Asymmetric Chromatid Segregation in Cardiac Progenitor Cells Is Enhanced by Pim-1 Kinase

Balaji Sundararaman, Daniele Avitabile, Mathias H. Konstandin, Christopher T. Cottage, Natalie Gude, Mark A. Sussman

Rationale: Cardiac progenitor cells (CPCs) in the adult heart are used for cell-based treatment of myocardial damage, but factors determining stemness, self-renewal, and lineage commitment are poorly understood. Immortal DNA strands inherited through asymmetric chromatid segregation correlate with self-renewal of adult stem cells, but the capacity of CPCs for asymmetric segregation to retain immortal strands is unknown. Cardioprotective kinase Pim-1 increases asymmetric cell division in vivo, but the ability of Pim-1 to enhance asymmetric chromatid segregation is unknown.

Objective: We aimed to demonstrate immortal strand segregation in CPCs and the enhancement of asymmetric chromatid distribution by Pim-1 kinase.

Methods and Results: Asymmetric segregation is tracked by incorporation of bromodeoxyuridine. The CPC DNA was labeled for several generations and then blocked in second cytokinesis during chase to determine distribution of immortal versus newly synthesized strands. Intensity ratios of binucleated CPCs with bromodeoxyuridine of ≥70:30 between daughter nuclei indicative of asymmetric chromatid segregation occur with a frequency of 4.57%, and asymmetric chromatid segregation is demonstrated at late mitotic phases. Asymmetric chromatid segregation is significantly enhanced by Pim-1 overexpression in CPCs (9.19% versus 4.79% in eGFP-expressing cells; P=0.006).

Conclusions: Asymmetric segregation of chromatids in CPCs is increased nearly two-fold with Pim-1 kinase overexpression, indicating that Pim-1 promotes self-renewal of stem cells. (Circ Res. 2012;110:1169-1173.)

Key Words: asymmetric cell division ■ asymmetric chromatid segregation ■ bromodeoxyuridine ■ cytochalasin B ■ immortal DNA strand hypothesis
phenotype. ACS in CPCs is enhanced two-fold in our study by overexpression of Pim-1, a kinase that enhances cardiac repair,18 increases asymmetric cell division,19 and promotes CPC self-renewal.

Methods
Cell culture, treatments, BrdU immunostaining, and image quantification are described in the detailed Methods provided in the Online Supplement. Experimental design for label release and retention assay are explained and depicted in Online Figure I.

Label Release Assay
“Immortal” strands are never labeled, as per the initial postulate of Cairns.1 Short-time BrdU labeling for a single S-phase results in CPCs with one labeled and one unlabeled DNA strand (Online Figure IA). After completion of mitosis when cells are chased in BrdU free media, chromatids with unlabeled stands segregate from chromatids with newly made BrdU-labeled strands. Therefore, stem cells release the label because of retention of the oldest immortal chromatids with newly made BrdU-labeled strands.7,11 Label Release Assay is devised to demonstrate presence of unlabeled “immortal stands.” Asymmetric segregation of unlabeled immortal strands from BrdU-labeled newly synthesized strands is revealed by rare binucleated CPCs with one daughter nucleus having little or no BrdU staining, whereas the other nucleus is positive for BrdU (Figure 1A). CPCs exhibiting daughter nuclei with equal BrdU intensity indicate symmetrical segregation (Figure 1B).

Label retention assay (Online Figure IB) validates results of the release assay that is limited to CPCs undergoing DNA synthesis at the time of labeling. Both strands are BrdU-labeled in the label retention assay, in which CPCs are continuously labeled and passaged at least four times to dilute slowly cycling and exclusively asymmetrically segregating CPCs.6–8,10 Daughter nuclei with approximately 50:50 BrdU intensity ratio (only symmetric segregation) are observed in CPCs blocked at first mitosis during chase (Figure 2A), consistent with model predictions (Online Figure IB). Both symmetric and asymmetric BrdU staining is observed at second mitosis (Figure 2B–D). Asymmetric segregation of BrdU-labeled chromatids is demonstrated by binucleated cells having daughter nuclei with disproportionate BrdU intensity (Figure 2B, D). CPCs never exhibited a completely negative paired with positive BrdU daughter nuclei pair, consistent with previous reports of punctate BrdU staining.10,20 Therefore, ACS is defined as a daughter nuclei pair having BrdU ratio of $\leq$0:70, similar to chromosome in situ hybridization experiment,5 instead of absolute ratio (100:0), confirming that not all chromatids are asymmetrically segregated.

Results
Cell-cycle distribution of CPCs after drug-induced mitotic synchronization of the cell population is shown in Online Figure II. Use of a label release assay (Online Figure IA) was devised to test the capacity of Pim-1 to

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Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ACS</td>
<td>asymmetric chromatid segregation</td>
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<tr>
<td>BrdU</td>
<td>5-bromo-2’-deoxyuridine</td>
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<tr>
<td>CPC</td>
<td>cardiac progenitor cell</td>
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<tr>
<td>CPCe</td>
<td>CPC expressing eGFP</td>
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<tr>
<td>CPCeP</td>
<td>CPC expressing eGFP and Pim-1</td>
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Figure 1. Cardiac progenitor cells (CPCs) retain unlabeled immortal strands in label release assay. A. Binucleated cell with one daughter nucleus exhibiting low BrdU immunostaining (red arrowhead) and another showing intense BrdU signal. B. Binucleated cell with daughter nuclei exhibiting symmetric BrdU immunostaining. Binucleated CPCs are confirmed by reflection scanning (cell). Scale bar=10 μm.
enhance ACS by label retention assay. Morphological differences were not observed between CPCs, CPCe, CPCeP, indicating that the genetic modification does not significantly affect cell function. Binucleated CPCs with daughter nuclei exhibiting marked differential BrdU intensity are observed in both CPCe and CPCeP (Figure 4A). Asymmetric segregation of chromatids is increased nearly two-fold in CPCeP (9.19%; n=5; 72–135 binucleated cells per experiment) compared to CPCe (4.79%; P=0.006; Figure 4B–D) with no significant difference between nontransduced control versus CPCe (4.59% versus 4.79%; P=0.375).

**Discussion**

Autologous cell therapy with CPC is an efficacious treatment for heart failure.\textsuperscript{15} Beneficial effects of naïve cells are improved by modification and selection to promote reparative potential in the harsh milieu of a postinfarct heart.\textsuperscript{16} Modification with Pim-1 facilitates CPC survival and proliferation after infarction injury,\textsuperscript{18,23} in addition to numerous other signaling molecules and pathways that facilitate stem cell–mediated repair.\textsuperscript{16} However, CPC-mediated regeneration could be further augmented by identification of a cell subset that possesses enhanced potential for self-renewal as evidenced by ACS. Therefore, there is a need to identify signal transduction cascades that enhance ACS to confer superior self-renewal properties.

The presence of immortal/template strands and ACS in adult stem cells remains controversial.\textsuperscript{2–4} The immortal strand hypothesis assumes ability of stem cells to retain old strands to minimize transformational risk associated with DNA replication errors.\textsuperscript{1} Our findings of predominant rather than total ACS are consistent with direct observation of chromatid segregation\textsuperscript{5} and punctate BrdU retention in non-myocardial stem cell studies,\textsuperscript{10,20} indicating partial ACS might be specific to stem cell type. The silent sister hypothesis presumes ACS to regulate differential gene expression profile to determine cell fate and self-renewal,\textsuperscript{3,4,24} which is strengthened by the observation of cosegregation of template strands with self-renewal markers in stem cells\textsuperscript{7–10} and of
conformational changes in histone variant H2A.Z on template strands.\textsuperscript{20} Functional significance of ACS through study of live cells is technically challenging because of protocols of fixation and immunolabeling required for detection by fluorescent cell sorting to isolate asymmetrically segregating cells, although utilization of directly labeled thymidine analogues recently has been used in cancer stem cells to circumvent fixation requirements.\textsuperscript{25,26} Future studies will capitalize on the functional characterization of asymmetrically segregating CPCs to determine their regenerative potential relative to comparable CPCs undergoing symmetric segregation, with the expectation that asymmetric segregation correlates with enhanced myocardial repair.

Previous research in our laboratory established multiple beneficial roles for Pim-1, including enhancement of proliferation, survival, and commitment of CPCs without causing tumor formation after transplantation.\textsuperscript{18,19} Pim-1-mediated increase in ACS can be explained by the multifaceted participation of Pim-1 in mitosis and transcriptional regulation. Pim-1 colocalizes at spindle poles during mitosis to phosphorylate NuMA and promote complex formation with HP1\textsubscript{γ}/H9252, dynein, and dynactin during chromosome segregation.\textsuperscript{27} The left–right dynein motor is necessary for selective segregation of mouse chromosome 7.\textsuperscript{22} Pim-1 also binds and phosphorylates HP1\textsubscript{γ} on serine-rich clusters, modulating its transcriptional repression activity.\textsuperscript{28} Pim-1–mediated phosphorylation of HP1 proteins, which are involved in chromatin structure, indicates that Pim-1 likely modulates chromatin dynamics. Chromatin dynamic regulation by Pim-1 could explain the functional significance of ACS in conjunction with the silent sister hypothesis and stem cell self-renewal. Pim-1 increases asymmetrically dividing CPCs in the heart after pathological injury to balance self-renewing versus differentiated cell populations.\textsuperscript{19} Pim-1 decreases spontaneous differentiation of embryonic stem cells\textsuperscript{29} and is highly expressed in long-term populating hematopoietic stem cells,\textsuperscript{30} supporting the premise that Pim-1 regulates stemness. Future studies are warranted to address the direct role of Pim-1 in ACS and stem cell self-renewal to further enhance the regenerative potential of CPCs and other stem cell types.

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Disclosures

None.

References

What Is Known?
- Preferential partitioning of old versus newly synthesized DNA strands known as asymmetric segregation is hypothesized to regulate self-renewal and aging in several cell types.
- The cardioprotective kinase Pim-1 increases proliferation, asymmetric cell division, and regenerative potential of cardiac progenitor cells (CPCs).

What New Information Does This Article Contribute?
- Asymmetric partitioning of old versus newly synthesized DNA formed during mitosis occurs in a small subpopulation of CPCs.
- Asymmetric segregation of chromatids into daughter CPCs is enhanced by Pim-1 kinase overexpression.

CPCs are potentially effective as agents of cell-based regenerative treatment for myocardial damage. However, factors that determine self-renewal and lineage commitment of CPCs are poorly understood. The regenerative potential of aged CPCs may be compromised in elderly patients with chronic heart disease. Identification and characterization of subsets of CPCs that exhibit preservation of proliferative potential and enhanced self-renewal might be valuable for use in the clinical setting to augment stem cell–based repair. Superior self-renewal properties and protection from phenotypic characteristics of aging are associated with asymmetric chromatid segregation wherein old DNA strands preferentially distribute to a single daughter cell during mitosis. This study confirms that asymmetric DNA segregation occurs in CPCs at a low frequency consistent with rates observed for other adult stem cell types. Moreover, the frequency of asymmetric DNA segregation in CPCs can be significantly increased by overexpression of Pim-1 kinase. The observation of increased asymmetric segregation with Pim-1 overexpression may help explain the enhanced regenerative properties of CPCs engineered to overexpress Pim-1 and provide another mechanistic basis for increasing self-renewal of CPCs that might be essential for effective myocardial repair.
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Supplemental Material

Detailed Methods

Cell culture

Mouse CPCs are isolated, maintained and lenti virally engineered to stably over express either eGFP alone (CPCe) or eGFP and human Pim-1 kinase (CPCeP) together as bicistronic transcripts by previously established methods\(^1\). For label release and retention assays cells were plated at 250 cells/mm\(^2\). Cells were labeled with 10 μmol/L BrdU for one generation or with 1 μmol/L BrdU for longer labeling period. BrdU concentration used to label DNA replication is well below typical concentrations used to track chromosomal activities such as DNA repair (30-100 μmol/L).

Cell cycle analysis and treatments

Cells were fixed in 70% ethanol and stored overnight at -20°C. Cell cycle distribution was analyzed by staining with propidium idodie (PI) RNase staining buffer (BD) at 37°C for 3 hours using BD FACSCanto and data processed by FlowJo software. Cells were incubated with 2.5 mmol/L thymidine for indicated times in full medium to block in G1-S phase. Cells were released by trypsinizing and washing twice with PBS and plated in full medium for synchronized progression of cell cycle. 2 μmol/L cytochalasin B was used whenever necessary to block in cytokinesis. For mitotic shake-off cells were grown at 70% confluence, blocked at S phase by 2.5 mmol/L thymidine blocking for 24 hours, released in thymidine free media for 18 hours. Loosely adherent mitotic cells were collected by gently knocking the plate against a hard surface once in every 1.30 hours for up to 5-6 times after thymidine release and cells were fixed in 70% ethanol.

Cell cycle analysis of CPCs indicated most of the cells complete mitosis 12-18 hours after thymidine removal. Thymidine block for 30 hours and releasing immediately in cytochalasin B for up to 24 hours blocks cells in first cytokinesis. Release in thymidine free medium for first 24-30 hours followed by cytochalasin B for another 30 hours block cells in second cytokinesis. These conditions are followed to block cells in first and second mitosis during chase period in BrdU free media to see asymmetric DNA segregation.
**BrdU staining**

Cells were fixed in 70% ethanol, stored at 4°C until use. Cells were denatured with 2N HCl/0.2% Triton-X100 for 1 hour at RT, then neutralized with 0.1 mol/L TrisCl pH 8.5 and blocked with 10% horse serum. For detecting BrdU, mouse-anti-BrdU (clone B44, BD # 347580) antibody was used at 1:100 dilution, 1 hour at RT followed by donkey-anti-mouse IgG-549 (1:150) as secondary antibody. Cells were mounted in Vectashield containing 1:1000 of Sytox-blue nuclear dye and scanned using Leica SP2 confocal microscope. Images were acquired using 20X lens with 2X electronic zoom for BrdU measurement and 15X electronic zoom for high resolution single cell panels. Differential interference contrast (DIC) images were acquired to confirm binucleated cells.

**Image processing and density measurements**

All images and panels were identically processed. ImageJ software was used for BrdU intensity measurements. Threshold levels of the single scan images were set using auto adjust then the image color was inverted. Mean gray value (d) of two daughter nuclei of a binucleated cell was measured separately and multiplied by their corresponding area (A). Fraction of BrdU intensity of individual daughter nuclei to the total (T= A₁*d₁ + A₂*d₂) was plotted (D₁= A₁*d₁/T versus D₂=1-D₁).

**Method of deriving tumor free Pim-1 CPCs**

Recent results from our lab indicate that Pim-1-mediated proliferation of CPCeP is transient. Increased proliferation of CPCeP observed at early passages diminishes to normal levels with repeated cell passage without loss of Pim-1 expression (manuscript in preparation). We are also exploring alternative non-lentiviral based methods for Pim-1 gene delivery including episomal minicircle vectors and cell penetrant proteins.
Online Figure I. Experimental design for label release and retention assay. A, In label release assay, cells are labeled for 6hrs and then chased in BrdU free media for 24hrs followed by a cytochalasin B treatment to induce binucleated cells. By retaining the unlabeled ‘immortal strands’, the stem cell will lose the BrdU labeled chromosomes. B, In the label retention assay cell are labeled for up to 15-25 days and chased for two consecutive cells divisions. Stem cells will retain the BrdU labeled immortal strands for ever while the daughter cells dilute the label.
Online Figure II. Cell cycle distribution of CPCs with drug treatments. Establishing CPCs’ cell cycle kinetics is necessary to successfully block at specific mitosis. Cell cycle distribution is determined by propidium iodide staining and flow cytometry. Population distribution of DNA content is plotted to examine different phases of cell cycle. CPCs grown without any drugs or treatment showed that more than 70% are in G1 and 5% of cells in G2 phase of cell cycle (Ctrl). Thymidine treatment for 30 hours blocked almost all cells in G1-S phase boundary (T30C0). Cells grown with thymidine (T) are washed and plated immediately with medium containing 2μM cytochalasin B (C) and fixed at six hour intervals. Cytochalasin blocks cells in cytokinesis preventing from further G1 phase, creating binucleated cells (cells with 4n DNA content). Six hours after thymidine removal, cells progressed to S phase (T30C6) and by 12 hours a small percentage of cells entered G2 phase. Eighteen hours after thymidine removal the majority of cells entered G2/M phase and by 24 hours more than 85% cells completed mitosis but are binucleated (T30C24). Analysis at thirty hours after thymidine removal and cytochalasin treatment confirmed that cells are permanently blocked in G2.

Supplementary references