P2Y$_2$ Nucleotide Receptor Prompts Human Cardiac Progenitor Cell Activation by Modulating Hippo Signaling

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Abstract

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 HF patients with genetic predispositions and/or comorbidities of chronic diseases exhibit poor proliferative and migratory capabilities, which impairs overall reparative potential for injured myocardium. Therefore, empowering functionally compromised hCPCs with pro-regenerative molecules *ex vivo* is crucial for improving the therapeutic outcome in HF patients.

Objective—To improve hCPC proliferation and migration responses that are critical for regeneration by targeting pro-regenerative P2Y$_2$ nucleotide receptor (P2Y$_2$R) activated by extracellular ATP and UTP molecules released following injury/stress.

Methods and Results—c-Kit$^+$ hCPCs were isolated from cardiac tissue of HF patients undergoing left ventricular assist device (LVAD) implantation surgery. Correlations between P2 nucleotide receptor expression and hCPC growth kinetics revealed downregulation of select P2 receptors, including P2Y$_2$R, in slow-growing hCPCs compared to fast-growers. hCPC proliferation and migration significantly improved by overexpressing or stimulating P2Y$_2$R. Mechanistically, P2Y$_2$R-induced proliferation and migration were dependent upon activation of yes-associated protein (YAP), the downstream effector of Hippo signaling pathway.

Conclusions—Proliferation and migration of functionally impaired hCPCs are enhanced by P2Y$_2$R-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.

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Lack of P2Y$_2$R 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 HF patients.

Keywords

Cellular stress; extracellular nucleotides; P2Y$_2$R; Hippo signaling; cardiac progenitor cells

Subject Terms

Basic Science Research; Cell Signaling/Signal Transduction; Mechanisms; Myocardial Regeneration; Stem Cells

INTRODUCTION

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 following myocardial infarction (MI) in animal models [1, 2]. In comparison, adoptive transfer of autologous c-Kit$^+$ CPCs into patients with pathologically injured myocardium yields modest and more variable outcomes in clinical trials [3, 4]. Inconsistent findings in the clinical setting are likely due, at least in part, to severely compromised regenerative potential of stem cells isolated from HF patients with genetic predispositions, comorbidities of chronic diseases such as hypertension and diabetes, and/or daily life stressors such as smoking and alcoholism. Therefore, enhancing regenerative capacity of stem cells *ex vivo* prior to transplantation is an interventional strategy to improve outcome of stem cell therapy as exemplified by empowering stem cells from diverse origins with pro-survival and anti-apoptotic genes [5, 6]. Regenerative capacity of stem cells also depends upon their ability to communicate with and adapt to the extracellular environment. To date, molecular mechanisms by which stem cells detect stress signals to initiate 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 seven P2X ligand-gated ion channels (P2X1-7) and eight P2Y G protein-coupled receptors (P2Y$_{1,2,4,6,11,12,13,14}$) [7, 8]. While some P2 receptors initiate early inflammatory responses, others mediate later regenerative responses required for the healing process.

P2Y$_2$ receptor (P2Y$_2$R) is a pro-regenerative Gαq protein-coupled receptor activated by ATP and UTP, which are released during cardiac ischemia [9–12]. P2Y$_2$R plays a central role in intracellular signaling by enabling extracellular ATP and UTP to promote regenerative responses in a variety of tissues. P2Y$_2$R regulates corneal epithelia wound healing [13] and

salivary gland reconstitution [14] by inducing cell migration, liver regeneration by promoting hepatocyte proliferation [15], and reepithelialization following experimental colitis [16] and inflammatory bowel disease [17]. On the stem cell level, UTP is a potent stimulant of human hematopoietic stem cell (hHSC) migration [18]. Herein, we hypothesize that $P2Y_2R$ induces proliferative and migratory responses in functionally compromised human CPCs (hCPCs) derived from HF patients.

$G\alpha q$ protein-coupled signaling regulates cell proliferation and migration through activation of yes-associated protein (YAP), the downstream effector of Hippo signaling [19]. However, it is not known whether $G\alpha q$ protein-coupled $P2Y_2R$-induced proliferative and migratory responses are dependent upon YAP activation (Figure 1). YAP activity is tightly regulated by multiple upstream kinases including mammalian MST1 (Hippo homolog) and large tumor suppressor kinase 1 (LATS1) [20]. MST1 activates LATS1, which in turn phosphorylates and represses YAP activity by promoting cytoplasmic retention. Conversely, LATS1 inhibition leads to YAP dephosphorylation (activation) and shuttling into the nucleus where it acts as a transcriptional co-activator to induce expression of genes that promote cell proliferation, migration and survival [20].

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 HF patients and P2 purinergic receptor expression. Indeed, expression of select P2 receptors, including the pro-regenerative $P2Y_2R$, directly correlated with hCPC growth kinetics. Proliferation and migration of functionally compromised hCPCs were improved by $P2Y_2R$ activation or overexpression and impaired by $P2Y_2R$ knockdown. $P2Y_2R$-induced proliferation and migration were mediated by YAP activation, introducing a novel downstream component in the $P2Y_2R$ intracellular signaling cascade. These findings are discussed in the context of manipulating hCPCs to enhance their phenotypic properties.

METHODS

Human cardiac progenitor cell isolation

Human CPCs were isolated from cardiac tissue specimens derived from patients undergoing LVAD implantation surgeries as previously described [21]. 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 (ES FBS), 1% penicillin/streptomycin/glutamine (PSG), 5 mU/mL human erythropoietin (Sigma Aldrich), 10 ng/mL basic FGF (Peprotech), and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. hCPCs with doubling time 24 hours were considered as fast-growers (F-hCPCs) while 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 (qRT-PCR) using iQ SYBER Green (Bio Rad). Primer sequences are listed in Online Table I.

Calcium imaging

Cultured cells on glass-bottom plates grown to 60–70% confluency were loaded for 1 hour with the calcium-dependent ratiometric fluorescent dye Fura-2AM (4μM) in Krebs-Ringer solution (KRH) (125mM NaCl, 5mM KCl, 1.2mM Mg_2SO_4, 2mM $CaCl_2$, 10mM glucose, 25mM HEPES and pH was adjusted to 7.4 with NaOH). Afterwards, cells were washed in KRH for 30 minutes to allow for de-esterification of Fura-2AM dye. Ca^{2+} imaging data were collected using an inverted fluorescent microscope (Leica) where the excitation wavelength was altered between 340 and 387nm (F340 and F387) and emission was detected at 510nm. Data is represented as the ratio of fluorescence intensities at 340nm excitation (F340) [Ca^{2+}-bound Fura-2] and 387nm (F387) excitation [Ca^{2+}-free Fura-2]. In inhibitor studies, cells were pretreated with $P2Y_2R$ selective antagonist (AR-C 118925XX; 0.1, 1 and 10μM) (Tocris Bioscience) for the indicated times prior to UTP treatment.

Cell proliferation assay

hCPCs were cultured in serum-starved medium (Hams F12 media supplemented with 2.5% ES FBS) in a 96-well plate (500 cells/well) then treated with CyQuant fluorescent nucleic-acid based dye (Life Technologies) that labels live cells where the fluorescence intensity directly correlates with cell number. After 1 hour, fluorescence intensity was measured using a plate reader and considered as baseline (day 0) reading. Then cells were treated with or without UTP (100μM) and 24 hours later CyQuant reagent was added and day 1 reading was recorded. Population doubling times were calculated based on readings from CyQuant assay using a population doubling time online calculator (http://www.doubling-time.com/compute.php). In inhibitor studies, cells were pretreated with YAP selective inhibitor (verteporfin; 100nM) (Tocris Bioscience) for the indicated times prior to UTP treatment.

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 (GFR) Matrigel (BD Biosciences) (1600 cells/well) and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 2 hours. Then the cell culture plate was mounted on a DMI6000 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% CO_2 and 95% air). A field of cells was located within each well with a 5X objective and marked for monitoring over 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; 100nM) (Tocris Bioscience) for the indicated times prior to UTP treatment.

Protein isolation, SDS-PAGE and immunoblot analysis

hCPCs were seeded in a 6-well plate (30,000 cells/well). Next day, cells were treated with or without UTP (100μM) 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 4–12% NuPage Novex Bis Tris gel (Invitrogen), transferred on to a polyvinylidene fluoride (PVDF) membrane, blocked in 5% skim milk in Tris-buffered saline Tween-20 (TBST) 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 TBST. 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 100mm dishes (180,000 cells/well). The following day, cells were treated with or without UTP (100μM) 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 3–5 minutes then trypsin was inactivated with hCPC growth medium. Cells were pelleted, culture medium was aspirated, cell pellet was gently resuspended in 100μL ice-cold cell fractionation buffer and incubated on ice for 5–10 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 as well as cytoplasmic fraction and samples were analyzed using SDS-PAGE 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 (100μM) in serum-starved medium for the indicated times. Cells were fixed with 4% paraformaldehyde (PFA) 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 min 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 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 quantitation of nuclear signal intensity 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 cardiac progenitor cells

hCPCs were cultured in a 6-well plate (30,000 cells/well). The following day, hCPCs were transduced with lentivirus (0.2 MOI) encoding either monomeric green fluorescent protein (hCPC-mGFP) or P2Y$_2$R fused to mGFP (hCPC-Y2) (lentiviral plasmids were purchased from Origene; SKU: RC223931L2). To knockdown P2Y$_2$R, hCPCs were transduced with lentiviral particles encoding P2Y$_2$R shRNA and mGFP (20 MOI) (lentiviral plasmid was purchased from Origene; SKU: TL302717; Gene ID: 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 three experiments. Two-tailed Student's *t* test or ANOVA followed by Dunnett's 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 heart failure patients exhibit variation in growth rate previously characterized as fast-growing (F-hCPC) or slow-growing (S-hCPC) [21]. F-hCPCs are characterized by a spindly morphology and lower levels of senescence markers, while S-hCPCs exhibit a flat morphology associated with higher levels of senescence markers [21]. P2 receptor mRNA expression levels were assessed by qRT-PCR-based analysis in representative fast- and slow-growing hCPC lines. 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 to F-hCPCs (0.031±0.015 fold change, P=0.012; 0.22±0.046 fold change, P=0.0112; and 0.058±0.014 fold change, P=0.0014, respectively) (Figure 2). P2Y$_2$R was particularly intriguing based upon prior reports of involvement in regeneration using various experimental models [13–15, 23, 24] and in stem cells from diverse origins [18, 25]. Higher P2Y$_2$R mRNA expression levels corresponded with faster hCPC growth rates indicated by shorter doubling times (R=0.7101, P=0.0369) (Online Figure IA). P2Y$_2$R was also downregulated at the protein level in S-hCPCs compared to F-hCPCs (0.56±0.047 fold change, P=0.0026).

Improving CPC proliferation and migration by P2Y$_2$R overexpression

hCPC proliferation and migration potential was improved by increasing P2Y$_2$R levels. hCPCs were infected with lentiviral particles encoding P2Y$_2$R 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 qRT-PCR showing increased mRNA levels of P2Y$_2$R in hCPC-Y2 (3.52±0.95 fold change) (Online Figure IIB) as well as by immunoblotting showing expression of P2Y$_2$R-mGFP fused construct (Online Figure IIC). Expression of GFP alone did not alter hCPC proliferation (Online Figure IID).

P2Y$_2$R overexpression improved basal hCPC proliferation (1.47±0.1 fold change, P=0.0031) (Figure 3A and Online Figure IIIA) and migration indicated by increased distance travelled from origin (1.37±0.14 fold change, P=0.047) (Figure 3B, Online Figures IIIB and IIII). P2Y$_2$R 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).

P2Y$_2$R overexpression enhances YAP activation

Yes-associated protein (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 Gαq protein-coupled receptors [19], upstream regulatory extracellular signals are still largely unknown. Overexpression of the Gαq protein-coupled P2Y$_2$R 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 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 S^{127} residue (0.86±0.0097 fold change, P=0.005) (Figure 3C–F). Total YAP levels were not significantly impacted by P2Y$_2$R overexpression (Figure 3G). Importantly, expression of GFP alone did not alter basal YAP activity (Online Figure IIE).

CPC activation by the P2Y$_2$R agonist UTP

Gαq protein-coupled receptor activation results in calcium release from intracellular stores through phospholipase C/inositol 1,4,5-trisphosphate (PLC/IP3) signaling pathway [31]. Therefore, Gαq protein-coupled P2Y$_2$R function was assessed by measurement of intracellular calcium $[Ca^{2+}]_i$ levels in response to ligand stimulation by UTP. Stimulation of hCPCs with UTP enhanced $[Ca^{2+}]_i$ levels as indicated by a calcium transient (Figure 4A and Online Figure VI). P2Y$_2$R inhibition using the selective antagonist AR-C 118925XX impaired UTP-mediated calcium transients in a dose-dependent manner (0.1μM: 0.71±0.095 fold change; 1μM: 0.44±0.14 fold change; 10μM: 0.12±0.019 fold change, P=0.0005) (Figure 4B), indicating UTP-induced responses are primarily mediated by P2Y$_2$R.

P2Y$_2$R is a potent stimulator of cell proliferation and migration [13–15, 32, 33]. Consistent with these findings, hCPC stimulation with P2Y$_2$R 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 GFR Matrigel as shown by increased distance travelled from origin (from 47326±2029 to 67145±4173 nm, P=0.0001) (Figure 4D) as well as cell velocity (from 2.21±0.09 to 3.12±0.19 nm/sec, P=0.0001) (Figure 4E). The effect of UTP stimulation upon proliferation and migration was assessed in 6 hCPC lines with varying doubling times (Online Figure VII).

UTP signals through two P2 receptors: P2Y$_2$ and P2Y$_4$. To validate involvement of P2Y$_2$R in UTP-induced responses, P2Y$_2$R knockdown was performed in hCPCs using lentivirus encoding P2Y$_2$R shRNA (hCPC-Y2SH) or scrambled shRNA (hCPC-ScrSH) as a control. Transduction efficiency was assessed by flow cytometry for percentage of GFP$^+$ cells (68.23±8.09% for hCPC-Y2SH and 59±10.48% for hCPC-ScrSH) (Online Figure VIIIA).

P2Y$_2$R knockdown was confirmed by qRT-PCR showing reduced P2Y$_2$R mRNA levels (0.51±0.046 fold change) (Online Figure VIIIB). P2Y$_2$R 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–D) confirming that UTP acts primarily via P2Y$_2$R.

UTP prompts YAP activation and nuclear localization

The role of P2Y$_2$R in regulating Hippo signaling was validated via assessment of LATS1 kinase and YAP activity resulting from P2Y$_2$R stimulation. hCPC treatment with UTP for 5, 10 or 15 minutes significantly inhibited phosphorylation of Hippo signaling upstream kinase LATS1 compared to untreated control (5 min: 0.61±0.1218 fold change; 10 min: 0.47±0.06 fold change; 15 min: 0.59±0.089 fold change, P=0.0173) while total LATS1 levels were unchanged (Figure 5A and 5C). Moreover, UTP treatment for 5, 10, 15 or 30 minutes reduced YAP phosphorylation at S^{127} residue (5 min: 0.69±0.028 fold change; 10 min: 0.62±0.079 fold change; 15 min: 0.69±0.1147 fold change; 30 min: 0.66±0.088 fold change, P=0.0007) (Figure 5B and 5D) resulting from inhibition of upstream kinase LATS1. YAP de-phosphorylation leads to activation and shuttling into the nucleus, so YAP nuclear localization was assessed following 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 (pYAP^{S127}) was only detected in the cytoplasmic fraction and excluded from the nucleus (Online Figure IX). Collectively, these findings support P2Y$_2$R-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 migration [34] that is known to crosstalk with both P2Y$_2$R [35–38] and YAP signaling pathways [39–42].

UTP enhances expression of YAP target genes

activation, YAP shuttles to the nucleus where it serves as a transcriptional co-activator for induction of gene expression to promote cell proliferation and migration [20]. Expression of canonical YAP target genes following 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, 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), *NEXN* (2.35±0.49 fold change, P < 0.05) and *ANKRD1* (1.38±0.13 fold change, P < 0.05) (Figure 7A–G). Peak expression of *CTGF*, *INHBA*, *CYR61*, *AMOTL2, NPPB and NEXN* occurred 2 hours after UTP stimulation, but *ANKRD1* expression peaked 24 hours post-treatment (Figure 7A–G). UTP-mediated induction of two 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 (100nM) for 1 hour prior to UTP treatment. A dose response of 1–1000nM of verteporfin was performed and 100nM 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 migration (from 1.57±0.218 to 0.94±0.058 fold change, P < 0.05) (Figure 8B and 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 heart failure (HF). However, stem cells derived from HF patients exhibit impaired proliferative and migratory capabilities [43], that could be addressed by identifying molecular components regulating these critical phenotypic characteristics of hCPCs. Findings in this study point to $P2Y_2R$ as an important regulator of hCPC proliferation and migration and delineate underlying mechanisms. $P2Y_2R$ was significantly downregulated in slow-growing hCPCs (S-hCPCs) compared to fast-growers (F-hCPCs). Augmenting $P2Y_2R$ levels or $P2Y_2R$ stimulation with UTP in hCPCs efficiently improved proliferation and migration potential. $P2Y_2R$-induced responses involved downstream activation of YAP signaling, introducing a novel component into the $P2Y_2R$ intracellular signaling network.

A primary role of stem cells emerges following injury and subsequent contribution to tissue repair and regeneration. Nucleotides accumulate in the extracellular milieu following injury/stress and activate purinergic receptors to initiate physiological responses required for the repair process [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. $P2Y_2R$ endogenous agonists ATP and UTP accumulate in large levels in the extracellular space in response to cellular stress [9–12] as a signal to prompt cellular reaction to injury. However, $P2Y_2R$ was significantly downregulated in S-hCPCs isolated from cardiac biopsies of HF patients (Figure 2E). Restoration of $P2Y_2R$ levels by lentiviral-mediated overexpression augments their proliferative and migratory capabilities (Figure 3). These results reinforce the emerging view of $P2Y_2R$ as pro-proliferative in various experimental models including hepatocytes [15, 44], corneal endothelial cells [45] and pancreatic duct epithelial cells [46]. In addition, $P2Y_2R$-induced migration in hCPCs is consistent with pro-migratory responses of $P2Y_2R$ in fibroblasts [24], salivary [14] and corneal epithelial cells [13]. Whether *ex vivo* manipulation of hCPCs by UTP preconditioning or $P2Y_2R$ overexpression improves transplanted hCPC homing, expansion and overall reparative potential for injured myocardium remains to be assessed.

$P2Y_2R$ was implicated in mediating enhanced proliferation and differentiation following preconditioning of atrial-derived hCPCs with ATP for 30 minutes *in vitro* and *in vivo* [25], but involvement of $P2Y_2R$ 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 upon YAP activation (Figure 8). Despite extensive study of Hippo signaling pathway as well as the downstream effector YAP, upstream regulatory extracellular signals and their membrane receptors have remained elusive. Recently, two independent studies reported that G protein-coupled receptors play a major role in regulating Hippo pathway [19, 47]. $G\alpha q/11$- and $G\alpha i/o$-coupled signals induce YAP activity, while $G\alpha s$-coupled signals repress YAP [20]. Concordantly, our data show $G\alpha q$ protein-coupled $P2Y_2R$ 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 [48]. Whether crosstalk occurs between YAP and other signaling molecules acting downstream of $P2Y_2R$, such as calcium, growth factor receptors, RGD-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 [26–30] where YAP inhibition was proposed as a potential therapeutic target to halt tumorigenesis.

Differential expression of P2 purinergic receptors between fast- and slow-growing hCPCs was not restricted to $P2Y_2R$. The ADP receptor $P2Y_1$ ($P2Y_1R$) was also significantly downregulated in S-hCPCs compared to F-hCPCs (Figure 2D). $P2Y_1R$ regulates regenerative responses in several tissues. $P2Y_1R$ mediates chondrocyte proliferation and cartilage repair in osteoarthritis [49], neuronal fibre outgrowth in organotypic brain slice co-cultures [50], expression of wound healing regulator cyclooxygenase-2 in intestinal subepithelial myofibroblasts [51] in addition to regulating proliferation and repair of retinal tissue in response to cytotoxic injury [52]. The UDP-sugar $P2Y_{14}$ receptor ($P2Y_{14}R$) is another interesting target downregulated in S-hCPCs compared to fast-growing cells (Figure 2H). Cumulative data over the past decade demonstrate involvement of $P2Y_{14}R$ in inducing proliferation and migration of human keratinocytes [53], chemotaxis of hHSCs [54] and human neutrophils [55]. In addition, $P2Y_{14}R$ enhances mouse HSC (mHSC) resistance to stress-induced senescence and maintains regenerative capacity after injury [56]. Future studies will aim to assess whether $P2Y_1R$ and $P2Y_{14}R$ mediate pro-regenerative roles in hCPCs. Overall, establishing physiological responses downstream of individual members of the P2 receptor family represents the first step towards understanding unexplored roles of purinergic signaling in hCPCs. Purinergic receptors with validated pro-regenerative roles could be used as cell surface markers for initial isolation of hCPCs from tissue specimens of HF patients to enrich for potentiated stem cells with enhanced responsiveness to purinergic drive in the extracellular environment. Many P2 receptors share common agonists indicates 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 HF patients. These findings fit with a growing body of supportive studies focused upon addressing inherent deficits of cardiac stem cells with the ultimate goal of boosting their phenotypic properties. $P2Y_2R$ is

part of the regulatory network of proliferation and migration responses impaired in CPCs isolated from human HF patients. Restoration of a youthful phenotype to CPCs, possibly including purinergic signaling, can augment engraftment and survival as noted in previous publications from our laboratory [21]. Our findings can shed light upon underlying impairment of endogenous stem cell repair in the aged or pathologically damaged myocardium.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Nonstandard Abbreviations and Acronyms

AMOTL2	angiomotin like 2
ANKRD1	ankyrin repeat domain 1
CTGF	connective tissue growth factor
CYR61	cysteine rich angiogenic inducer 61
ES FBS	embryonic stem cell screened fetal bovine serum
F-hCPCs	fast-growing human cardiac progenitor cells
GFR Matrigel	growth factor reduced Matrigel
hCPCs	human cardiac progenitor cells
hCPC-mGFP	human cardiac progenitor cells expressing monomeric green fluorescent protein
hCPC-Y2	human cardiac progenitor cells overexpressing $P2Y_2$ receptor and mGFP
hHSCs	human hematopoietic stem cells
INHBA	inhibin beta a subunit
LATS1	large tumor suppressor kinase 1
LVAD	left ventricular assist device
mHSCs	mouse hematopoietic stem cells
NEXN	nexilin F-actin binding protein

NPPB	natriuretic peptide B
P2Y$_1$R	P2Y$_1$ nucleotide receptor
P2Y$_2$R	P2Y$_2$ nucleotide receptor
P2Y$_{14}$R	P2Y$_{14}$ nucleotide receptor
PBS	phosphate-buffered saline
PFA	paraformaldehyde
qRT-PCR	quantitative reverse-transcriptase polymerase chain reaction
RGD	Arg-Gly-Asp
S-hCPCs	slow-growing human cardiac progenitor cells
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBST	Tris-buffered saline Tween-20 solution
YAP	yes-associated protein

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## NOVELTY AND SIGNIFICANCE

What Is Known?

- Aged/diseased cardiac progenitor cells (CPCs) derived from heart failure (HF) patients display functional impairment due to 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 pro-regenerative $P2Y_2$ receptor ($P2Y_2R$) correlate with altered phenotypic properties of CPCs isolated from cardiac biopsies of HF patients.

- Proliferative and migratory responses of functionally compromised CPCs are improved by $P2Y_2R$ activation or overexpression that is associated with modulating YAP activity.

Cardiac Progenitor Cell (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 HF patients and expression levels of a subset of P2 purinergic receptors. Specifically, expression of several P2 receptors including $P2Y_2R$, known to mediate regenerative responses in various tissues, is diminished in slow-growing CPCs. CPC proliferation and migration was improved by augmenting $P2Y_2R$ levels or $P2Y_2R$ stimulation with UTP via inhibiting Hippo signaling and activating the concordant downstream effector YAP. 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 $P2Y_2R$ as a novel upstream regulator of Hippo signaling revealing a link between extracellular nucleotides released during injury/stress and YAP that is critical for CPC growth and myocardial repair in response to injury.
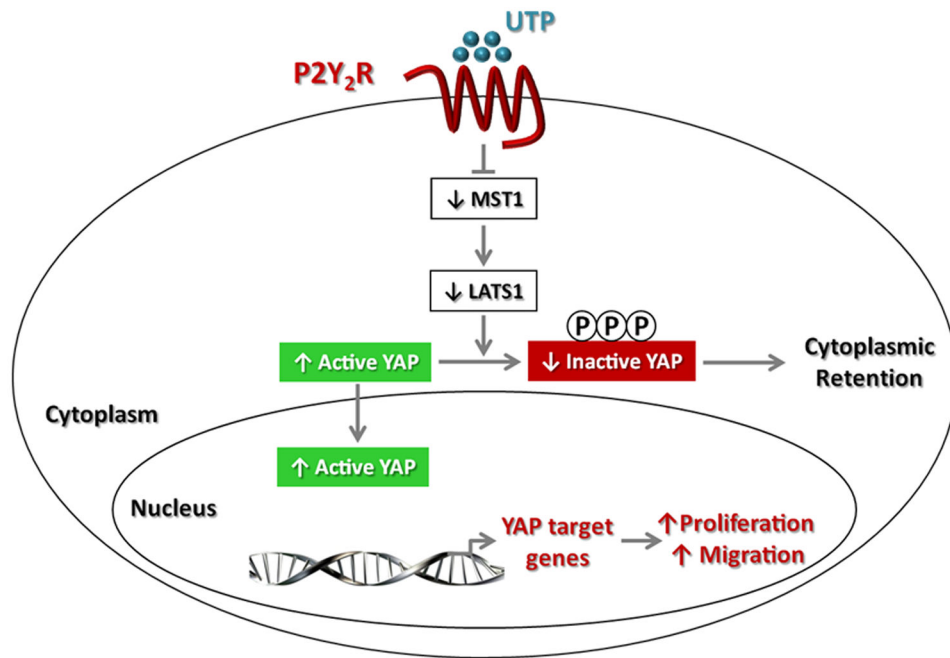
**Figure 1. P2Y$_2$R proposed mechanisms in CPCs**

Schematic representation of P2Y$_2$R proposed mechanisms for inducing hCPC proliferation and migration through YAP activation resulting from inhibition of Hippo upstream kinases MST1 and LATS1.
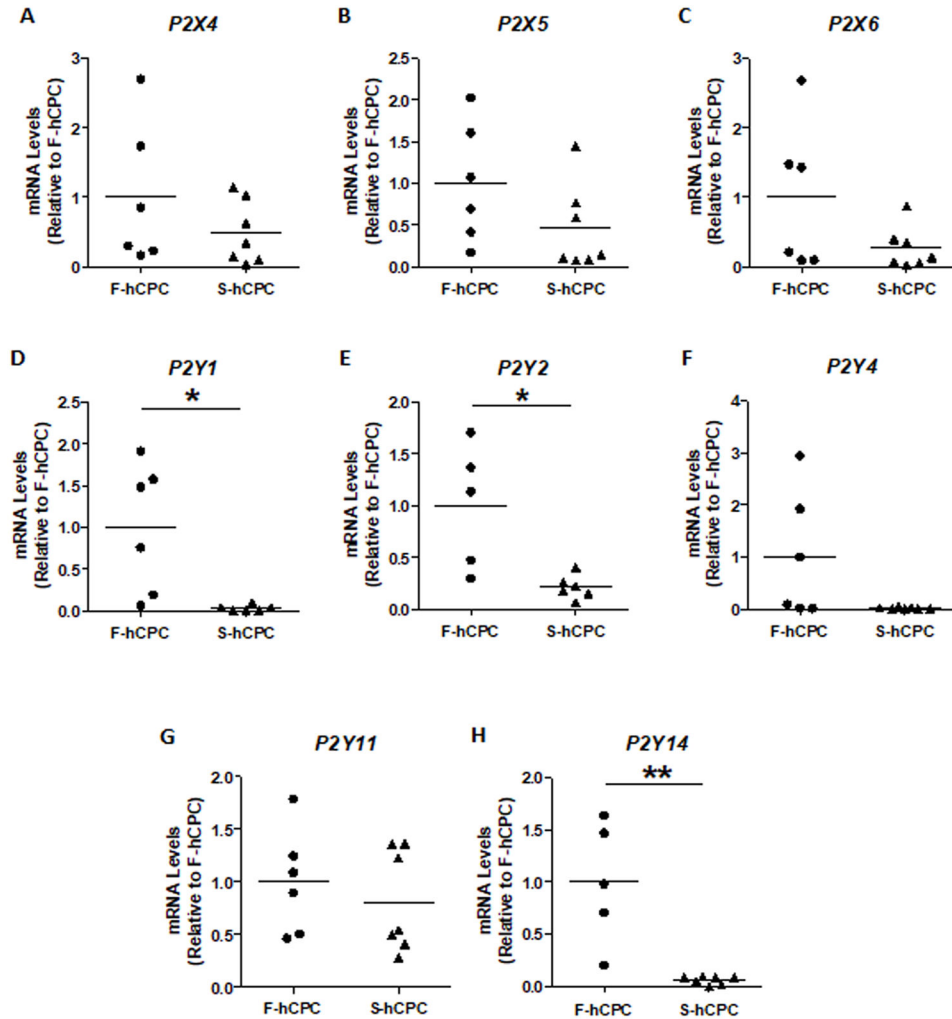
Figure 2. Differential P2 receptor subset expression in fast- and slow-growing CPCs
Expression of **(A)** *P2X4*, **(B)** *P2X5*, **(C)** *P2X6*, **(D)** *P2Y1*, **(E)** *P2Y2*, **(F)** *P2Y4*, **(G)** *P2Y11* and **(H)** *P2Y14* receptor mRNA by qRT-PCR analysis in fast-growing hCPCs (F-hCPC) and slow-growing hCPCs (S-hCPC). Cycle numbers were normalized to 18S and data is represented relative to F-hCPC. *P2Y1*, *P2Y2* and *P2Y14* mRNA expression levels are significantly downregulated in S-hCPC compared to F-hCPC (n=5–7). *P < 0.05 indicates significant difference from F-hCPC as measured by unpaired Student *t* test.
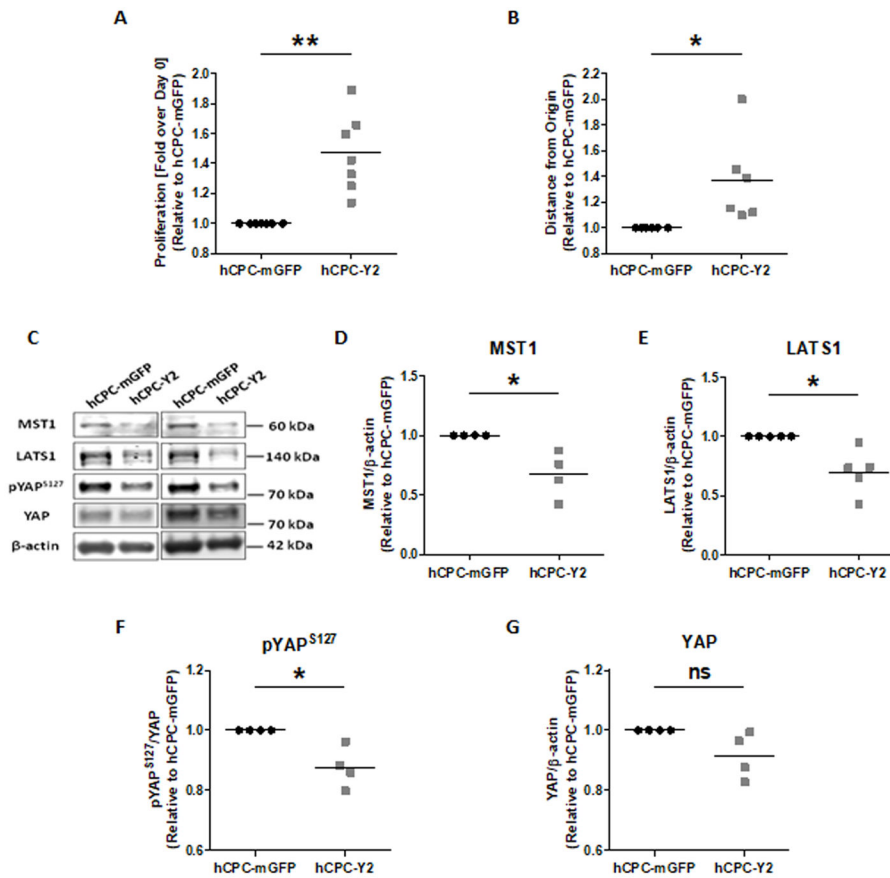
Figure 3. Enhancing CPC proliferation, migration and YAP activation by P2Y$_2$R overexpression
(A) Proliferation (n=7) and **(B)** migration (n=6) analysis showing that hCPC-Y2 exhibit enhanced proliferative and migratory capabilities compared to control hCPC-mGFP. Cell proliferation was measured using CyQuant assay and cell migration on GFR Matrigel was assessed by measuring the distance that cells traveled from origin after monitoring by time-lapse live cell imaging for 6 hours. hCPC immunoblotting analysis **(C)** and corresponding quantitation **(D, E, F, 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 to control hCPC-mGFP. pYAP^{S127} was normalized to total YAP. Total MST1, LATS1 and YAP levels were normalized to β-actin (loading control). *P < 0.05 and **P < 0.01 indicate significant difference from hCPC-mGFP as measured by paired Student t test.
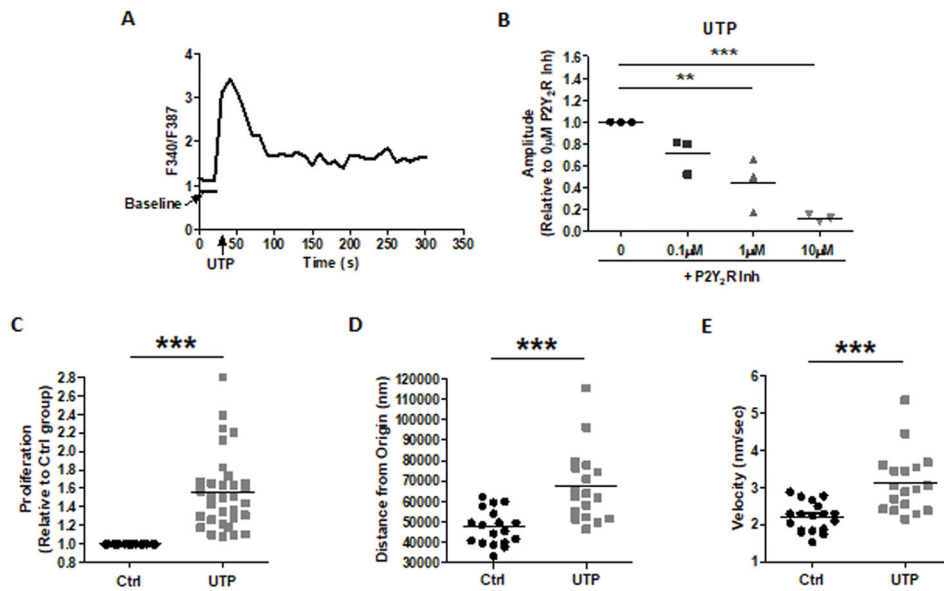
Figure 4. CPC activation by the P2Y$_2$R agonist UTP

(**A**) Intracellular calcium [Ca^{2+}]$_i$ transient in hCPCs in response to stimulation with UTP (100μM). [Ca^{2+}]$_i$ transients were measured using the calcium-dependent fluorescent dye Fura-2AM (4μM). Data is represented as the ratio of fluorescence intensities at 340nm excitation (F340) [Ca^{2+}-bound Fura-2] and 387nm (F387) excitation [Ca^{2+}-free Fura-2]. (**B**) P2Y$_2$R is the primary mediator of UTP-induced [Ca^{2+}]$_i$ transients as indicated by a decrease in [Ca^{2+}]$_i$ amplitude following hCPC treatment with selective P2Y$_2$R inhibitor AR-C 118925XX (0.1, 1 and 10μM) for 2 hours. Data is represented relative to 0μM P2Y$_2$R inhibitor. **$P < 0.01$ and ***$P < 0.001$ indicate significant difference from 0μM P2Y$_2$R inhibitor as measured by one-way ANOVA followed by Dunnett's post hoc test. (**C**) UTP induces hCPC proliferation as assessed by CyQuant assay on hCPCs treated with or without UTP (100μM) for 24 hours (n=32). ***$P < 0.001$ indicates significant difference from control group as measured by paired Student t test. (**D**) hCPC stimulation with UTP increases migration on GFR Matrigel as assessed by measuring the distance traveled from origin and (**E**) cell velocity 6 hours following UTP (100μM) treatment. Cell migration was monitored using time-lapse live cell imaging (n=18). ***$P < 0.001$ indicates significant difference from control group as measured by unpaired Student *t* test.
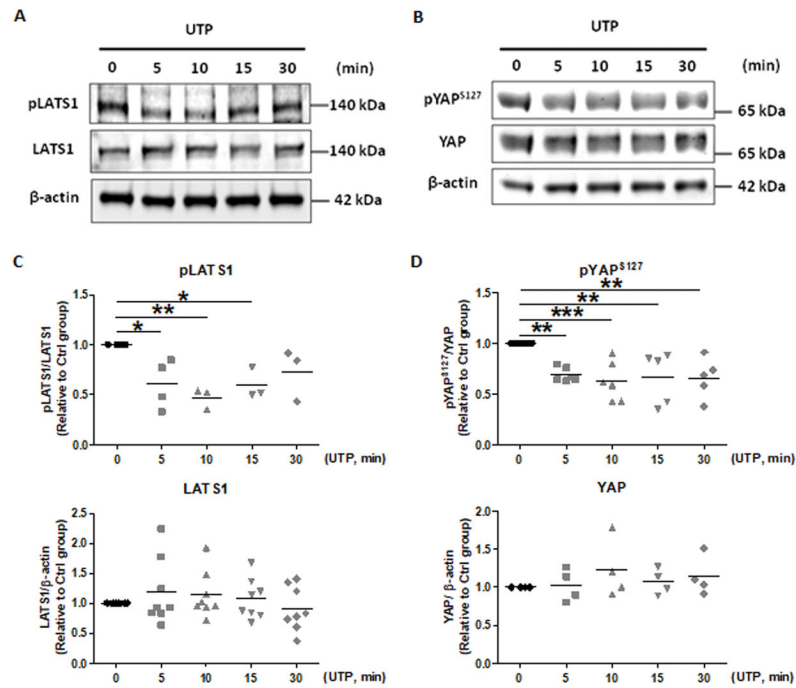
Figure 5. UTP inhibits LATS1 and activates YAP in CPCs

hCPC immunoblotting analysis **(A, B)** and corresponding quantitation **(C, D)** showing reduced phosphorylation of LATS1 (indicating inhibition) and YAP^{S127} (indicating activation) in response to UTP (100μM) 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 is represented relative to 0 min (no UTP treatment). *P < 0.05 and **P < 0.01 indicate significant difference from 0 min as measured by one-way ANOVA followed by Dunnett's post hoc test.
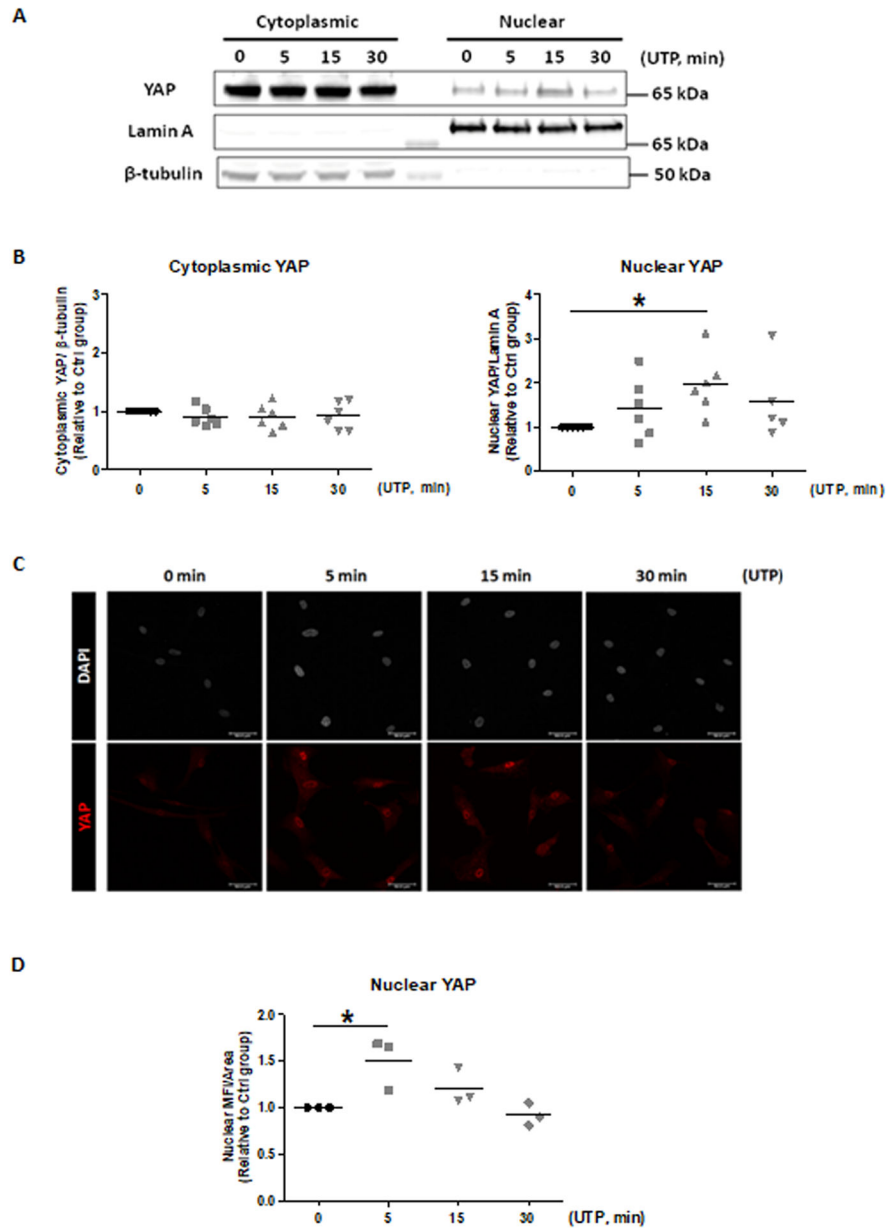
Figure 6. UTP enhances YAP nuclear localization in CPCs

Cytoplasmic and nuclear extracts of hCPCs on immunoblot **(A)** with corresponding quantitation **(B)** shows increased nuclear levels of YAP 15 minutes after UTP (100μM) stimulation. Cytoplasmic YAP was normalized to 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 quantitation showing increased nuclear levels of YAP 5 minutes after UTP (100μM) treatment (n=3). YAP nuclear signal intensity was normalized to nuclear area. DAPI nuclear stain: white; YAP: red. Scale bar = 50μm. Data is represented relative to 0 min (no UTP treatment). *P < 0.05 indicates significant difference from 0 min as measured by one-way ANOVA followed by Dunnett's post hoc test.
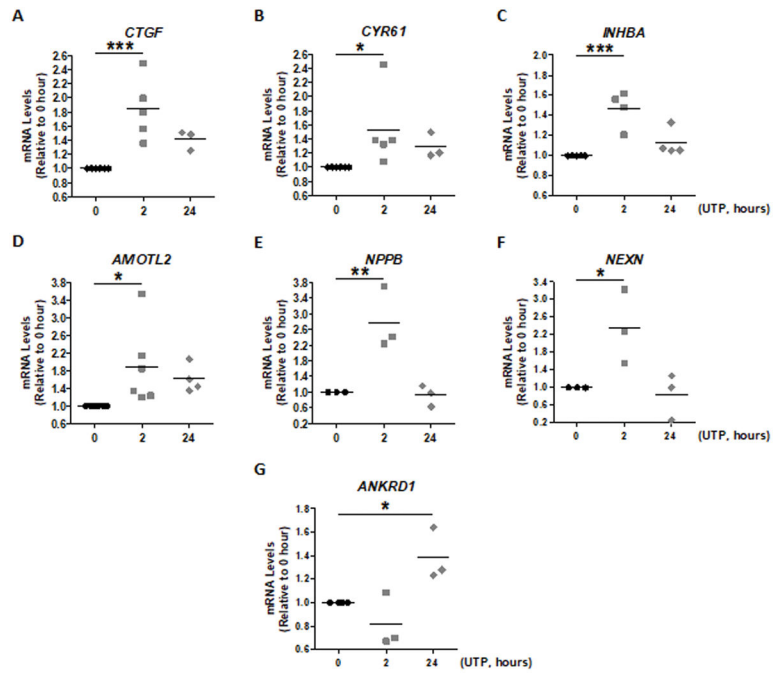
Figure 7. UTP enhances expression of YAP target genes in CPCs

Enhanced mRNA expression of YAP canonical target genes by qRT-PCR analysis for **(A)** *CTGF*, **(B)** *INHBA*, **(C)** *CYR61*, **(D)** *AMOTL2*, **(E)** *NPPB*, **(F)** *NEXN* and **(G)** *ANKRD1* after UTP (100μM) treatment. mRNA levels of *CTGF*, *INHBA*, *CYR61*, *AMOTL2*, *NPPB* and *NEXN* peaked 2 hours following UTP stimulation while increase in *ANKRD1* mRNA levels was observed 24 hours post UTP treatment (n=3–6/time point/gene). Data is represented relative to 0 hour (no UTP treatment). *P < 0.05, **P < 0.05 and ***P < 0.001 indicate significant difference from 0 hour as measured by one-way ANOVA followed by Dunnett's post hoc test.
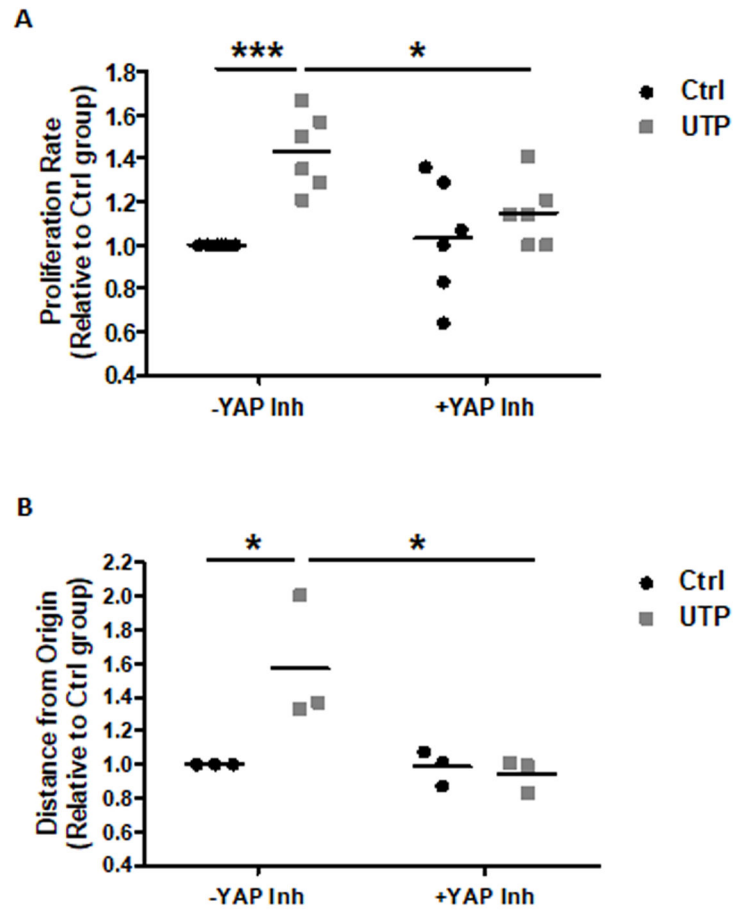
Figure 8. UTP-induced CPC proliferation and migration are dependent on YAP activation
hCPC proliferation (n=6) **(A)** and migration (n=3) **(B)** analysis showing that UTP-mediated responses require YAP activation as assessed by stimulating hCPCs with UTP (100µM) following treatment with or without YAP selective inhibitor verteporfin (100nM) for 1 hour. Cell proliferation and migration were measured as described in Figure 4. *P < 0.05 indicates significant difference between UTP groups treated versus non-treated with YAP inhibitor as measured by two-way ANOVA followed by Bonferroni post hoc test.

Table 1

Clinical Profile of Patients Used for Stem Cell Isolation

Patient ID	hCPC Doubling Time (hr)	P2Y₂R mRNA Levels (C(t))	Age (yr)	Sex	EF (%)	Cardiac Index	Diabetes	Hypercholesterolemia	Smoking	Infarct	Ischemia	Ace Inhibitor	β-blocker	Anticoagulant	NYHA
H10-014	21	20.5	73	M	17	1.6	x			-		x	x		IV
H11-020	22	20.9	68	F	20	1.7	x	x	x	x	x	x	x		IV
H10-001	24	23.1	68	M	11	1.6		x	x	x	x		x		IV
H12-053	24	21.0	59	M	15	1.7			x						IV
H12-047	25	23.8	72	M	8	1.1		x	x	x	x	x	x		IV
H10-004	28	21.0	81	M	8	2.0							x		IV
H13-067	30	22.6	84	M	-	-				-	-	-	-	-	-
H13-073	31	23.4	73	M	-	-	-	-	-	-	-	-	-	-	-
H13-064	41	23.5	57	M	15–25	-	-	x	-			-			IV

EF, ejection fraction; NYHA, New York Heart Association; patient information: ( ) positive; (X) negative; (-) unavailable.