# Spontaneous Calcium Oscillations Regulate Human Cardiac Progenitor Cell Growth

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**Rationale: The adult heart possesses a pool of progenitor cells stored in myocardial niches, but the mechanisms involved in the activation of this cell compartment are currently unknown.**

**Objective: $Ca^{2+}$ promotes cell growth raising the possibility that changes in intracellular $Ca^{2+}$ initiate division of c-kit–positive human cardiac progenitor cells (hCPCs) and determine their fate.**

**Methods and Results: $Ca^{2+}$ oscillations were identified in hCPCs and these events occurred independently from coupling with cardiomyocytes or the presence of extracellular $Ca^{2+}$. These findings were confirmed in the heart of transgenic mice in which enhanced green fluorescent protein was under the control of the c-kit promoter. $Ca^{2+}$ oscillations in hCPCs were regulated by the release of $Ca^{2+}$ from the endoplasmic reticulum through activation of inositol 1,4,5-triphosphate receptors (IP3Rs) and the reuptake of $Ca^{2+}$ by the sarco-/endoplasmic reticulum $Ca^{2+}$ pump (SERCA). IP3Rs and SERCA were highly expressed in hCPCs, whereas ryanodine receptors were not detected. Although $Na^{+}$-$Ca^{2+}$ exchanger, store-operated $Ca^{2+}$ channels and plasma membrane $Ca^{2+}$ pump were present and functional in hCPCs, they had no direct effects on $Ca^{2+}$ oscillations. Conversely, $Ca^{2+}$ oscillations and their frequency markedly increased with ATP and histamine which activated purinoceptors and histamine-1 receptors highly expressed in hCPCs. Importantly, $Ca^{2+}$ oscillations in hCPCs were coupled with the entry of cells into the cell cycle and 5-bromodeoxyuridine incorporation. Induction of $Ca^{2+}$ oscillations in hCPCs before their intramyocardial delivery to infarcted hearts was associated with enhanced engraftment and expansion of these cells promoting the generation of a large myocyte progeny.**

**Conclusion: IP3R-mediated $Ca^{2+}$ mobilization control hCPC growth and their regenerative potential. (*Circ Res*. 2009;105:764-774.)**

**Key Words:** human cardiac progenitor cells ■ calcium oscillations ■ cell growth



The recognition that the adult heart in animals and humans possesses a pool of stem/progenitor cells[1–3] has raised the critical question concerning the mechanisms involved in the activation of this cell compartment and the modulation of cardiac homeostasis and repair. During the course of life before the manifestations of myocardial aging become apparent,[4] dying parenchymal cells are continuously replaced by newly formed myocytes[5,6] through activation and commitment of quiescent cardiac progenitor cells (CPCs) stored in myocardial niches.[7,8] However, the signals responsible for the initiation of the cell cycle in CPCs, cardiomyocyte generation and preservation of the steady state of the organ are currently unknown. Calcium has 2 fundamental functions in the heart: it activates growth processes[9,10] and modulates the mechanical behavior of cardiomyocytes.[11,12] Critical for understanding physiological cell turnover and myocardial regeneration following injury is the identification of the mechanisms by which CPCs divide and acquire the myocyte phenotype. Changes of calcium levels in CPCs may occur and trigger a cascade of events that dictate their ultimate fate. Therefore, the objectives of the present study were: (1) to determine the pathways that regulate intracellular $Ca^{2+}$ in human CPCs (hCPCs); (2) to establish whether $Ca^{2+}$ oscillations in hCPCs condition cell replication; and (3) to assess whether $Ca^{2+}$ oscillations are intrinsic to the cells or are triggered by interaction of hCPCs with cardiomyocytes. This cell-to-cell communication may favor the translocation of $Ca^{2+}$ from myocytes to quiescent hCPCs initiating a cellular growth response. Moreover, transmembrane $Ca^{2+}$ fluxes may contribute to rapid and transient rise in cytosolic $Ca^{2+}$ promoting the entry of hCPCs into the cell cycle. These variables include a functional endoplasmic reticulum (ER) where $Ca^{2+}$ is stored, the activity of ER channels that promote $Ca^{2+}$ release, and the membrane systems modulating the exchange of $Ca^{2+}$ with the extracellular compartment.

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## Methods

hCPCs were isolated from myocardial specimens obtained from patients who underwent cardiac surgery. The study was approved by the Partners Human Research Committee. Cytosolic $Ca^{2+}$ levels in cultured hCPCs were measured using the $Ca^{2+}$ indicator Fluo-3 and 2-photon microscopy. Cell proliferation in vivo and in vitro was evaluated by 5-bromodeoxyuridine (BrdUrd) incorporation. Results are shown as means±SEM. An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Non-standard Abbreviations and Acronyms**

| | |
|---|---|
| **2-APB** | 2-aminoethyl diphenylborinate |
| **BrdUrd** | 5-bromodeoxyuridine |
| **CPC** | cardiac progenitor cell |
| **DiI** | 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine |
| **EGFP** | enhanced green fluorescent protein |
| **ER** | endoplasmic reticulum |
| **H1** | histamine receptor-1 |
| **hCPC** | human cardiac progenitor cell |
| **IGF** | insulin-like growth factor |
| **IGF-1R** | insulin-like growth factor-1 receptor |
| **IP3** | inositol 1,4,5-triphosphate |
| **IP3R** | inositol 1,4,5-triphosphate receptor |
| **IRS** | insulin receptor substrate protein |
| **LV** | left ventricular |
| **mCPC** | mouse cardiac progenitor cell |
| **P2Y2** | P2-purinoceptor |
| **PLC** | phospholipase-C |
| **RyR** | ryanodine receptor |
| **SERCA** | sarco-/endoplasmic reticulum $Ca^{2+}$ pump |

## Results

### Intracellular $Ca^{2+}$ in hCPCs

Changes in $[Ca^{2+}]_i$ occur in excitable and nonexcitable cells raising the possibility that a similar phenomenon is present in hCPCs and may have a functional role. Thus, hCPCs were loaded with the $Ca^{2+}$-sensitive dye Fluo-3 and the intensity of the fluorescent signal was monitored over a period of ≈30 minutes. During this interval, 79% hCPCs maintained stable levels of $[Ca^{2+}]_i$, whereas 21% displayed one or more consecutive $Ca^{2+}$ oscillations. Repetitive events were restricted to a small percentage of cells and were comparable in amplitude and duration (Figure 1A through 1C). The fraction of hCPCs displaying $Ca^{2+}$ oscillations increased with time up to 2 hours, although the frequency of these episodes remained low (Figure 1D). These cells were all positive for the stem cell antigen c-kit (Online Figure I). $Ca^{2+}$ oscillations increased in hCPCs at the $G_1$-S phase transition but decreased at $G_2$-M (Figure 1E and Online Figure II).

### Cell-to-Cell Interaction and $Ca^{2+}$ Oscillations in hCPCs

The next objective was to establish whether $Ca^{2+}$ oscillations in hCPCs are modulated at the single cell level or are mediated by adjacent cells. hCPCs are nested in myocardial niches and connexins are found between hCPCs and myocytes, which operate as supporting cells.[3,7] These intercellular communications may account for the generation of $Ca^{2+}$ oscillations in hCPCs, a process that is commonly observed in cardiomyocytes (Online Figure III and Online Movie 1). Therefore, to identify the origin of $Ca^{2+}$ oscillations in hCPCs, these cells were cultured alone or together with neonatal cardiomyocytes. Initially, dye transfer assays were performed[3,7,13] to document the formation of functional gap junctions between hCPCs. The fluorescent dye cascade blue

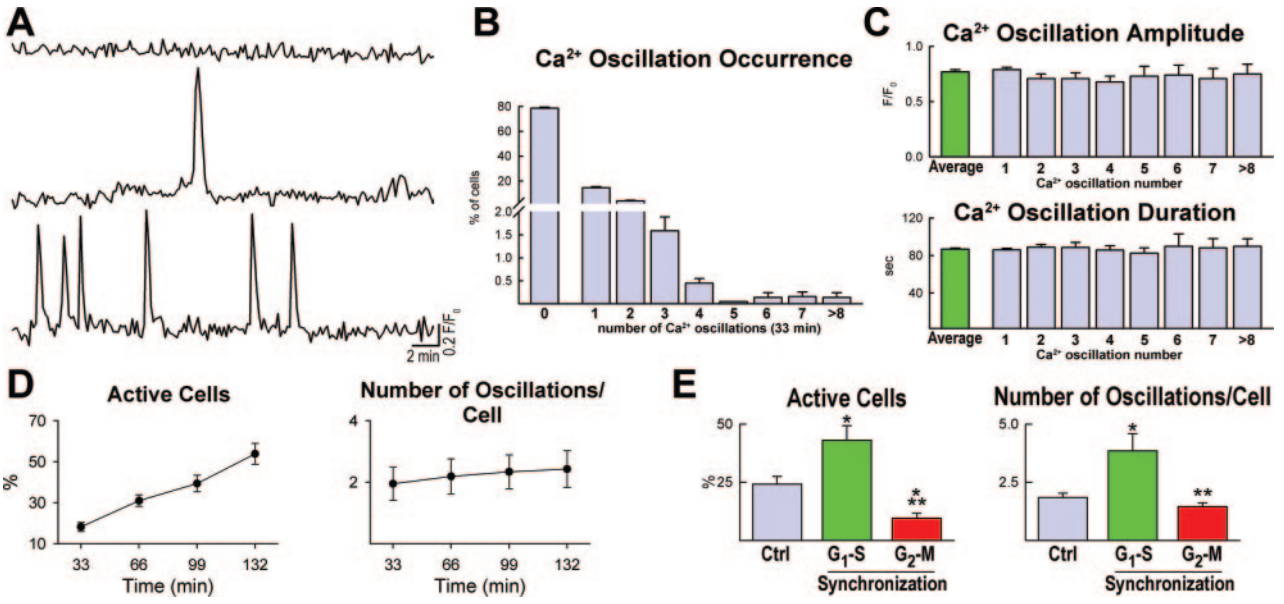


**Figure 1.** Intracellular $Ca^{2+}$ in hCPCs. A, Cytosolic $Ca^{2+}$ levels in a quiescent hCPC (upper trace) and in 2 hCPCs showing single (middle trace) and multiple (lower trace) $Ca^{2+}$ oscillations. B, Distribution of $Ca^{2+}$ oscillations, from 1 to more than 8, in hCPCs over a period of 33 minutes. C, Amplitude and duration of $Ca^{2+}$ events in hCPCs. D, $Ca^{2+}$ oscillations in hCPCs (Active Cells) analyzed for a period of 132 minutes. E, $Ca^{2+}$ oscillations in hCPCs in control condition (Ctrl) and at the $G_1$-S and $G_2$-M transition.

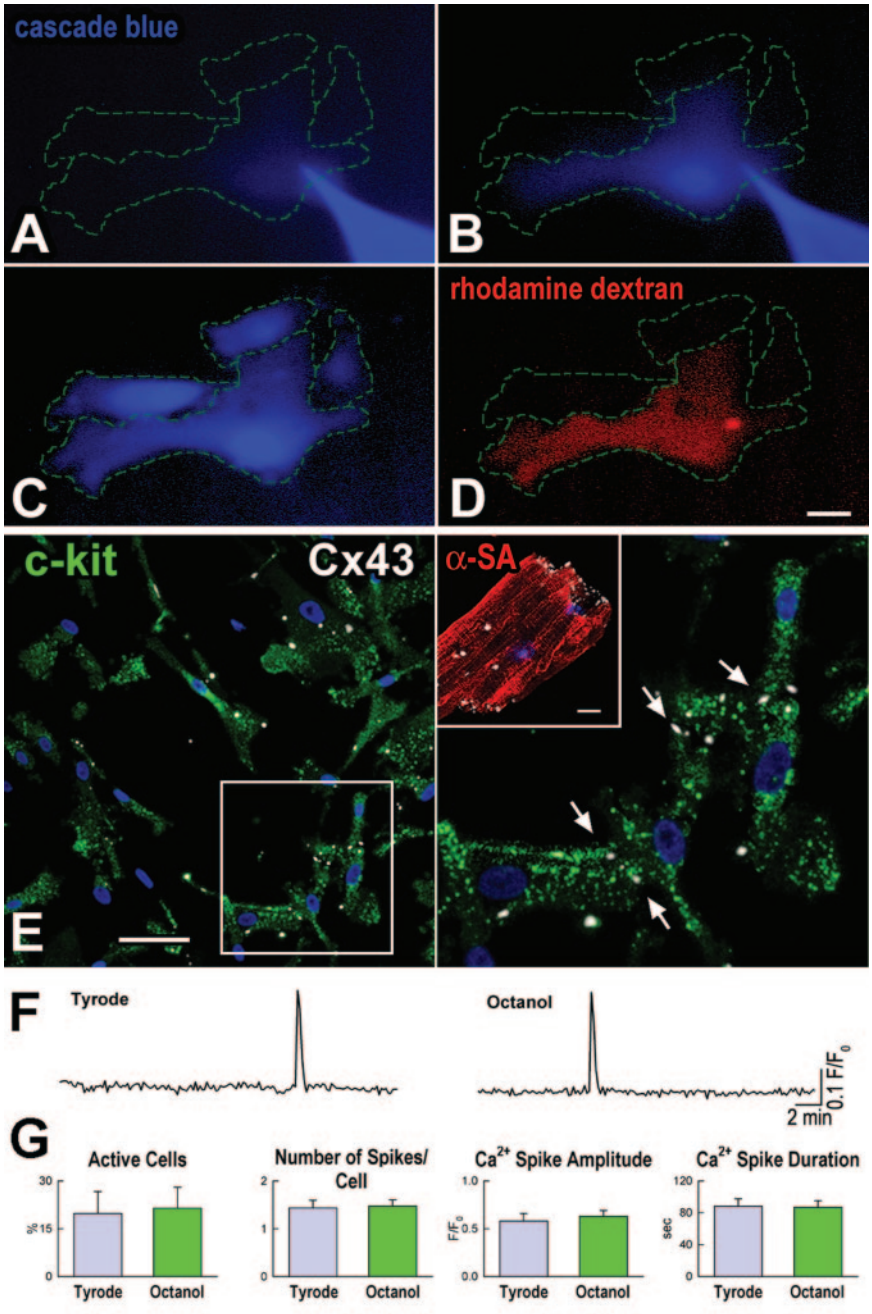


**Figure 2.** Cell-to-cell interaction and $Ca^{2+}$ oscillations. A through C, Cascade blue (blue) microinjected in a single hCPC (A) translocated spontaneously to adjacent cells (B and C). D, Rhodamine-labeled dextran (red), delivered simultaneously with cascade blue, remained confined to the injected cell. Scale bar: 20 $\mu$m. Cascade blue translocation was detected in 6 experiments. E, Connexin 43 (Cx43) (white) is present between hCPCs (c-kit, green). Nuclei are stained by DAPI (blue). Scale bar: 20 $\mu$m. A group of cells is shown at higher magnification on the right image. The inset shows connexin 43 labeling in a myocyte ($\alpha$-sarcomeric actin [$\alpha$-SA]; red). Scale bar: 10 $\mu$m. F, Intracellular $Ca^{2+}$ in hCPCs before (left trace) and after (right trace) exposure to octanol. G, Effects of uncoupling on $Ca^{2+}$ oscillations.

was microinjected in individual hCPCs and found to rapidly migrate to neighboring cells through gap junction channels expressing connexin 43 (Figure 2A through 2E). However, the high molecular weight rhodamine-labeled dextran, injected simultaneously with cascade blue, failed to translocate to adjacent hCPCs. Additionally, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI)-labeled hCPCs loaded with calcein were cultured with untreated cells. After ≈12 hours, calcein was detected in unlabeled hCPCs structurally connected to DiI-calcein–positive cells (Online Figure IV). Intracellular $Ca^{2+}$ was then measured before and after exposure of hCPCs to the connexin hemi-gap-junction channel blocker octanol. Octanol did not affect the frequency and properties of $Ca^{2+}$ oscillations in hCPCs (Figure 2F and 2G).

Subsequently, the effect of $Ca^{2+}$ cycling in myocytes on hCPC function was assessed by plating together DiI-labeled hCPCs and unlabeled cardiomyocytes. Despite the presence of functional gap junctions between these 2 cell populations (Figure 3A and Online Figure V), spontaneous or electrically stimulated $Ca^{2+}$ transients in myocytes had no detectable consequence on $[Ca^{2+}]_i$ of hCPCs (Figure 3B and 3C). In fact, $Ca^{2+}$ oscillations in hCPCs persisted in the presence of cadmium which abolished $Ca^{2+}$ transients in myocytes.

## Cell-to-Cell Interaction and $Ca^{2+}$ Oscillations in Mouse CPCs

To strengthen these in vitro results, a transgenic mouse model in which enhanced green fluorescent protein (EGFP) was

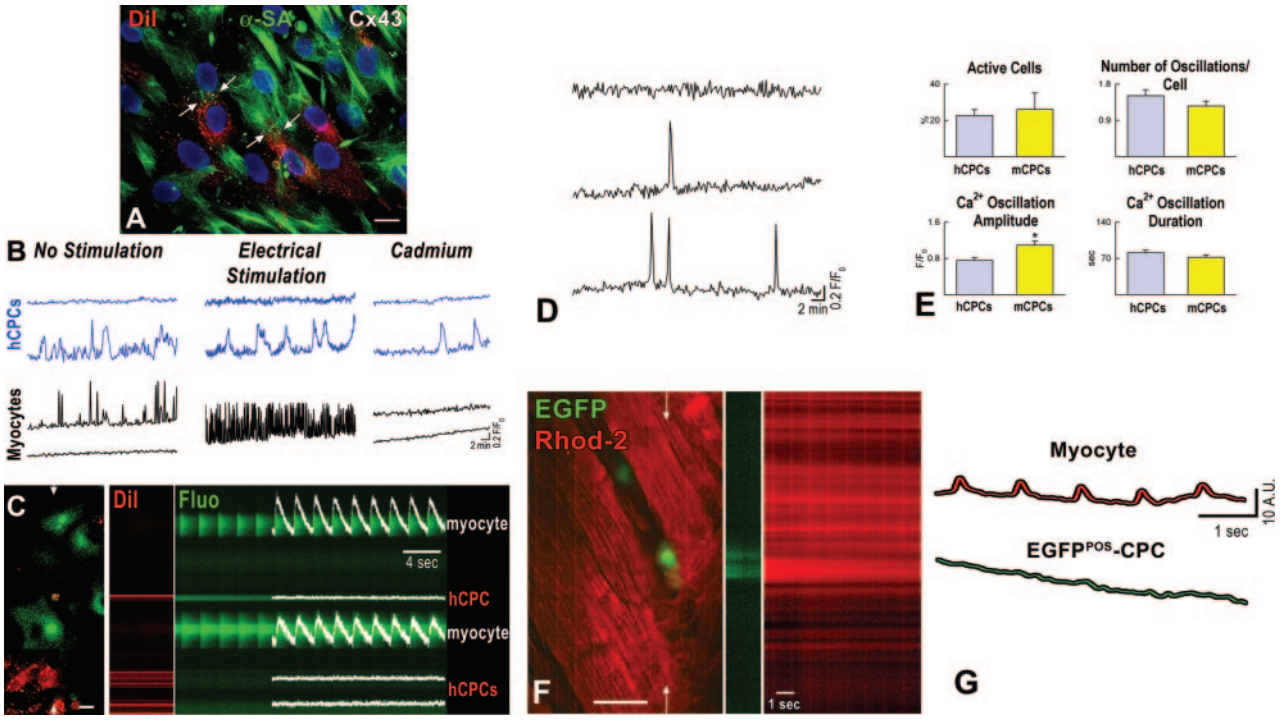


**Figure 3.** $Ca^{2+}$ cycling in myocytes and CPCs. A, Connexin 43 (Cx43) (white) between DiI-labeled hCPCs (DiI) (red) and myocytes ($\alpha$-SA) (green). Nuclei are identified by DAPI. Scale bars: 10 $\mu$m. B, Intracellular $Ca^{2+}$ in hCPCs (blue traces) and adjacent cocultured neonatal myocytes (black traces). The effects of electric stimulation and cadmium chloride are also shown. Different cells were used in the 3 conditions. C, Intracellular $Ca^{2+}$ in hCPCs and neonatal myocytes in line scan mode. Red identifies hCPCs loaded with DiI, and green corresponds to Fluo-3; scale bar: 20 $\mu$m. D, Cytosolic $Ca^{2+}$ in a quiescent mCPC (upper trace) and 2 mCPCs showing a single (middle trace) and multiple (lower trace) $Ca^{2+}$ oscillations. E, Properties of $Ca^{2+}$ oscillations in hCPCs and mCPCs. F, c-kit-EGFP mouse heart loaded with Rhod-2 (red), stimulated at 1 Hz and analyzed in line-scan mode (arrows). CPCs were identified by EGFP (green). G, $Ca^{2+}$ transients in myocytes (red trace) did not affect $Ca^{2+}$ levels in the neighboring EGFP-positive CPC (green trace). Identical results were obtained in 7 other experiments. Scale bars: 20 $\mu$m.

under the control of the c-kit promoter[14] was used to test whether $Ca^{2+}$ cycling in myocytes triggers $Ca^{2+}$ oscillations in CPCs in situ within the myocardium. Preliminary studies were conducted to evaluate whether EGFP-positive mouse CPCs (mCPCs) in vitro showed spontaneous $Ca^{2+}$ oscillations, mimicking the behavior of hCPCs. The percentage of mCPCs exhibiting $Ca^{2+}$ oscillation was comparable to that measured in human cells. Similarly, the rate of these events and their duration did not differ in these 2 cell classes, whereas the amplitude was larger in mCPCs (Figure 3D and 3E).

The heart of these transgenic mice was then examined ex vivo by 2-photon microscopy[3,13] following perfusion of the coronary circulation with the $Ca^{2+}$ indicator Rhod-2. The possibility that EGFP may interfere with the detection of Rhod-2 was excluded in preliminary studies conducted in EGFP-positive mouse myocytes (Online Figure VI). Based on these observations, the mouse heart was stimulated at 1 Hz and the $Ca^{2+}$ levels in EGFP-positive mCPCs were found not to be affected by the changes in $Ca^{2+}$ transients in neighboring cardiomyocytes (Figure 3F and 3G). Thus, human and mouse CPCs appear to possess an intracellular $Ca^{2+}$ regulatory system that is independent from that of terminally differentiated parenchymal cells.

## Intracellular $Ca^{2+}$ Control in hCPCs

We then determined whether activation of inositol 1,4,5-triphosphate (IP3) receptors (IP3Rs) and/or ryanodine receptors (RyRs) resulted in the release of $Ca^{2+}$ from the ER and $Ca^{2+}$ oscillatory events. Moreover, sarco-/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) is responsible for the reuptake of $Ca^{2+}$ into the ER and restoration of $Ca^{2+}$ stores.[10,11] Before conducting the functional studies, quantitative RT-PCR and immunolabeling were used to document in hCPCs the presence of transcripts and proteins for IP3Rs and SERCA. Both IP3Rs and SERCA were highly expressed in hCPCs. However, RyRs were not identified in these cells (Figure 4A through 4D).

Subsequently, (IP3) binding to IP3Rs was enhanced by thimerosal and this intervention markedly increased the number of hCPCs displaying $Ca^{2+}$ oscillations and the frequency of $Ca^{2+}$ oscillatory episodes per cell (Figure 4E and 4F). An opposite response was observed by inhibition of IP3R function with 2-amino-ethoxydiphenyl borate (2-APB) or xestospongin-C or attenuation of IP3 formation by phospholipase (PL)C blockade with U-73122 (Figure 4G and 4H). Similarly, inhibition of SERCA with cyclopiazonic acid reduced the fraction of activated hCPCs (Figure 4I and 4J). Additionally, $Ca^{2+}$ oscillations were smaller in amplitude and prolonged in duration, pointing to SERCA as a relevant component of hCPC function. Consistent with the lack of identification of RyRs in hCPCs, their agonists ryanodine and caffeine had no effects on $Ca^{2+}$ oscillations; the number of active cells and the frequency, amplitude, and duration of $Ca^{2+}$ events did

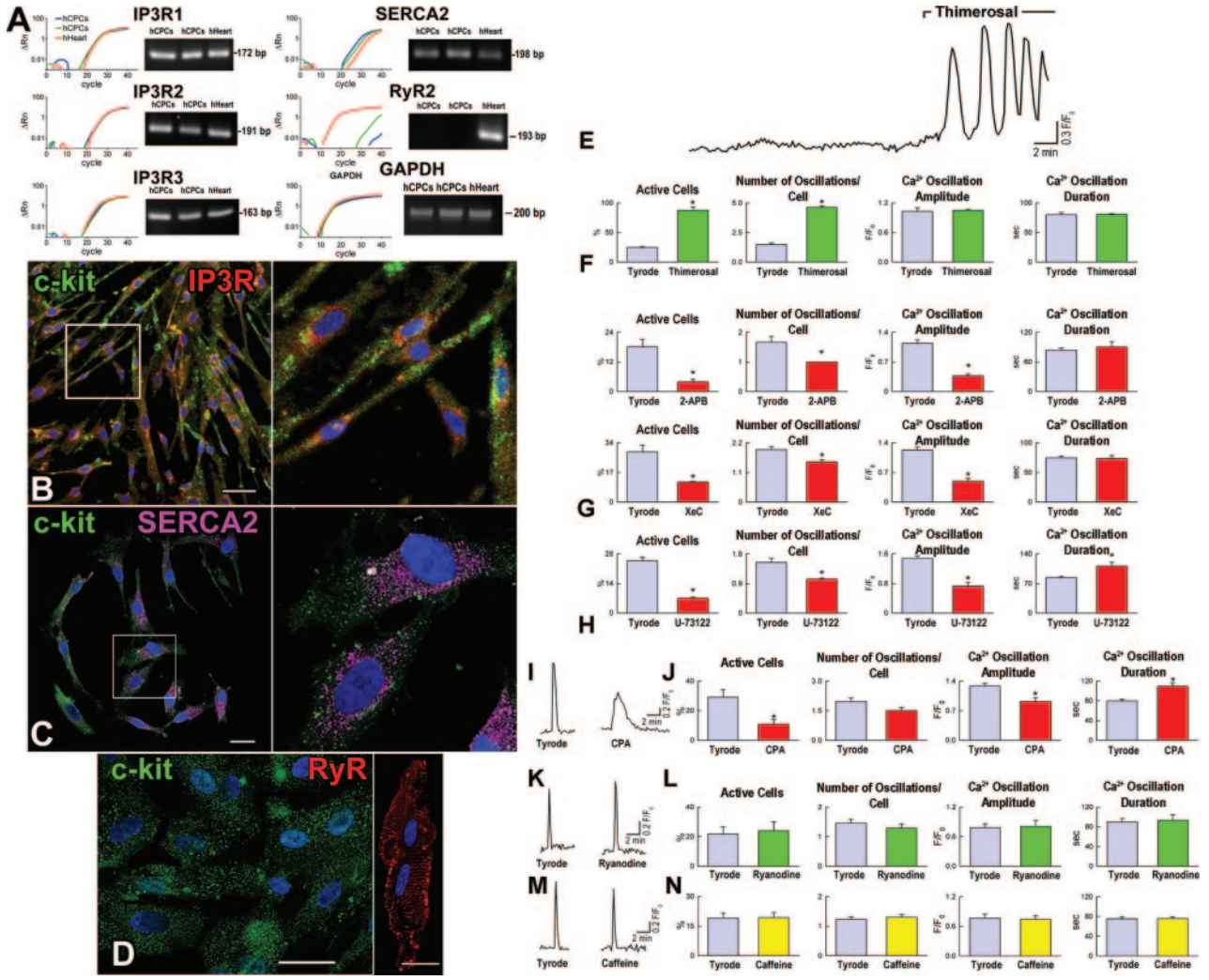


**Figure 4.** $Ca^{2+}$ regulatory proteins in hCPCs. A through D, Expression at the mRNA (A) and protein (B through D) levels of the components of the ER that are implicated in $Ca^{2+}$ homeostasis. Myocytes were used as positive control for RyRs. Human heart (hHeart) was used as positive control. Scale bars: 20 $\mu$m. E, Repetitive $Ca^{2+}$ oscillations in hCPCs in the presence of IP3R agonist. F through H, $Ca^{2+}$ oscillations in hCPCs at baseline (Tyrode) and in the presence of activation (F) and inhibition (G and H) of IP3R function. XeC indicates xestospongin-C. I through N, $Ca^{2+}$ in hCPCs in the presence of modulators of SERCA (I and J) and RyRs (K through N). *$P<0.05$ vs Tyrode.

not differ from baseline (Figure 4K through 4N). These data point to the IP3-IP3R system and SERCA as the predominant modulators of $Ca^{2+}$ in hCPCs.

ATP-specific P2-purinoceptors (P2Y2) and histamine receptor-1 (H1) are $G_q$ protein–coupled receptors that are implicated in the release of intracellular $Ca^{2+}$ from the ER by activation of PLC, IP3 synthesis, and, ultimately, IP3R binding.[15,16] P2Y2 and H1 receptors were expressed at the mRNA and protein level in hCPCs (Figure 5A through 5C), suggesting that they may be implicated in $Ca^{2+}$ cycling of these progenitor cells. In the presence of ATP or histamine, a more than 3-fold increase in the number of hCPCs exhibiting oscillations was detected. Similarly, the number of events per cell markedly increased, whereas the amplitude and duration of $Ca^{2+}$ elevations did not change (Figure 5D through 5G). Inhibition of IP3Rs or IP3 formation prevented the effects of ATP and histamine on $Ca^{2+}$ mobilization (Online Figure VII).

Because PLC-$\beta$3 may be modulated by P2Y2 receptor activation,[17] the expression of PLC-$\beta$ subunits was evaluated in hCPCs. Additionally, the functional role of PLC-$\beta$3 in mediating intracellular $Ca^{2+}$ mobilization on ATP stimulation was established. PLC-$\beta$3 mRNA in hCPCs was significantly higher than that of the other subunits (Online Figure VIII, A through C). Thus, siRNA strategy was used to downregulate PLC-$\beta$3 in hCPCs before their stimulation with ATP. This intervention markedly attenuated the ability of ATP to dramatically increase the pool of hCPCs displaying $Ca^{2+}$ oscillations (Online Figure VIII, D and E).

Although IP3R-mediated $Ca^{2+}$ mobilization from the ER is responsible for the generation of $Ca^{2+}$oscillations in hCPCs, transmembrane $Ca^{2+}$ fluxes maybe operative contributing to intracellular calcium cycling. Exposure of hCPCs to $Ca^{2+}$-free medium did not alter the frequency and characteristics of $Ca^{2+}$ oscillations (Online Figure IX), strengthening the role of intracellular stores as the source of $Ca^{2+}$ for oscillatory events. Immunolabeling and PCR data, together with patch-clamp and cytosolic $Ca^{2+}$ imaging (Online Figures X and XI) revealed that store-operated channels, $Na^+/Ca^{2+}$ exchanger,

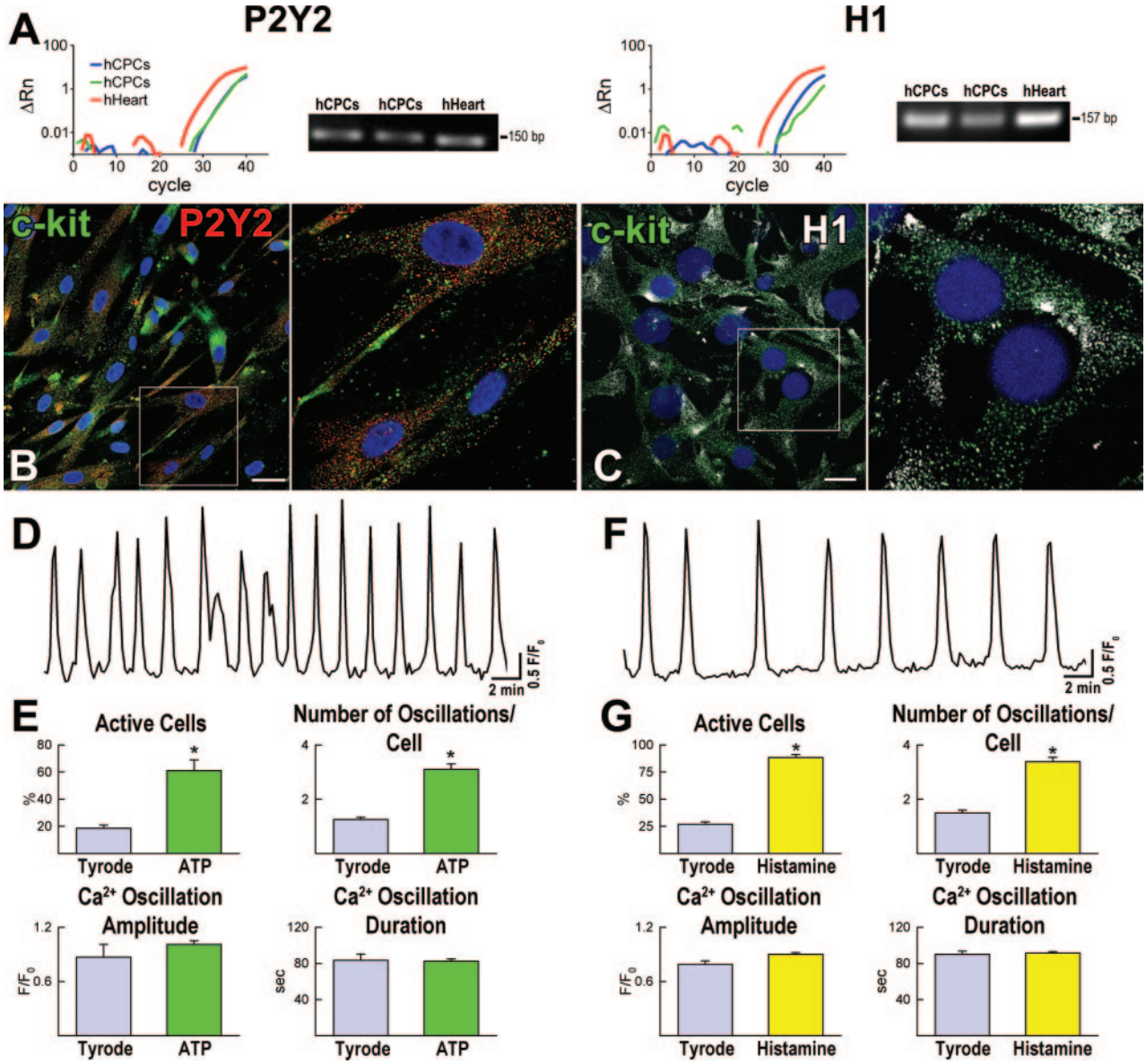


**Figure 5.** $G_q$ protein–coupled receptors and intracellular $Ca^{2+}$ in hCPCs. A through C, Expression at the mRNA (A) and protein (B and C) levels of P2Y2 and H1 receptors in hCPCs. Human heart (hHeart) was used as positive control. Scale bars: 10 $\mu$m. D through G, $Ca^{2+}$ oscillations in hCPCs in the presence of ATP (D and E) or histamine (F and G). *$P<0.05$ vs Tyrode.

and plasma membrane $Ca^{2+}$ pump were functional in hCPCs. However, these systems did not appear to participate in the generation of $Ca^{2+}$ oscillations in these primitive cells.

## $Ca^{2+}$ Oscillations and hCPC Growth

To evaluate the functional import of $Ca^{2+}$ oscillations in hCPC replication, these cells were cultured in serum-free medium for 24 hours and were exposed to either ATP or histamine to induce $Ca^{2+}$ release from the ER and oscillatory events. BrdUrd was added to the medium and its incorporation in hCPCs was measured 24 hours later. To exclude the potential confounding effect of cell death on the evaluation of cell proliferation, apoptosis was also determined. With either ATP or histamine, a nearly 2-fold increase in BrdUrd labeling of hCPCs was detected (Figure 6A and 6B). Conversely, inhibition of $Ca^{2+}$ release from the ER led to a decrease in BrdUrd incorporation in hCPCs to values lower than those seen at baseline (Figure 6A through 6C). Moreover, purinergic stimulation failed to promote proliferation of hCPCs when PLC-$\beta$3 was downregulated and the P2Y2-IP3R axis was disrupted (Online Figure XII). ATP and histamine had no influence on hCPC apoptosis (Figure 6D and 6E), suggesting that increases in $Ca^{2+}$ oscillations were not coupled with the activation of the cell death program.

To strengthen the possibility of a cause and effect relationship between $Ca^{2+}$ oscillations and cell cycle activation in hCPCs, the impact of a well-established activator of progenitor cell division, insulin-like growth factor (IGF)-1, was determined. Resident cardiac progenitors express IGF-1 receptors (Figure 6F and 6G) and synthesize and secrete the ligand.[6] IGF-1 increased the percentage of hCPCs showing $Ca^{2+}$ oscillations by nearly 3-fold (Figure 6H and 6I).

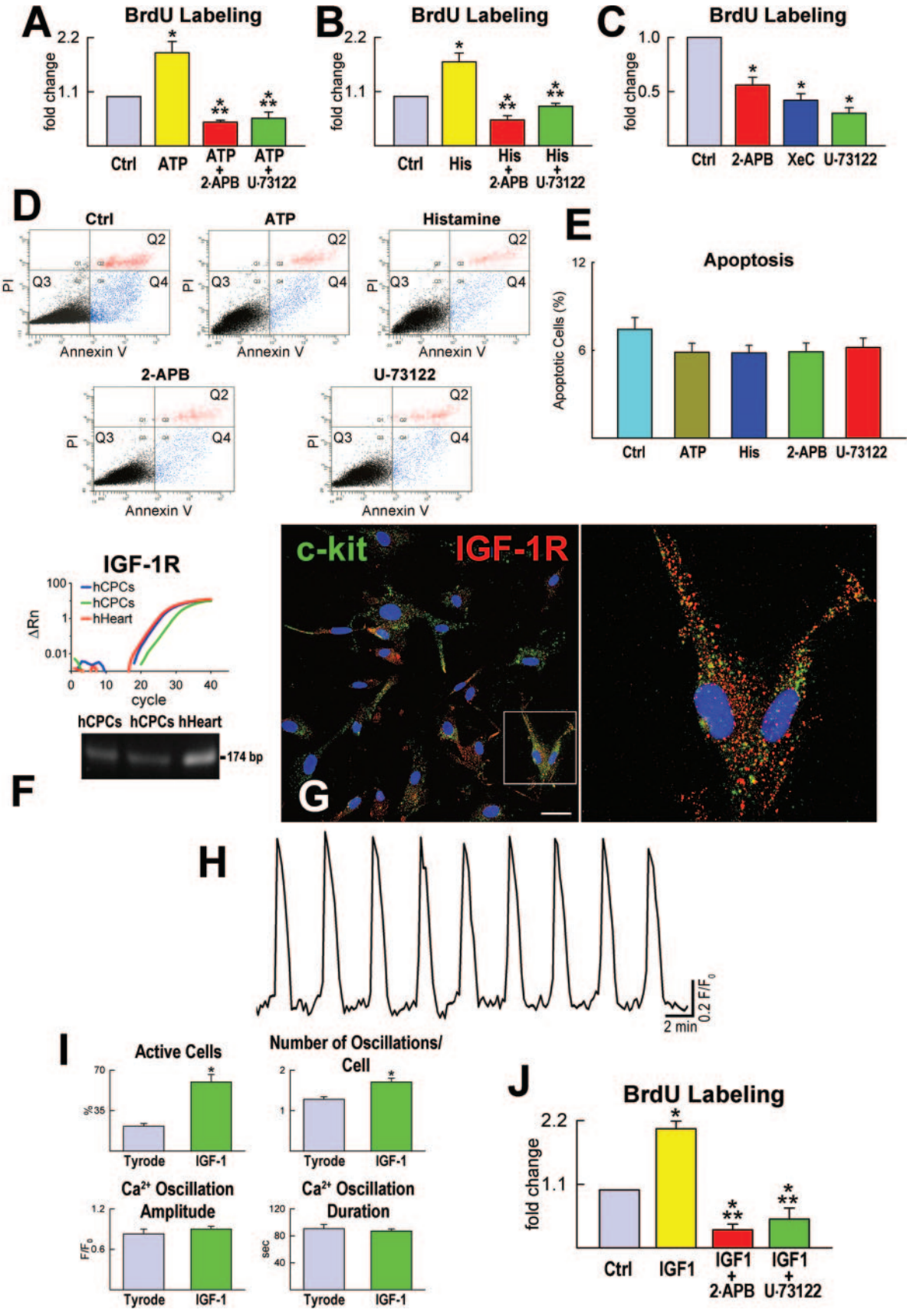


**Figure 6.** hCPC growth and apoptosis. A through C, ATP and histamine increase $Ca^{2+}$ oscillations and proliferation of hCPCs. Inhibitors of $Ca^{2+}$ oscillations prevent the effects of ATP and histamine. Ctrl indicates control; His, histamine. *$P<0.05$ vs Ctrl, **$P<0.05$ vs agonist. D and E, Apoptosis of hCPCs measured by Annexin V labeling and fluorescence-activated cell-sorting analysis. PI indicates propidium iodide; Q2,

Similarly, there was an increase in the number of $Ca^{2+}$ oscillations per cell, whereas the amplitude and duration of $Ca^{2+}$ events remained essentially constant. When $Ca^{2+}$ oscillations were blocked, the growth promoting effects of IGF-1 on hCPCs were completely prevented. IGF-1 increased hCPC proliferation by ≈2-fold and blockade of $Ca^{2+}$ release from the ER decreased cell replication to levels below baseline values (Figure 6J). Similarly, $Ca^{2+}$ oscillations mediated by IGF-1 were abrogated by PLC and IP3R antagonists (supplemental Figure XIII).

To establish whether $Ca^{2+}$ oscillations in hCPCs favor the acquisition of the myocyte lineage, these cells were cultured in differentiating medium[3] in the absence or presence of ATP or histamine. After 1 week, the fraction of primitive cells expressing $\alpha$-sarcomeric actin was comparable in stimulated and nonstimulated hCPCs (Online Figure XIV), suggesting that these agents did not impact on the differentiation of hCPCs into the myocyte phenotype.

### $Ca^{2+}$ Oscillations in hCPCs and Myocardial Regeneration

In both animals and humans, shortly after ischemic myocardial injury, there is an increase of resident progenitors mostly restricted to the border zone of the infarcted heart.[1] These cells rapidly acquire the myocyte lineage and result in small foci of cardiac repair.[18] The possibility that factors naturally released in the damaged area facilitate this process was confirmed here in our transgenic mouse model in which EGFP labeling made rather easy the identification of c-kit–positive CPCs. Myocardial infarction at 2 days led to a 15- and 5-fold increase in CPCs in the region bordering and remote from the infarction, respectively (Online Figure XV).

However, spontaneous regeneration is severely limited and only a minimal fraction of a variety of progenitor cells delivered to the infarcted myocardium survives and integrates in the unfavorable environment of the necrotic tissue.[13,19] Activation of hCPCs with ATP and histamine may enhance their engraftment, growth and formation of a myocyte progeny. EGFP-labeled hCPCs were exposed to ATP or histamine 30 minutes before their injection in the area bordering an acute infarct in immunosuppressed mice. Untreated hCPCs were used as control. All cell preparations were serum-starved for 24 hours before ATP or histamine exposure. Animals were examined 2 days later when cell engraftment is completed, cell death is markedly attenuated and the number of cells available for cardiac repair is established.[13,20] Mice were exposed to BrdUrd to obtain cumulative values of cell regeneration. Additional groups of mice were euthanized at 7 days to asses the impact of this protocol on myocardial regeneration and cardiac function.

Of the $60\times10^3$ hCPCs injected in each heart, 7000 EGFP-positive cells were found in control hearts, whereas nearly 19 000 cells ($P<0.002$) were detected in hearts in which hCPCs were treated with ATP or histamine. Cell engraftment was confirmed by the detection of connexin 43 and N-cadherin at the interface of hCPCs and spared myocytes (Online Figure XVI). Moreover, the number of EGFP-positive cells labeled by BrdUrd was 10- to 12-fold higher in infarcts injected with ATP or histamine activated hCPCs (Figure 7A through 7C). Most importantly, the aggregate number of EGFP-positive cells expressing the myocyte transcription factors Nkx2.5 was ≈4-fold larger in hearts treated with cells exposed to ATP or histamine.

Seven days after coronary artery occlusion and cell implantation, the extent of myocardial regeneration associated with the delivery of activated hCPCs was markedly superior to that obtained with untreated cells. The number of newly formed cardiomyocytes with ATP-histamine treatment was ≈3-fold greater than in controls. However, the volume of cardiomyocytes was similar in the 2 groups (Figure 8A and 8B). Importantly, left ventricular (LV) hemodynamics revealed that pretreatment of hCPCs with ATP or histamine enhanced the effects of cell transplantation and positively interfered with the deterioration of cardiac function following myocardial injury (Figure 8C).

## Discussion

The results of the present study indicate that hCPCs display spontaneous elevations in intracellular $Ca^{2+}$ attributable to IP3R-mediated $Ca^{2+}$ release from the ER. Reuptake of $Ca^{2+}$ into the ER is accomplished by SERCA, which replenishes the $Ca^{2+}$ stores, allowing repetitive oscillations with preserved amplitude and duration. The $Ca^{2+}$ handling molecules $Na^+/Ca^{2+}$ exchanger, plasma membrane $Ca^{2+}$ pump, and store-operated channels are functional and contribute to $Ca^{2+}$ homeostasis in hCPCs but are not implicated in the initiation and incidence of $Ca^{2+}$ oscillations in these undifferentiated cells. Agonists of $G_q$ protein–coupled receptors and histamine and ATP stimulate PLC and IP3 formation, leading to an increase in the number of activated hCPCs and frequency of $Ca^{2+}$ oscillatory episodes per cell in vitro. These $Ca^{2+}$ oscillations promote hCPC proliferation, documenting that cytosolic $Ca^{2+}$ plays a primary role in hCPC growth. Induction of $Ca^{2+}$ oscillatory events in hCPCs before their intramyocardial delivery in vivo was coupled with enhanced engraftment of these cells within the infarcted heart, their expansion in the unfavorable environment of the necrotic tissue, and the generation of a myocyte progeny.

### Origin of $Ca^{2+}$ Oscillations in hCPCs

In the present study, a fundamental issue in need of resolution involved the recognition whether $Ca^{2+}$ oscillations in hCPCs represent an intrinsic property of these primitive cells or the consequence of $Ca^{2+}$ entry from cardiomyocytes and/or the extracellular compartment. Collectively, our results on the regulation of $Ca^{2+}$ in hCPCs in vitro and in mouse CPCs within the myocardium in ex vivo preparations, suggest that cell-to-cell communication and the interstitial milieu are not implicated in the rapid and transient elevations of $Ca^{2+}$ in CPCs. Under our experimental conditions, $Ca^{2+}$ cycling in myocytes appears to have no influence on $Ca^{2+}$ oscillations in CPCs. However, changes in the rate and amplitude of $Ca^{2+}$ tran-

**Figure 6 (Continued).** late apoptotic or necrotic cells; Q3, alive cells; Q4, cells undergoing apoptosis. F and G, IGF-1R transcript and protein in hCPCs and human heart (hHeart). Scale bar: 20 $\mu$m. Right image in G illustrates selected cells at higher magnification. H and I, Intracellular $Ca^{2+}$ in hCPCs exposed to IGF-1. *$P<0.05$ vs Tyrode. J, Proliferation of hCPCs in the presence of IGF-1 alone or in combination with inhibitors of $Ca^{2+}$ oscillations. *$P<0.05$ vs Ctrl; **$P<0.05$ vs IGF-1.

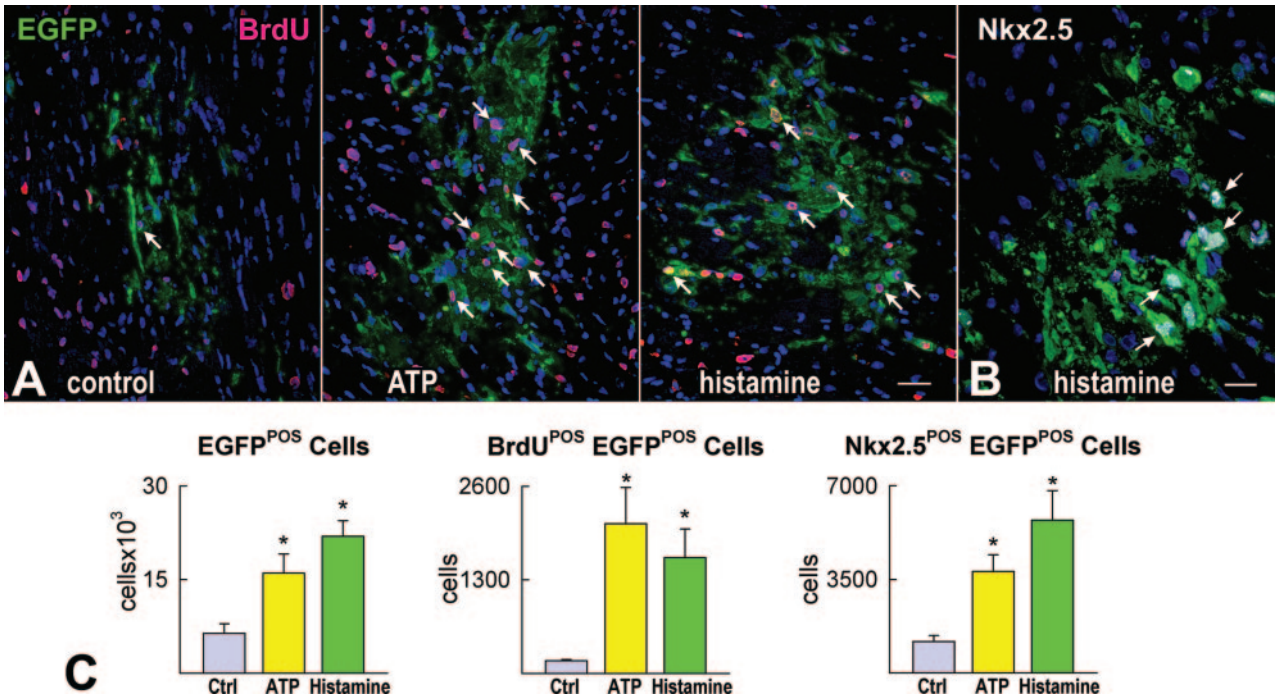


**Figure 7.** $Ca^{2+}$ oscillations and growth of hCPCs in vivo. A, EGFP-positive hCPCs 48 hours after implantation in the infarcted mouse heart under control conditions and following activation of hCPCs with ATP or histamine. Proliferation of EGFP-positive cells (green) is documented by BrdUrd labeling (magenta, arrows). B, Nkx2.5 (white) is present in several EGFP-positive cells (arrows). Scale bars: 20 $\mu$m. C, Results are shown as means±SEM. *$P<0.05$ vs control.

sients in myocytes may affect $Ca^{2+}$ loading in CPCs. Cardiomyocytes function as supporting cells in myocardial niches[7] and are connected by gap and adherens junctions to CPCs making them the ideal candidate for the translocation of $Ca^{2+}$ and the initiation of oscillatory processes in CPCs. Although this was not found to be the case, this phenomenon may occur later during differentiation; the intercellular passage of $Ca^{2+}$ may activate in lineage committed CPCs the release of $Ca^{2+}$ from the ER conditioning the acquisition of the adult cardiomyocyte phenotype and contractile function.

The most significant regulator of IP3R-mediated $Ca^{2+}$ release from the ER is $Ca^{2+}$ itself. IP3R open channel probability is stimulated at low $[Ca^{2+}]_i$, whereas high $[Ca^{2+}]_i$ exerts an inhibitor effect.[16] Accordingly, changes in $[Ca^{2+}]_i$ may initiate and end $Ca^{2+}$ oscillations in hCPCs. IP3 regulates IP3R channels mainly by enhancing their sensitivity to $Ca^{2+}$. Two receptor ligand systems, P2Y2-ATP and H1-histamine, were identified in hCPCs and their importance in the modulation of intracellular $Ca^{2+}$ and progenitor cell growth was defined in vitro and in vivo to characterize their potential function in cardiac homeostasis and regeneration. Importantly, the doses of ATP and histamine used here have previously been shown to exert a powerful effect on $Ca^{2+}$ mobilization in other cell systems.[21,22]

In physiological conditions, cell loss by normal wear and tear may result in an increase in the local level of ATP[15] leading to $Ca^{2+}$ oscillations in neighboring hCPCs, which, in turn, activate cell replication and expansion. Additionally, the release of ATP from synaptic vesicles in terminal nerves[15] may produce a comparable role in the hCPC compartment. These 2 mechanisms of ATP accumulation in the interstitial space are enhanced by prolonged myocardial ischemia and myocyte death.[15,23] Cardiac pathology potentiates the load on the spared myocardium and mechanical stretch further enhances exocytosis of ATP from cardiomyocytes.[15] In humans and animals, myocardial infarction is associated with CPC division and the generation of functionally competent cardiomyocytes,[1] supporting the notion that ATP-mediated CPC growth may be critical for cardiac repair.

Mast cells are the predominant source of histamine in the myocardium.[24] The number of mast cells and CPCs is comparable in the rodent and human heart.[3,7,25,26] Additionally, tissue damage and inflammation recruit mast cells and CPCs, suggesting that histamine released from mast cells may be implicated in the activation of cardiac progenitors and the creation of myocytes. ATP is formed largely by cardiomyocytes that represent ≈85% of the myocardium, whereas histamine is synthesized by a very small number of mast cells, ≈2 to 3/mm$^2$ of tissue.[15,25,26] However, extracellular ATP is rapidly degraded to inactive ADP. Conversely, histamine has a longer half-life time,[15,27] indicating that these 2 molecules may have complementary function in the modulation of hCPC function.

As shown here for hCPCs, IP3R-mediated $Ca^{2+}$ mobilization from the ER has been reported in human mesenchymal stem cells[28] and mouse embryonic stem cells.[29,30] In both cases, the increases in intracellular $Ca^{2+}$ have been linked to cell growth and lineage specification. Embryonic stem cells and hCPCs differentiate into cardiomyocytes and the characteristics of $Ca^{2+}$ cycling in these cells is regulated by the $Ca^{2+}$-induced $Ca^{2+}$-release mechanism that controls myocyte mechanics and ventricular function in vivo.[11] Whether mes-

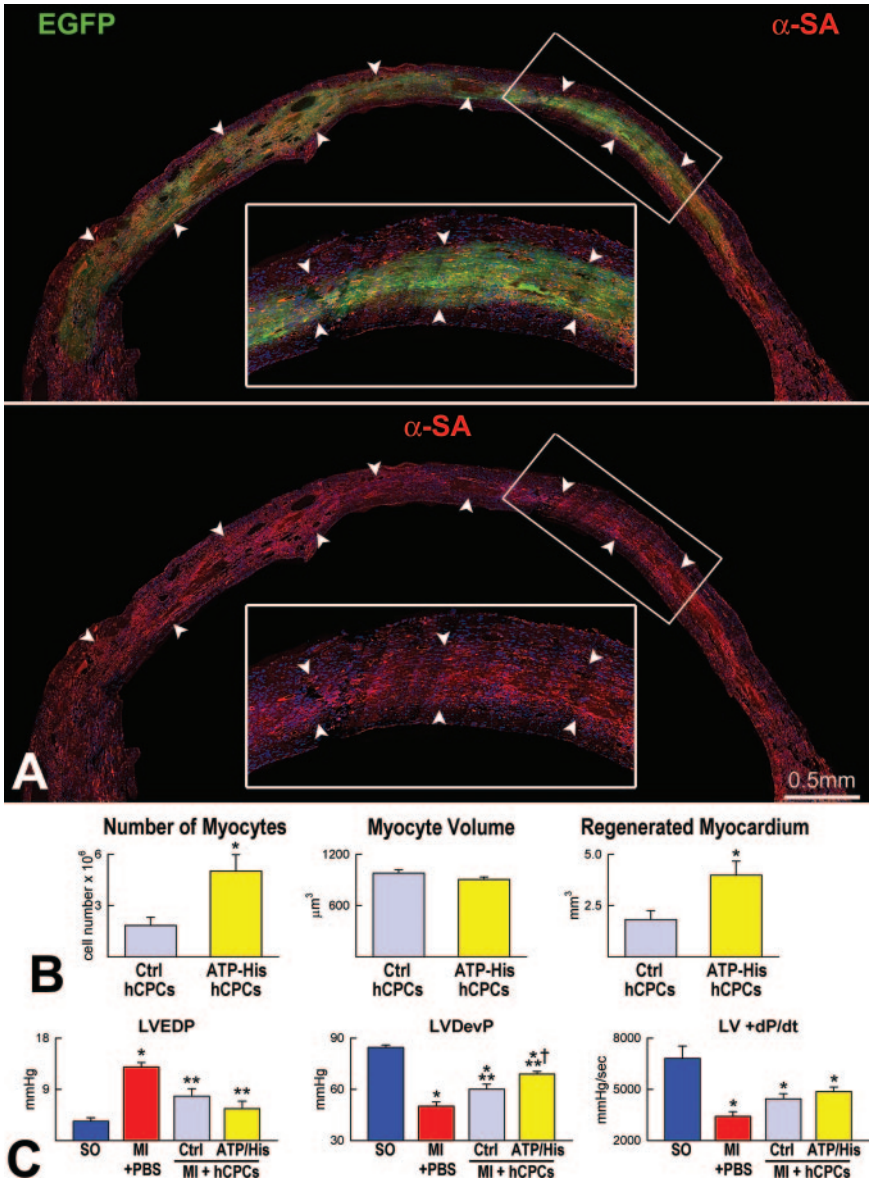


**Figure 8.** Myocardial regeneration by activated hCPC. A, Mouse heart treated with histamine-stimulated hCPCs. The midportion of the infarct is replaced by EGFP-positive (upper, green) and α-SA–positive (lower, red) cardiomyocytes. The area in the rectangle is shown at higher magnification in the inset. B, Extent of regeneration mediated by nonactivated hCPCs (Ctrl hCPCs) or hCPCs exposed to ATP or histamine (ATP-His hCPCs). C, LV function in sham-operated (SO), infarcted untreated (MI+PBS), and infarcted hCPC-treated (MI+hCPCs) mice 7 days after coronary ligation. Ctrl, ATP, and His indicate nonstimulated, ATP-stimulated, and histamine-stimulated hCPCs, respectively; LVEDP, LV end-diastolic pressure; LVDevP, LV developed pressure. *$P<0.05$ vs SO; **$P<0.05$ vs MI+PBS; †$P<0.05$ vs MI injected with untreated hCPCs.

enchymal stem cells have a similar capacity is currently debatable.

## IGF-1 and $Ca^{2+}$ Oscillations in hCPCs

The function of IGF-1 is largely mediated by binding to the receptor tyrosine kinase IGF-1 receptor (IGF-1R). Phosphorylation of IGF-1R leads to recruitment of the insulin receptor substrate protein (IRS)-1 that modulates the effects of IGF-1R on cellular responses in the heart. The recruitment of IRS-1 upregulates phosphatidylinositol 3-kinase, which phosphorylates Akt; Akt activation favors cell differentiation, hypertrophy, or proliferation.[31,32] IRS-1 also promotes the interaction of Ras, Raf, and extracellular signal-regulated kinase, which may lead to cellular hypertrophy or division.[33] Surprisingly, in the present study, IGF-1 induced $Ca^{2+}$ oscillatory episodes in hCPCs through the activation of IP3Rs and the release of $Ca^{2+}$ from the ER. Whether this was a direct effect or was mediated by the generation of IP3 is currently unclear. However, the mitogenic properties of IGF-1 appear to be mediated, at least in part, by the release of $Ca^{2+}$ from the ER, strengthening the notion that $Ca^{2+}$ mobilization via IP3R is involved in cell cycle progression and growth of hCPCs.

## $Ca^{2+}$ Oscillations and hCPC In Vivo

A critical component of cell therapy is related to the recognition of the variables implicated in the engraftment and expansion of the delivered cells within the damaged myocardium.[34] The function of progenitor cells is determined by causes inherent to the cells and risk factors for cardiovascular diseases. The former includes the telomere–telomerase axis, DNA damage, and the expression of genes implicated in the forced entry of cells into in an irreversible quiescent state and/or activation of the endogenous cell death program.[6,35] The latter involves several pathological states such as diabetes, hypertension, coronary artery disease, valvular defects, dilated cardiomyopathy, and myocardial aging.[34]

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## Disclosures

None.

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