferation and Differentiation
Conrad Hodgkinson, Jose Gomez, James A Payne, Lunan Zhang, Xiaowen Wang, Sophie Dal-Pra, Richard E Pratt and Victor J Dzau

Circ Res. published online October 8, 2014;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2014/10/08/CIRCRESAHA.115.304216

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2014/10/08/CIRCRESAHA.115.304216.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
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

**Immunostaining**

<table>
<thead>
<tr>
<th>Name</th>
<th>Company</th>
<th>Cat no</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>αMHC</td>
<td>Abcam</td>
<td>ab15</td>
<td>1:100</td>
</tr>
<tr>
<td>Cardiac Troponin-T FITC</td>
<td>Abcam</td>
<td>ab105439</td>
<td>1:100</td>
</tr>
<tr>
<td>c-Kit</td>
<td>R&amp;D Systems</td>
<td>BAF1356</td>
<td>1:100</td>
</tr>
<tr>
<td>Gata4-Alexa 647</td>
<td>BD Biosciences</td>
<td>560400</td>
<td>1:100</td>
</tr>
<tr>
<td>Mef2C</td>
<td>Abcam</td>
<td>ab64644</td>
<td>1:100</td>
</tr>
</tbody>
</table>

**Immunoblotting**

<table>
<thead>
<tr>
<th>Name</th>
<th>Company</th>
<th>Cat no</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abi3bp</td>
<td>Abcam</td>
<td>ab104510</td>
<td>1:500</td>
</tr>
<tr>
<td>Actin</td>
<td>Cell Signaling</td>
<td>4970</td>
<td>1:3000</td>
</tr>
<tr>
<td>Akt</td>
<td>Cell Signaling</td>
<td>4961</td>
<td>1:1000</td>
</tr>
<tr>
<td>αMHC</td>
<td>Abcam</td>
<td>ab15</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-rabbit HRP</td>
<td>Cell Signaling</td>
<td>7074</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-mouse HRP</td>
<td>Cell Signaling</td>
<td>7076</td>
<td>1:1000</td>
</tr>
<tr>
<td>βtubulin</td>
<td>Cell Signaling</td>
<td>2128</td>
<td>1:1000</td>
</tr>
<tr>
<td>Cardiac troponin-I</td>
<td>Cell Signaling</td>
<td>13083</td>
<td>1:1000</td>
</tr>
<tr>
<td>Gata4</td>
<td>Abcam</td>
<td>ab84593</td>
<td>1:1000</td>
</tr>
<tr>
<td>p-Akt</td>
<td>Cell Signaling</td>
<td>9271</td>
<td>1:1000</td>
</tr>
<tr>
<td>p-PKCζ</td>
<td>Cell Signaling</td>
<td>9378</td>
<td>1:1000</td>
</tr>
<tr>
<td>PKCζ</td>
<td>Abcam</td>
<td>ab59364</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

**FACS**

<table>
<thead>
<tr>
<th>Name</th>
<th>Company</th>
<th>Cat no</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-rabbit PE</td>
<td>eBioscience</td>
<td>12-4739</td>
<td>0.4μg/10⁶ cells</td>
</tr>
<tr>
<td>BrdU-Alexa 647</td>
<td>BD Biosciences</td>
<td>560209</td>
<td>0.4μg/10⁶ cells</td>
</tr>
<tr>
<td>c-Kit PE</td>
<td>eBioscience</td>
<td>12-1171</td>
<td>0.4μg/10⁶ cells</td>
</tr>
<tr>
<td>CD29 APC</td>
<td>eBioscience</td>
<td>17-0291</td>
<td>0.4μg/10⁶ cells</td>
</tr>
<tr>
<td>Gata4-Alexa 647</td>
<td>BD Biosciences</td>
<td>560400</td>
<td>0.4μg/10⁶ cells</td>
</tr>
<tr>
<td>Mef2C</td>
<td>Abcam</td>
<td>ab64644</td>
<td>0.4μg/10⁶ cells</td>
</tr>
</tbody>
</table>
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 ~1mm 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).
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 F510383 (shRNA 83): 5'CACCAGCGACTCCATCCTCTTGAAGTTCC3'. c-Kit+ CPCs at passage 2 (5x10⁴) 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 75cm² 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 5x10⁴ cells per cm² 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 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 FACSCantoII (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 al.

**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 Rneasy 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 Immunblotting**

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 resuspended 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 IV 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 set 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, Mirotosou 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
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

![Graph showing expression relative to GAPDH for scrambled shRNA and Abi3bp shRNA.]

Differentiation media (14 days)

![Graph showing expression relative to GAPDH for scrambled shRNA and Abi3bp shRNA.]

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

![Graph showing expression relative to GAPDH for unmodified CPCs and scrambled shRNA CPCs for Abi3bp, Mef2C, and Gata4.]

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

![Graph showing expression of Abi3bp relative to GAPDH for KO and WT with and without Abi3bp.]

Online Figure III
Online Figure IV

A

WT CPC

KO CPC

Mef2C
Cardiac Troponin-T
αMHC

Cells positive for protein marker (%)

WT-CPC KO-CPC

*** *** ***

B

Gata4

Gata4 expression

WT KO

0d 14d 0d 14d

WT KO

0.0 0.5 1.0 1.5 2.0 2.5

***

CTN-I

CTN-I expression

WT KO

0 0 14 14

0.0 0.5 1.0 1.5

***

αMHC

αMHC expression

WT KO

0 0 14 14

0.0 0.5 1.0 1.5

***

Actin

C

D

Mef2C

Percent cells Mef2C positive

WT KO

WT KO

0 20 40 60 80

***

Fold change over 0d

WT KO

Gata4 Gata6 Mef2C

* ** ***

***
Wild-type

Knockout

Online Figure V
Online Figure VI
Online Figure VII
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.

<table>
<thead>
<tr>
<th></th>
<th>IVS;d (mm)</th>
<th>LVID;d (mm)</th>
<th>LVID;s (mm)</th>
<th>LVPW;d (mm)</th>
<th>EF (%)</th>
<th>FS (%)</th>
<th>LV Mass (mg)</th>
<th>LV Vol;d (µl)</th>
<th>LV Vol;s (µl)</th>
<th>Heart Rate (bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-Echo</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>0.917±0.028</td>
<td>3.246±0.058</td>
<td>1.305±0.045</td>
<td>1.208±0.039</td>
<td>89.94±0.62</td>
<td>59.90±0.95</td>
<td>102.7±4.6</td>
<td>42.85±1.8</td>
<td>4.406±0.405</td>
<td>619.1±16.2</td>
</tr>
<tr>
<td>Knockout</td>
<td>0.875±0.037</td>
<td>3.176±0.090</td>
<td>1.347±0.056</td>
<td>1.166±0.035</td>
<td>88.50±0.83</td>
<td>57.71±1.12</td>
<td>93.11±4.61</td>
<td>40.96±2.91</td>
<td>4.790±0.515</td>
<td>635.0±20.3</td>
</tr>
<tr>
<td><strong>Sham</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>0.909±0.071</td>
<td>3.179±0.081</td>
<td>1.220±0.111</td>
<td>1.112±0.079</td>
<td>90.90±2.73</td>
<td>61.49±2.99</td>
<td>92.81±8.43</td>
<td>40.86±2.65</td>
<td>3.660±1.423</td>
<td>668.5±41.5</td>
</tr>
<tr>
<td>Knockout</td>
<td>0.951±0.030</td>
<td>3.156±0.164</td>
<td>1.294±0.173</td>
<td>1.241±0.135</td>
<td>89.61±2.47</td>
<td>59.02±2.90</td>
<td>102.5±12.3</td>
<td>39.85±6.27</td>
<td>4.172±2.430</td>
<td>620.7±35.4</td>
</tr>
<tr>
<td><strong>MI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>0.760±0.076</td>
<td>5.196±0.144</td>
<td>3.867±0.174</td>
<td>0.984±0.076</td>
<td>52.07±2.74</td>
<td>27.19±1.84</td>
<td>173.5±18.9</td>
<td>137.5±10.8</td>
<td>66.60±6.86</td>
<td>574.3±28.7</td>
</tr>
<tr>
<td>Knockout</td>
<td>0.671±0.085</td>
<td>5.434±0.380</td>
<td>4.359±0.376</td>
<td>0.918±0.061</td>
<td>40.70±2.65</td>
<td>20.11±1.40</td>
<td>158.0±19.9</td>
<td>147.3±24.1</td>
<td>73.15±10.21</td>
<td>589.6±55.1</td>
</tr>
</tbody>
</table>