P2Y₂ Nucleotide Receptor Prompts Human Cardiac Progenitor Cell Activation by Modulating Hippo Signaling

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Rationale: Autologous stem cell therapy using human c-Kit⁺ cardiac progenitor cells (hCPCs) is a promising therapeutic approach for treatment of heart failure (HF). However, hCPCs derived from aged patients with HF with genetic predispositions and comorbidities of chronic diseases exhibit poor proliferative and migratory capabilities, which impair overall reparative potential for injured myocardium. Therefore, empowering functionally compromised hCPCs with proregenerative molecules ex vivo is crucial for improving the therapeutic outcome in patients with HF.

Objective: To improve hCPC proliferation and migration responses that are critical for regeneration by targeting proregenerative P2Y₂ nucleotide receptor (P2Y₂R) activated by extracellular ATP and UTP molecules released following injury/stress.

Methods and Results: c-Kit⁺ hCPCs were isolated from cardiac tissue of patients with HF undergoing left ventricular assist device implantation surgery. Correlations between P2 nucleotide receptor expression and hCPC growth kinetics revealed downregulation of select P2 receptors, including P2Y₂R, in slow-growing hCPCs compared with fast growers. hCPC proliferation and migration significantly improved by overexpressing or stimulating P2Y₂R. Mechanistically, P2Y₂R-induced proliferation and migration were dependent on activation of YAP (yes-associated protein)—the downstream effector of Hippo signaling pathway.

Conclusions: Proliferation and migration of functionally impaired hCPCs are enhanced by P2Y₂R-mediated YAP activation, revealing a novel link between extracellular nucleotides released during injury/stress and Hippo signaling—a central regulator of cardiac regeneration. Functional correlations exist between hCPC phenotypic properties and P2 purinergic receptor expression. Lack of P2Y₂R and other crucial purinergic stress detectors could compromise hCPC responsiveness to presence of extracellular stress signals. These findings set the stage for subsequent studies to assess purinergic signaling modulation as a potential strategy to improve therapeutic outcome for use of hCPCs in patients with HF. (Circ Res. 2017;121:1224-1236. DOI: 10.1161/CIRCRESAHA.117.310812.)

Key Words: adult stem cells ■ heart failure ■ nucleotides

Heart failure (HF) secondary to cardiomyopathy is a leading cause of death in the US and worldwide, necessitating developing alternative therapeutic strategies to tackle the progression of HF and alleviate its symptoms. Autologous stem cell therapy has been implemented as a promising therapeutic approach for HF for over a decade. c-Kit⁺ cardiac-derived progenitor cells (CPCs) improve cardiac function after myocardial infarction in animal models. In comparison, adoptive transfer of autologous c-Kit⁺ CPCs into patients with pathologically injured myocardium yields modest and more variable outcomes in clinical trials. Inconsistent findings in the clinical setting are likely due, at least in part, to severely compromised regenerative potential of stem cells isolated from patients with HF with genetic predispositions, comorbidities of chronic diseases, such as hypertension and diabetes mellitus, and daily life stressors, such as smoking and alcoholism. Therefore, enhancing regenerative capacity of stem cells ex vivo before transplantation is an intervention strategy to improve outcome of stem cell therapy as exemplified by empowering stem cells from diverse origins with prosurvival and antiapoptotic genes. Regenerative capacity of...
Regenerative responses are poorly understood. Extracellular nucleotides represent a major class of stress signals that accumulate in the extracellular milieu in response to injury/stress. Extracellular nucleotides bind to and activate transmembrane purinergic receptors that are categorized into P1 receptors (activated by adenosine) and P2 receptors (activated by ATP, ADP, UTP, UDP, and UDP sugars). P2 receptors comprise 7 P2X ligand-gated ion channels (P2X1-7) and 8 P2Y G-protein-coupled receptors (P2Y1,2,4,6,11,12,13,14). Whereas some P2 receptors initiate early inflammatory responses, others mediate later regenerative responses required for the healing process.

P2Y2R nucleotide receptor (P2Y/R) is a prorregenerative Gqα protein-coupled receptor activated by ATP and UTP, which are released during cardiac ischemia.6–12 P2Y/R plays a central role in intracellular signaling by enabling extracellular ATP and UTP to promote regenerative responses in a variety of tissues. P2Y/R regulates corneal epithelia wound healing11 and salivary gland reconstitution14 by inducing cell migration, liver regeneration by promoting hepatocyte proliferation,15 and reepithelialization after experimental colitis16 and inflammatory bowel disease.17 On the stem cell level, UTP is a potent stimulant of human hematopoietic stem cell migration.18 Herein, we hypothesize that P2Y/R induces proliferative and migratory responses in functionally compromised human CPCs (hCPCs) derived from patients with HF.

Gαq protein-coupled signaling regulates cell proliferation and migration through activation of YAP (yes-associated protein)—the downstream effector of Hippo signaling.19 Given the importance of purinergic signaling in stress responses and sensing environmental damage, phenotypic associations should be present between growth potential of hCPCs derived from patients with HF and P2 purinergic receptor expression. Indeed, expression of select P2 receptors, including the prorregenerative P2Y/R, directly correlated with hCPC growth kinetics of human CPCs derived from patients with heart failure and expression levels of a subset of P2 purinergic receptors. Specifically, expression of several P2 receptors, including P2Y/R, known to mediate regenerative responses in various tissues, is diminished in slow-growing CPCs. CPC proliferation and migration was improved by augmenting P2Y/R levels or P2Y/R stimulation with UTP via inhibiting Hippo signaling and activating the concordant downstream effector yes-associated protein. Overall, impaired functional capacity of CPCs could be caused, in part, by lack of purinergic receptor expression that impairs responsiveness to extracellular nucleotides. Findings from this study suggest modulating purinergic signaling as part of a multifaceted approach to enhance CPC functional activity. Mechanistically, this report introduces P2Y/R as a novel upstream regulator of Hippo signaling revealing a link between extracellular nucleotides released during injury/stress and yes-associated protein that is critical for CPC growth and myocardial repair in response to injury.

### What Is Known?
- Aged/diseased cardiac progenitor cells (CPCs) derived from patients with heart failure exhibit functional impairment because of inherent molecular deficits.
- P2 purinergic receptors regulate crucial inflammatory and regenerative responses in cardiovascular system with largely undefined roles in CPCs.

### What New Information Does This Article Contribute?
- Expression levels for a subset of P2 receptors, including proregenerative P2Y2 receptor (P2Y2R), correlate with altered phenotypic properties of CPCs isolated from cardiac biopsies of patients with heart failure.
- Proliferative and migratory responses of functionally compromised CPCs are improved by P2Y2R activation or overexpression that is associated with modulating yes-associated protein activity.

CPC function deteriorates with age and is further compromised by chronic diseases and environmental stresses. Identification of inherent molecular deficits in aged/diseased CPCs will be valuable information to design interventional approaches to improve phenotypic characteristics that restore functional competency. Herein, we report significant correlations between growth kinetics of human CPCs derived from patients with heart failure and expression levels of a subset of P2 purinergic receptors. Specifically, expression of several P2 receptors, including P2Y/R, known to mediate regenerative responses in various tissues, is diminished in slow-growing CPCs. CPC proliferation and migration was improved by augmenting P2Y/R levels or P2Y/R stimulation with UTP via inhibiting Hippo signaling and activating the concordant downstream effector yes-associated protein. Overall, impaired functional capacity of CPCs could be caused, in part, by lack of purinergic receptor expression that impairs responsiveness to extracellular nucleotides. Findings from this study suggest modulating purinergic signaling as part of a multifaceted approach to enhance CPC functional activity. Mechanistically, this report introduces P2Y/R as a novel upstream regulator of Hippo signaling revealing a link between extracellular nucleotides released during injury/stress and yes-associated protein that is critical for CPC growth and myocardial repair in response to injury.
Human Cardiac Progenitor Cell Isolation

Human CPCs were isolated from cardiac tissue specimens derived from patients undergoing left ventricular assist device implantation surgeries as described previously. Briefly, tissue was minced, digested in collagenase (150 U mg/mL; Worthington Bio Corp) for 2 hours at 37°C, then incubated with c-Kit–labeled beads (Miltenyi Biotec), and sorted according to the manufacturer’s protocol. Pelleted cells were cultured in hCPC growth media: Hams F12 (Fisher Scientific), 10% embryonic stem cell screened fetal bovine serum, 1% penicillin/streptomycin/glutamine, 5 mU/mL human erythropoietin (Sigma-Aldrich), and 10 ng/mL basic fibroblast growth factor (Peprotech), and incubated at 37°C, then incubated with c-Kit–labeled beads (Miltenyi Biotec), and sorted according to the manufacturer’s protocol. Pelleted cells were cultured in hCPC growth media: Hams F12 (Fisher Scientific), 10% embryonic stem cell screened fetal bovine serum, 1% penicillin/streptomycin/glutamine, 5 mU/mL human erythropoietin (Sigma-Aldrich), 10 ng/mL basic fibroblast growth factor (Peprotech), and incubated at 37°C in a humidified atmosphere of 5% CO2 and 95% air. hCPCs with doubling time ≤24 hours were considered as fast growers (F-hCPCs), whereas hCPCs with doubling time >24 hours were considered as slow growers (S-hCPCs).

Real-Time Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was isolated from hCPCs using Quick-RNA MiniPrep (Zymo Research), and cDNA was synthesized using iScript cDNA Synthesis Kit (Bio Rad) according to manufacturers’ protocols. Samples were prepared for quantitative reverse transcriptase–polymerase chain reaction using iQ SYBER Green (Bio Rad). Primer sequences are listed in Online Table I.

Calcium Imaging

Cultured cells on glass-bottom plates grown to 60% to 70% confluency were loaded for 1 hour with the calcium-dependent ratiometric dye Fura-2 AM (4 µM) in Krebs–Ringer solution (125 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 2 mM CaCl2, 10 mM NaHCO3, 25 mM HEPES, and pH was adjusted to 7.4 with NaOH). Afterward, cells were washed in Krebs–Ringer solution for 30 minutes to allow for de-esterification of Fura-2 AM dye. Ca2+ imaging data were collected using an inverted fluorescent microscope (Leica) where the excitation wavelength was altered between 340 and 387 nm (F340 and F387), and emission was detected at 510 nm. Data were analyzed using iQ Image (Leica) where the excitation wavelength was altered between 340 and 387 nm (F340 and F387), and emission was detected at 510 nm.

Cell Migration Assay

hCPC single-cell suspensions were seeded in serum-free Hams F12 media in a 96-well plate coated with growth factor reduced Matrigel (BD Biosciences; 1600 cells/well) and incubated at 37°C in a humidified atmosphere of 5% CO2 and 95% air for 2 hours. Then the cell culture plate was mounted on a DM16000 live cell imaging microscope (Leica) equipped with a digital camera, an automatic shutter, a motorized x–y stage and an OKO stage top incubator (37°C, 5% CO2, and 95% air). A field of cells was located within each well with a 5× objective and marked for monitoring during the duration of the experiment. The exposure time was kept constant for all positions and all time points. Bright field images of cells were obtained every 30 minutes for 6 hours. Cell migration was assessed by measuring distance travelled from origin using Leica LAX software. Cell velocity was calculated by dividing distance travelled from origin over time. In inhibitor studies, cells were pretreated with YAP selective inhibitor (verteporfin; 100 nM; Tocris Bioscience) for the indicated times before UTP treatment.

Protein Isolation, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis, and Immunoblot Analysis

hCPCs were seeded in a 6-well plate (30000 cells/well). Next day, cells were treated with or without UTP (100 µM) in serum-starved medium for the indicated times. Samples were collected in 50 µL of sample buffer, sonicated, and boiled. Protein lysates were run on 4% to 12% NuPage Novex Bis Tris gel (Invitrogen), transferred to a polyvinylidene fluoride membrane, blocked in 5% skim milk in Tris-buffered saline Tween-20 solution for 1 hour at room temperature, then incubated with primary antibodies overnight at 4°C. Membranes were incubated with secondary antibodies (1:1000–1:5000) for 1 hour at room temperature after several washes with...
Tris-buffered saline Tween-20. Fluorescence signal was detected using Typhoon or LI-COR fluorescent scanners and quantitated using ImageJ software (Amersham Biosciences). Antibodies used are listed in Online Table II.

Nuclear/Cytoplasmic Fractionation
hCPCs were cultured in 100-mm dishes (180 000 cells/well). The following day, cells were treated with or without UTP (100 µM) in serum-starved medium for the indicated times. Preparation of separate nuclear and cytoplasmic lysates was performed using Paris Kit (Thermo Fisher Scientific) according to manufacturer's instructions. Briefly, adherent hCPCs were detached by trypsinizing for 3 to 5 minutes, and then trypsin was inactivated with hCPC growth medium. Cells were pelleted, culture medium was aspirated, cell pellet was gently resuspended in 100-µL ice-cold cell fractionation buffer and incubated on ice for 5 to 10 minutes. Samples were then centrifuged for 5 minutes at 4°C and 500 g. The supernatant (cytoplasmic fraction) was carefully transferred to a new tube. The pellet (nuclear fraction) was washed once with ice-cold cell fractionation buffer and centrifuged for 5 minutes at 4°C and 500 g. Fractionation buffer was aspirated. Sample buffer was added to nuclear pellet, and cytoplasmic fraction and samples were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by immunoblotting as described above. Antibodies used are listed in Online Table II.

Immunocytochemistry
hCPCs were cultured in 2-well chamber slides (15 000 cell/well). Next day, cells were treated with or without UTP (100 µM) in serum-starved medium for the indicated times. Cells were fixed with 4% paraformaldehyde for 10 minutes, washed twice (5 minutes each) with 1X phosphate-buffered saline (PBS), permeabilized with 1% Triton-X-100 for 10 minutes, washed twice (5 minutes each) with 1X PBS, then blocked with 10% horse serum for 45 minutes at 37°C. Cells were then washed in 1X PBS, then blocked with 10% horse serum for 45 minutes at 37°C. Then cells were treated with primary antibody in blocking solution (1:100) overnight at 4°C. The following day, cells were washed twice (5 minutes each) with 1X PBS, and then treated with secondary antibody in blocking solution (1:200; Invitrogen) for 1 hour at 37°C. Cells were then washed once (5 minutes) with 1X PBS, treated with the nuclear stain DAPI in 1X PBS (1:10 000; Sigma-Aldrich) for 1 minute. Finally, cells were rinsed in 1X PBS and mounted using VectaShield. Images were acquired in z-stacks using SP8 confocal microscope (Leica), and quantification of nuclear/cytoplasmic fraction was performed on maximum projection of stacked images and normalized to nuclear area. Antibodies used are listed in Online Table II.

Lentiviral-Mediated Transduction of Human CPCs
hCPCs were cultured in a 6-well plate (30 000 cells/well). The following day, hCPCs were transduced with lentivirus (0.2 multiplicity of infection [MOI]) encoding either monomeric green fluorescent protein (hCPC-mGFP) or P2Y2R fused to mGFP (hCPC-Y2; lentiviral plasmids were purchased from OriGene; SKU: RC22931L2). To knockdown P2Y2R, hCPCs were transduced with lentiviral particles encoding P2Y2R shRNA and mGFP (20 MOI; lentiviral plasmid was purchased from OriGene; SKU: TL302717; Gene identifier: 5029) or scrambled shRNA and enhanced GFP (eGFP; 2 MOI) as a control.22

Statistical Analysis
Quantitative results are presented as the means±SE of data from at least 3 experiments. Two-tailed Student t test or ANOVA followed by Dunnett or Bonferroni post hoc test was performed, as indicated, where P<0.05 represents a significant difference. Statistical analysis was performed using GraphPad prism, version 5.0 (GraphPad Software).

Results
Differential P2 Receptor Subset Expression in Fast- and Slow-Growing CPCs
hCPCs derived from multiple HF patients exhibit variation in growth rate previously characterized as fast growing (F-hCPC) or slow growing (S-hCPC).23 F-hCPCs are characterized by a spindly morphology and lower levels of senescence markers, whereas S-hCPCs exhibit a flat morphology associated with higher levels of senescence markers.24 P2 receptor mRNA expression levels were assessed by quantitative reverse transcriptase–polymerase chain reaction–based analysis in representative fast- and slow-growing hCPC lines (Table). Receptor mRNA expression was present for P2X4, P2X5, P2X6, P2Y1, P2Y2, P2Y4, P2Y11, and P2Y14 in all hCPC lines examined. However, several P2 receptors were differentially expressed between fast- and slow-growing lines: P2Y1, P2Y2, and P2Y14 were significantly downregulated in S-hCPCs compared with F-hCPCs (0.031±0.015-fold change, P=0.058±0.014-fold change, P=0.058±0.014-fold change, P=0.058±0.014-fold change, respectively; Figure 2). P2Y2R was particularly intriguing based on prior reports of involvement in regeneration using various

Table. Clinical Profile of Patients Used for Stem Cell Isolation

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<th>P2Y,R mRNA Levels (ΔCt)</th>
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Patient information: (+) positive; (−) negative; and (…) unavailable. EF indicates ejection fraction; hCPC, human cardiac progenitor cells; NYHA, New York Heart Association; and P2Y,R, P2Y, nucleotide receptor.
Higher P2Y2R mRNA expression levels corresponded with faster hCPC growth rates indicated by shorter doubling times ($R=0.7101; P=0.0369$; Online Figure IA). P2Y2R was also downregulated at the protein level in S-hCPCs compared with F-hCPCs (0.56±0.047-fold change; $P=0.0026$).

Improving CPC Proliferation and Migration by P2Y2R Overexpression

hCPC proliferation and migration potential was improved by increasing P2Y2R levels. hCPCs were infected with lentiviral particles encoding P2Y2R fused to mGFP (hCPC-Y2) or mGFP alone (hCPC-mGFP) as a control. Transduction efficiency was assessed by flow cytometric analysis for percentage of GFP + cells (56% for hCPC-mGFP and 50.1% for hCPC-Y2; Online Figure IIA), with confirmation of overexpression by quantitative reverse transcriptase–polymerase chain reaction showing increased mRNA levels of P2Y2R (3.52±0.95-fold change; Online Figure IIB), as well as by immunoblotting showing expression of P2Y2R-mGFP fused construct (Online Figure IIC). Expression of GFP alone did not alter hCPC proliferation (Online Figure IID). P2Y2R overexpression improved basal hCPC proliferation (1.47±0.1-fold change; $P=0.0031$; Figure 3A; Online Figure IIIA) and migration indicated by increased distance travelled from origin (1.37±0.14-fold change; $P=0.047$; Figure 3B; Online Figure IIIB and IIII). P2Y2R overexpression studies were performed in 3 representative lines with varying doubling times (H10-001, 24 hours; H13-073, 31 hours; and H13-064, 41 hours).

P2Y2R Overexpression Enhances YAP Activation

YAP—the downstream effector of Hippo signaling pathway—is a critical regulator of proliferation and migratory responses in several experimental models.26-30 Although YAP activity is modulated by Gτq protein-coupled receptors,19 upstream regulatory extracellular signals are still largely unknown. Overexpression of the Gτq protein-coupled P2Y2R resulted in significant downregulation of Hippo signaling pathway upstream kinases MST1 (0.67±0.097-fold change; $P=0.0419$) and LATS1 (0.64±0.055-fold change; $P=0.0224$). No significant differences were observed on phosphorylated

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**Figure 2.** Differential P2 receptor subset expression in fast- and slow-growing cardiac progenitor cells (CPCs). Expression of (A) P2X4, (B) P2X5, (C) P2X6, (D) P2Y1, (E) P2Y2, (F) P2Y4, (G) P2Y11, and (H) P2Y14 receptor mRNA by reverse transcriptase–polymerase chain reaction analysis in fast-growing human CPCs (F-hCPC) and slow-growing hCPCs (S-hCPC). Cycle numbers were normalized to 18S and data are represented relative to F-hCPC. P2Y1, P2Y2, and P2Y14 mRNA expression levels are significantly downregulated in S-hCPC compared with F-hCPC (n=5–7). *$P<0.05$ indicates significant difference from F-hCPC as measured by unpaired Student t test.
MST1/2 or phosphorylated LATS1 levels (Online Figure V). MST1 and LATS1 downregulation was associated with activation of YAP as indicated by reduced phosphorylation at S127 residue (0.86±0.0097-fold change; \(P=0.005\); Figure 3C through 3F). Total YAP levels were not significantly impacted by P2Y2R overexpression (Figure 3G). Importantly, expression of GFP alone did not alter basal YAP activity (Online Figure IIE).

**CPC Activation by the P2Y2R Agonist UTP**

G\(\alpha\)q protein-coupled receptor activation results in calcium release from intracellular stores through phospholipase C/inositol 1,4,5-trisphosphate signaling pathway.\(^{31}\) Therefore, G\(\alpha\)q protein-coupled P2Y2R function was assessed by measurement of intracellular calcium \([\text{Ca}^{2+}]\) levels in response to ligand stimulation by UTP. Stimulation of hCPCs with UTP enhanced \([\text{Ca}^{2+}]\) levels as indicated by a calcium transient (Figure 4A; Online Figure VI). P2Y2R inhibition using the selective antagonist AR-C 118925XX impaired UTP-mediated calcium transients in a dose-dependent manner (0.1 \(\mu\)M, 0.71±0.095-fold change; 1 \(\mu\)M, 0.44±0.14-fold change; 10 \(\mu\)M, 0.12±0.019-fold change; \(P=0.0005\); Figure 4B), indicating UTP-induced responses are primarily mediated by P2Y2R.

P2Y2R is a potent stimulator of cell proliferation and migration.\(^{13–15,32,33}\) Consistent with these findings, hCPC stimulation with P2Y2R agonist UTP for 24 hours significantly enhanced cell proliferation (1.56±0.073-fold change; \(P<0.0001\); Figure 4C). Additionally, UTP treatment enhanced hCPC migration on growth factor reduced Matrigel as shown by increased distance travelled from origin (from 47326±2029 to 67145±4173 nm; \(P=0.0001\); Figure 4D) and cell velocity (from 2.21±0.09 to 3.12±0.19 nm/sec; \(P=0.0001\); Figure 4E). The effect of UTP stimulation on proliferation and migration was assessed in 6 hCPC lines with varying doubling times (Online Figure VII).

UTP signals through 2 P2 receptors: P2Y2 and P2Y4. To validate involvement of P2Y2R in UTP-induced responses, P2Y2R knockdown was performed in hCPCs using lentivirus encoding P2Y2R shRNA or scrambled shRNA as a control. Transduction

![Figure 3. Enhancing cardiac progenitor cell (CPC) proliferation, migration, and yes-associated protein (YAP) activation by P2Y2R overexpression. A, Proliferation (n=7) and (B) migration (n=6) analysis showing that human CPC (hCPC)-Y2 exhibit enhanced proliferative and migratory capabilities compared with control hCPC-monomeric green fluorescent protein (mGFP). Cell proliferation was measured using CyQuant assay, and cell migration on growth factor reduced Matrigel was assessed by measuring the distance that cells traveled from origin after monitoring by time-lapse live cell imaging for 6 h. hCPC immunoblotting analysis (C) and corresponding quantification (D–G) showing downregulation of YAP repressors MST1 (n=4) and LATS1 (n=5) and decreased YAP\(^{S127}\) phosphorylation (indicating activation; n=4) and no significant change in total YAP levels (n=4) in hCPC-Y2 compared with control hCPC-mGFP. pYAP\(^{S127}\) was normalized to total YAP. Total MST1, LATS1, and YAP levels were normalized to \(\beta\)-actin (loading control). *\(P<0.05\) and **\(P<0.01\) indicate significant difference from hCPC-mGFP as measured by paired Student t test.]
efficiency was assessed by flow cytometry for percentage of GFP+ cells (68.23±8.09% for hCPCs using lentivirus encoding P2Y2R shRNA and 59±10.48% for hCPCs using scrambled shRNA; Online Figure VIIIA). P2Y2R knockdown was confirmed by quantitative reverse transcriptase–polymerase chain reaction showing reduced P2Y2R mRNA levels (0.51±0.046-fold change; Online Figure VIIIB). P2Y2R knockdown impaired UTP-induced hCPC proliferation by ≈70% (from 1.44±0.107 to 1.13±0.161-fold change) and migration (from 1.33±0.117 to 0.99±0.113-fold change; Online Figure VIIIC and VIIID) confirming that UTP acts primarily via P2Y2R.

**UTP Prompts YAP Activation and Nuclear Localization**

The role of P2Y2R in regulating Hippo signaling was validated via assessment of LATS1 kinase and YAP activity resulting from P2Y2R stimulation. hCPC treatment with UTP for 5, 10, or 15 minutes significantly inhibited phosphorylation of Hippo signaling upstream kinase LATS1 compared with untreated control (5 minutes, 0.61±0.1218-fold change; 10 minutes, 0.47±0.06-fold change; 15 minutes, 0.59±0.089-fold change; P=0.0173), whereas total LATS1 levels were unchanged (Figure 5A and 5C). Moreover, UTP treatment for 5, 10, 15, or 30 minutes reduced YAP phosphorylation at S127 residue (5 minutes, 0.69±0.027-fold change; 10 minutes, 0.62±0.079-fold change; 15 minutes, 0.69±0.1147-fold change; 30 minutes, 0.66±0.088-fold change; P=0.0007; Figure 5B and 5D) resulting from inhibition of upstream kinase LATS1. YAP dephosphorylation leads to activation and shuttling into the nucleus, so YAP nuclear localization was assessed after UTP treatment via immunoblotting on hCPC nuclear extracts. UTP stimulation increased YAP nuclear levels 15 minutes post-treatment (1.97±0.27-fold change; P<0.05; Figure 6A and 6B) as corroborated by confocal analysis showing higher levels of nuclear YAP in response to UTP stimulation for 5 minutes (1.51±0.16-fold change; P<0.05; Figure 6C and 6D). As expected, the phosphorylated inactive form of YAP (pYAPs127) was only detected in the cytoplasmic fraction and excluded from the nucleus (Online Figure IX).

Collectively, these findings support P2Y2R-mediated YAP activation and nuclear localization through inhibition of upstream YAP repressor LATS1.

In addition, hCPC stimulation with UTP activated ERK1/2 (Online Figure X)—a canonical inducer of cell proliferation and migration34 that is known to crosstalk with both P2Y2R35–38 and YAP signaling pathways39–42.

**UTP Enhances Expression of YAP Target Genes**

Upon activation, YAP shuttles to the nucleus where it serves as a transcriptional coactivator for induction of gene expression to promote cell proliferation and migration.20 Expression of canonical YAP target genes after UTP stimulation revealed significantly elevated mRNA levels of CTGF (1.84±0.19-fold change; P<0.05), INHBA (1.47±0.09-fold change;
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P<0.05), CYR61 (1.52±0.24-fold change; P<0.05), AMOTL2 (1.88±0.36-fold change; P<0.05), NPPB (2.77±0.46-fold change; P<0.05), and ANKRDI (1.38±0.13-fold change; P<0.05; Figure 7A through 7G). Peak expression of CTGF, INHBA, CYR61, AMOTL2, NPPB, and NEXN occurred 2 hours after UTP stimulation, but ANKRDI expression peaked 24 hours post-treatment (Figure 7A through 7G). UTP-mediated induction of 2 representative YAP target genes, CTGF and CYR61, was confirmed at the protein level (Online Figure XI). UTP-induced upregulation of target genes downstream of YAP signaling pathway does not exclude the potential involvement of other UTP-mediated regulatory mechanisms in the expression of those genes.

UTP-Induced CPC Proliferation and Migration Are Dependent on YAP Activation

Involvement of YAP in UTP-induced proliferation and migration responses in hCPCs was confirmed by pretreatment with the selective YAP inhibitor verteporfin (100 nM) for 1 hour before UTP treatment. A dose response of 1 to 1000 nM of verteporfin was performed, and 100 nM was the minimum dose required to significantly impair UTP-induced proliferation (from 1.43±0.072 to 1.15±0.062-fold change; P<0.05) without altering basal proliferation levels (Figure 8A). Verteporfin abolished UTP-induced proliferation and migration in hCPCs (from 1.57±0.218 to 0.94±0.058-fold change; P<0.05; Figure 8B; Online Figure XII). Therefore, UTP-induced proliferation and migration in hCPCs are YAP-dependent.

Discussion

Autologous stem cell therapy is a promising approach for treatment of HF. However, stem cells derived from patients with HF exhibit impaired proliferative and migratory capabilities, which could be addressed by identifying molecular components regulating these critical phenotypic characteristics of hCPCs. Findings in this study point to P2Y2R as an important regulator of hCPC proliferation and migration and delineate underlying mechanisms. P2Y2R was significantly downregulated in S-hCPCs compared with fast growers (F-hCPCs). Augmenting P2Y2R levels or P2Y2R stimulation with UTP in hCPCs efficiently improved proliferation and migration potential. P2Y2R-induced responses involved downstream activation of YAP signaling, introducing a novel component into the P2Y2R intracellular signaling network.

A primary role of stem cells emerges after injury and subsequent contribution to tissue repair and regeneration. Nucleotides accumulate in the extracellular milieu after injury/stress and activate purinergic receptors to initiate

Figure 5. UTP inhibits LATS1 and activates YAP (yes-associated protein) in cardiac progenitor cells (CPCs). Human CPC immunoblotting analysis (A and B) and corresponding quantification (C and D) showing reduced phosphorylation of LATS1 (indicating inhibition) and YAP(S127) (indicating activation) in response to UTP (100 µM) treatment. pLATS1 (n=3–4/time point) and pYAP(S127) (n=5–6/time point) were normalized to total LATS1 and total YAP levels, respectively. Total LATS1 (n=8) and total YAP levels (n=4) were normalized to β-actin (loading control). Data are represented relative to 0 min (no UTP treatment). *P<0.05 and **P<0.01 indicate significant difference from 0 min as measured by 1-way ANOVA followed by Dunnett post hoc test.
physiological responses required for the repair process.\textsuperscript{7,8} Stem cells with compromised ability to detect extracellular nucleotides could elicit impaired regenerative responses to injury. Thus, a major key to improving regenerative capacity of impaired stem cells would be to augment detection of extracellular nucleotides through modulating purinergic receptors. \textit{P2Y$_2$R} endogenous agonists ATP and UTP accumulate in large levels in the extracellular space in response to cellular stress\textsuperscript{9–12} as a signal to prompt cellular reaction to injury. However, \textit{P2Y$_2$R} was significantly downregulated in S-hCPCs isolated from cardiac biopsies of patients with HF (Figure 2E). Restoration of \textit{P2Y$_2$R} levels by lentiviral-mediated overexpression augments their proliferative and migratory capabilities (Figure 3). These results reinforce the emerging view of \textit{P2Y$_2$R} as proproliferative in various experimental models, including hepatocytes,\textsuperscript{13,44} corneal endothelial cells,\textsuperscript{45} and cardiac progenitor cells (CPCs).

\begin{figure}[h]
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\caption{UTP enhances YAP (yes-associated protein) nuclear localization in cardiac progenitor cells (CPCs). Cytoplasmic and nuclear extracts of human CPCs (hCPCs) on immunoblot (A) with corresponding quantification (B) shows increased nuclear levels of YAP 15 min after UTP (100 \textmu M) stimulation. Cytoplasmic YAP was normalized to \textit{β}-tubulin, and nuclear YAP was normalized to Lamin A (n=4–6/time point). C, Representative fluorescence microscopy images of hCPCs and (D) corresponding quantification showing increased nuclear levels of YAP 5 min after UTP (100 \textmu M) treatment (n=3). YAP nuclear signal intensity was normalized to nuclear area. DAPI nuclear stain, white; YAP, red. Scale bar=50 \textmu m. Data are represented relative to 0 min (no UTP treatment). *\textit{P}<0.05 indicates significant difference from 0 min as measured by 1-way ANOVA followed by Dunnett post hoc test.}
\end{figure}
and pancreatic duct epithelial cells. In addition, P2Y2R-induced migration in hCPCs is consistent with promigratory responses of P2Y2R in fibroblasts, salivary and corneal epithelial cells. Whether ex vivo manipulation of hCPCs by UTP preconditioning or P2Y2R overexpression improves transplanted hCPC homing, expansion, and overall reparative potential for injured myocardium remains to be assessed.

P2Y2R was implicated in mediating enhanced proliferation and differentiation after preconditioning of atrial-derived hCPCs with ATP for 30 minutes in vitro and in vivo, but involvement of P2Y2R in the ATP-induced responses was not confirmed through any inhibitor, loss- or gain-of-function studies. Additionally, ATP-mediated responses were primarily attributed to stimulating calcium signaling. Results obtained herein demonstrate that UTP-induced proliferative and migratory responses in hCPCs are dependent on YAP activation (Figure 8). Despite extensive study of Hippo signaling pathway and the downstream effector YAP, upstream regulatory extracellular signals and their membrane receptors have remained elusive. Recently, 2 independent studies reported that G protein-coupled receptors play a major role in regulating Hippo pathway. Gq/11- and Gs/o-coupled signals induce YAP activity, whereas Gs-coupled signals repress YAP. Concordantly, our data show Gq protein-coupled P2Y2R induces YAP activation (Figures 3F, 5, and 6) revealing a novel link between extracellular nucleotides released during injury/stress and Hippo signaling—a core component in mediating CPC proliferation and overall cardiac regeneration. Whether crosstalk occurs between YAP and other signaling molecules acting downstream of P2Y2R, such as calcium, growth factor receptors, Arg-Gly-Asp-binding integrins, and Rho GTPases, remains to be determined. Furthermore, involvement of YAP in promoting hCPC proliferation and migration supports previous literature demonstrating similar responses downstream of YAP, as extensively studied in several cancer models where YAP inhibition was proposed as a potential therapeutic target to halt tumorigenesis.

**Figure 7.** UTP enhances expression of YAP (yes-associated protein) target genes in cardiac progenitor cells. Enhanced mRNA expression of YAP canonical target genes by reverse transcriptase–polymerase chain reaction analysis for (A) CTGF, (B) INHBA, (C) CYR61, (D) AMOTL2, (E) NPPB, (F) NEXN, and (G) ANKR1 after UTP (100 µM) treatment. mRNA levels of CTGF, INHBA, CYR61, AMOTL2, NPPB, and NEXN peaked 2 h after UTP stimulation, whereas increase in ANKR1 mRNA levels was observed 24 h post-UTP treatment (n=3–6/time point/gene). Data are represented relative to 0 h (no UTP treatment). *P<0.05, **P<0.05, and ***P<0.001 indicate significant difference from 0 h as measured by 1-way ANOVA followed by Dunnett post hoc test.
Differential expression of P2 purinergic receptors between fast- and slow-growing hCPCs was not restricted to P2Y$_{14}$R. The ADP receptor P2Y$_2$R (P2Y$_{2R}$) was also significantly downregulated in S-hCPCs compared with F-hCPCs (Figure 2D). P2Y$_{14}$R regulates regenerative responses in several tissues. P2Y$_{14}$R mediates chondrocyte proliferation and cartilage repair in osteoarthritis, neuronal fiber outgrowth in organotypic brain slice cocultures, expression of wound healing regulator cyclooxygenase-2 in intestinal subepithelial myofibroblasts in addition to regulating proliferation and repair of retinal tissue in response to cytotoxic injury. The UDP-sugar P2Y$_{14}$R receptor (P2Y$_{14}$R) is another interesting target downregulated in S-hCPCs compared with fast-growing cells (Figure 2H). Cumulative data during the past decade demonstrate involvement of P2Y$_{14}$R in inducing proliferation and migration of human keratinocytes, chemotaxis of human hematopoietic stem cells, and human neutrophils. In addition, P2Y$_{14}$R enhances mouse hematopoietic stem cell resistance to stress-induced senescence and maintains regenerative capacity after injury. Future studies will aim to assess whether P2Y$_2$R and P2Y$_{14}$R mediate prorgerative roles in hCPCs. Overall, establishing physiological responses downstream of individual members of the P2 receptor family represents the first step toward understanding unexplored roles of purinergic signaling in hCPCs. Purinergic receptors with validated prorgerative roles could be used as cell surface markers for initial isolation of hCPCs from tissue specimens of patients with HF to enrich for potentiated stem cells with enhanced responsiveness to purinergic drive in the extracellular environment. Many P2 receptors share common agonists indicating a potential crosstalk among P2 family members in hCPCs, reinforcing the importance of understanding signaling interplay between various P2 receptors.

In summary, the present study demonstrates lack of expression of a subset of P2 purinergic receptors in functionally compromised hCPCs derived from patients with HF. These findings fit with a growing body of supportive studies focused on addressing inherent deficits of cardiac stem cells with the ultimate goal of boosting their phenotypic properties. P2Y$_{14}$R is part of the regulatory network of proliferation and migration responses impaired in CPCs isolated from human patients with HF. Restoration of a youthful phenotype to CPCs, possibly including purinergic signaling, can augment engraftment and survival as noted in previous publications from our laboratory. Our findings can shed light on underlying impairment of endogenous stem cell repair in the aged or pathologically damaged myocardium.

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Disclosures
M.A. Sussman is co-founder and chief scientific officer of CardioCreate, Inc.

References


P2Y₂ Nucleotide Receptor Prompts Human Cardiac Progenitor Cell Activation by Modulating Hippo Signaling

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Online Figure I: P2Y2R expression correlates with hCPC doubling time.
(A) P2Y2R mRNA expression in hCPCs derived from multiple heart failure patients plotted against hCPC doubling time showing that low doubling time (fast growth kinetics) corresponds to low cycle number (high P2Y2R mRNA expression levels). Data is represented as Δ C(t) (cycle numbers normalized to 18S). *P < 0.05 indicates significant correlation between the assessed variables as measured by two-tailed Spearman correlation analysis. (B) Immunoblot analysis and (C) corresponding quantitation showing P2Y2R downregulation in a slow-growing hCPC line (S-hCPC; H13-064 [Doubling Time 41 hours]) compared to relatively fast-growing hCPC line (F-hCPC; H10-001 [Doubling Time 24 hours]). **P < 0.01 indicates significant difference from F-hCPC as measured by paired Student t test.
Online Figure II: Lentiviral-mediated overexpression of P2Y₂R-mGFP in CPCs.

(A) Transduction efficiency of hCPCs with lentiviral particles encoding for P2Y₂R and mGFP (hCPC-Y2) or mGFP alone (hCPC-mGFP) by flow cytometry analysis with percentage of GFP⁺ cells (56% for hCPC-mGFP and 50.1% for hCPC-Y2). (B) Elevated P2Y₂R mRNA levels by qRT-PCR analysis in hCPC-Y2 compared to control hCPC-mGFP. (C) A representative blot showing a 28 kDa band corresponding to mGFP in hCPC-mGFP and a shifted ~75 kDa mGFP band in hCPC-Y2 validating overexpression of the P2Y₂R-mGFP fused construct at the protein level. (D) Proliferation assay showing that mGFP expression does not impact hCPC proliferation (n=6). (E) Immunoblot analysis showing that mGFP expression does not alter YAP activation in hCPCs.
Online Figure III: Enhancing proliferation and migration of individual hCPC lines by P2Y₂R overexpression.
Enhanced proliferation (n=2-3/line) (A) and migration (n=2-3/line) (B) of 3 representative hCPC lines by P2Y₂R overexpression. Cell proliferation and migration were assessed as described in Figure 3.
Online Figure IV: Improving hCPC migration by P2Y$_2$R overexpression. Representative images from time-lapse live cell imaging showing enhanced migration of hCPCs overexpressing P2Y$_2$R (hCPC-Y2) on GFR matrigel as assessed by increased distance travelled by single cells from origin (denoted by red circle).
Online Figure V: MST1/2 or LATS1 phosphorylation is not impacted by P2Y$_2$R overexpression. (A, B) Immunoblot analysis showing no difference in levels of phosphorylated MST1/MST2 or phosphorylated LATS1 in hCPCs overexpressing P2Y$_2$R.
Online Figure VI: Functional response to P2Y$_2$R agonist UTP in hCPCs.
A dose response of P2Y$_2$R agonist UTP (1, 10 and 100μM) showing increase in intracellular calcium [Ca$^{2+}$] levels, indicated by increased amplitude of [Ca$^{2+}$] transient, in a dose-dependent manner.
Online Figure VII: Enhancing proliferation and migration of individual hCPC lines by P2Y$_2$R agonist UTP.
Enhanced proliferation (n=2-11/line) (A) and migration (n=3-5/line) (B) of 6 hCPC lines in response to UTP treatment (100µM). Cell proliferation and migration were assessed as described in Figure 3.
Online Figure VIII: UTP-induced hCPC proliferation is primarily mediated by P2Y₂R. (A) shRNA-mediated P2Y₂R knockdown using lentiviral particles encoding for P2Y₂R shRNA (hCPC-Y2SH) or scrambled shRNA (hCPC-ScrSH). Transduction efficiency was 59% for hCPC-ScrSH and 68% for hCPC-Y2SH as assessed by flow cytometry analysis for percentage of GFP⁺ cells. (B) Lower P2Y₂R mRNA levels by qRT-PCR analysis in hCPC-Y2SH compared to control hCPC-ScrSH confirming P2Y₂R knockdown. (C, D) Proliferation and migration assays showing impairment of UTP-induced responses in hCPC-Y2SH (n=4) validating that UTP acts primarily via P2Y₂R. Proliferation and migration assays were performed as described in Figure 4. *P < 0.05 indicates significant difference as measured by two-way ANOVA followed by Bonferroni post hoc test.
Online Figure IX: Exclusion of inactive pYAP$^{5127}$ from the nuclear fraction of hCPCs.
Representative immunoblot showing exclusion of the inactive phosphorylated form of YAP from the nuclear extracts of hCPCs. Nuclear protein Lamin A was used as additional control to validate the purity of nuclear fraction.
Online Figure X: Induction of ERK1/2 activation by UTP treatment. Representative immunoblot showing enhanced ERK1/2 phosphorylation in response to stimulation with UTP (100μM) in hCPCs.
Online Figure XI: Upregulation of CTGF and CYR61 expression by UTP treatment.
Representative immunoblot showing enhanced CTGF and CYR61 expression following stimulation with UTP (100μM) for 4 hours in hCPCs.
Online Figure XII: Impairment of UTP-induced hCPC migration by YAP inhibition. Representative images from time-lapse live cell imaging showing impaired UTP-induced migration of hCPCs in the presence of YAP inhibitor verteporfin (100nM) as indicated by diminished distance travelled by single cells from origin (denoted by red circle).
### Online Table I. List of primers

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