$P2Y_2$ 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 $P2Y_2$ nucleotide receptor ($P2Y_2R$) 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 $P2Y_2R$, in slow-growing hCPCs compared with fast growers. hCPC proliferation and migration significantly improved by overexpressing or stimulating $P2Y_2R$. Mechanistically, $P2Y_2R$-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 $P2Y_2R$-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 $P2Y_2R$ 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.[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 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 interventional strategy to improve outcome of stem cell therapy as exemplified by empowering stem cells from diverse origins with prosurvival and antiapoptotic genes.[5,6] Regenerative capacity of

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Novelty and Significance

What Is Known?

- Aged/diseased cardiac progenitor cells (CPCs) derived from patients with heart failure display 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 P2Y$_2$ receptor (P2Y$_2$R), 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 P2Y$_2$R 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$_2$R, known to mediate regenerative responses in various tissues, is diminished in slow-growing CPCs. CPC proliferation and migration was improved by augmenting P2Y$_2$R levels or P2Y$_2$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$_2$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.

Nonstandard Abbreviations and Acronyms

CPCs	cardiac-derived progenitor cells
F-hCPCs	fast-growing human cardiac progenitor cells
hCPCs	human cardiac progenitor cells
HF	heart failure
LATS1	large tumor suppressor kinase 1
mGFP	monomeric green fluorescent protein
P2Y$_2$R	P2Y$_2$ nucleotide receptor
PBS	phosphate-buffered saline
S-hCPCs	slow-growing human cardiac progenitor cells
YAP	yes-associated protein

stem cells also depends on 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 7 P2X ligand-gated ion channels (P2X1-7) and 8 P2Y G protein-coupled receptors (P2Y$_{1,2,4,6,11,12,13,14}$).[7,8] Whereas some P2 receptors initiate early inflammatory responses, others mediate later regenerative responses required for the healing process.

P2Y$_2$ nucleotide receptor (P2Y$_2$R) is a proregenerative 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 after experimental colitis[16] 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$_2$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] However, it is not known whether Gαq protein-coupled P2Y$_2$R-induced proliferative and migratory responses are dependent on YAP activation (Figure 1). YAP activity is tightly regulated by multiple upstream kinases, including mammalian MST1 (Hippo homolog) and LATS1 (large tumor suppressor kinase 1).[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 coactivator 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 patients with HF and P2 purinergic receptor expression. Indeed, expression of select P2 receptors, including the proregenerative P2Y$_2$R, directly correlated with hCPC growth kinetics. Proliferation and migration of functionally compromised hCPCs were improved by P2Y$_2$R activation or overexpression and impaired by P2Y$_2$R knockdown. P2Y$_2$R-induced proliferation and migration were mediated by YAP activation, introducing a novel downstream component in the P2Y$_2$R intracellular signaling cascade. These findings are discussed in the context of manipulating hCPCs to enhance their phenotypic properties.

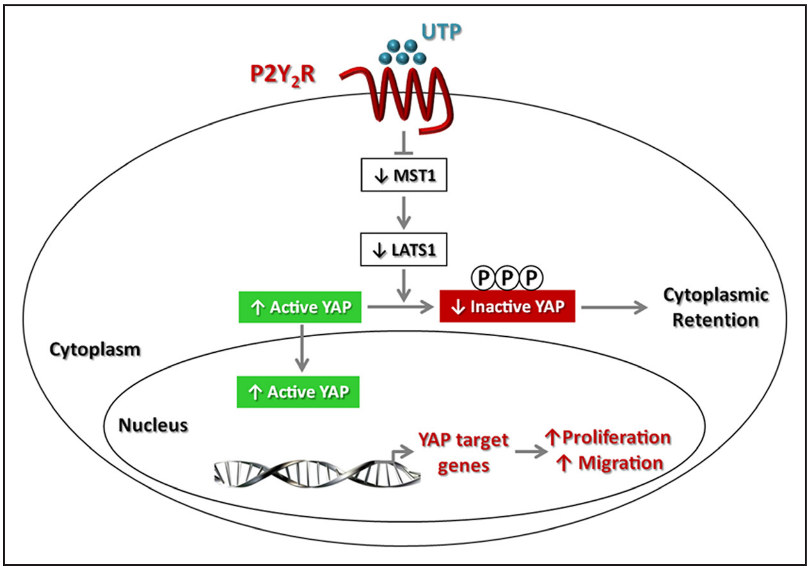

Figure 1. P2Y$_2$R proposed mechanisms in cardiac progenitor cells (CPCs). Schematic representation of P2Y$_2$R proposed mechanisms for inducing human CPC proliferation and migration through YAP (yes-associated protein) activation resulting from inhibition of Hippo upstream kinases MST1 and LATS1.

Methods

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.[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, 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% CO_2 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 fluorescent dye Fura-2 AM (4 μM) in Krebs–Ringer solution (125 mmol/L NaCl, 5 mmol/L KCl, 1.2 mmol/L Mg_2SO_4, 2 mmol/L $CaCl_2$, 10 mmol/L glucose, 25 mmol/L 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. Ca^{2+} 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 are represented as the ratio of fluorescence intensities at 340- (F340) [Ca^{2+}-bound Fura-2] and 387-nm (F387) excitation [Ca^{2+}-free Fura-2]. In inhibitor studies, cells were pretreated with P2Y$_2$R selective antagonist (AR-C 118925XX; 0.1, 1, and 10 μM; Tocris Bioscience) for the indicated times before UTP treatment.

Cell Proliferation Assay

hCPCs were cultured in serum-starved medium (Hams F12 media supplemented with 2.5% embryonic stem cell screened fetal bovine serum) in a 96-well plate (500 cells/ well) and 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 (100 μM), 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; 100 nM; Tocris Bioscience) for the indicated times before 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 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 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 (30 000 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 on 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. 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 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 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 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 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).[21] 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.[21] 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=0.012; 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 on prior reports of involvement in regeneration using various

Table. Clinical Profile of Patients Used for Stem Cell Isolation

Patient Identifier	hCPC Doubling Time, h	P2Y$_2$R mRNA Levels (ΔC(t))	Age, y	Sex	EF, %	Cardiac Index	Diabetes Mellitus	Hypercholesterolemia	Smoking	Infarct	Ischemia	Ace Inhibitor	β-Blocker	Anticoagulant	NYHA
H10-014	21	20.5	73	M	17	1.6	–	+	+	…	+	–	–	+	IV
H11-020	22	20.9	68	F	20	1.7	–	–	–	–	–	–	–	+	IV
H10-001	24	23.1	68	M	11	1.6	+	–	–	–	–	+	–	+	IV
H12-053	24	21.0	59	M	15	1.7	+	+	–	+	+	+	+	+	IV
H12-047	25	23.8	72	M	8	1.1	+	–	–	–	–	–	–	+	IV
H10-004	28	21.0	81	M	8	2.0	+	+	+	+	+	+	–	+	IV
H13-067	30	22.6	84	M	…	…	+	+	+	…	…	…	…	…	…
H13-073	31	23.4	73	M	…	…	…	…	…	…	…	…	…	…	…
H13-064	41	23.5	57	M	15–25	…	…	–	…	+	+	…	+	+	IV

Patient information: (+) positive; (–) negative; and (…) unavailable. EF indicates ejection fraction; hCPC, human cardiac progenitor cells; NYHA, New York Heart Association; and P2Y$_2$R, P2Y$_2$ nucleotide receptor.

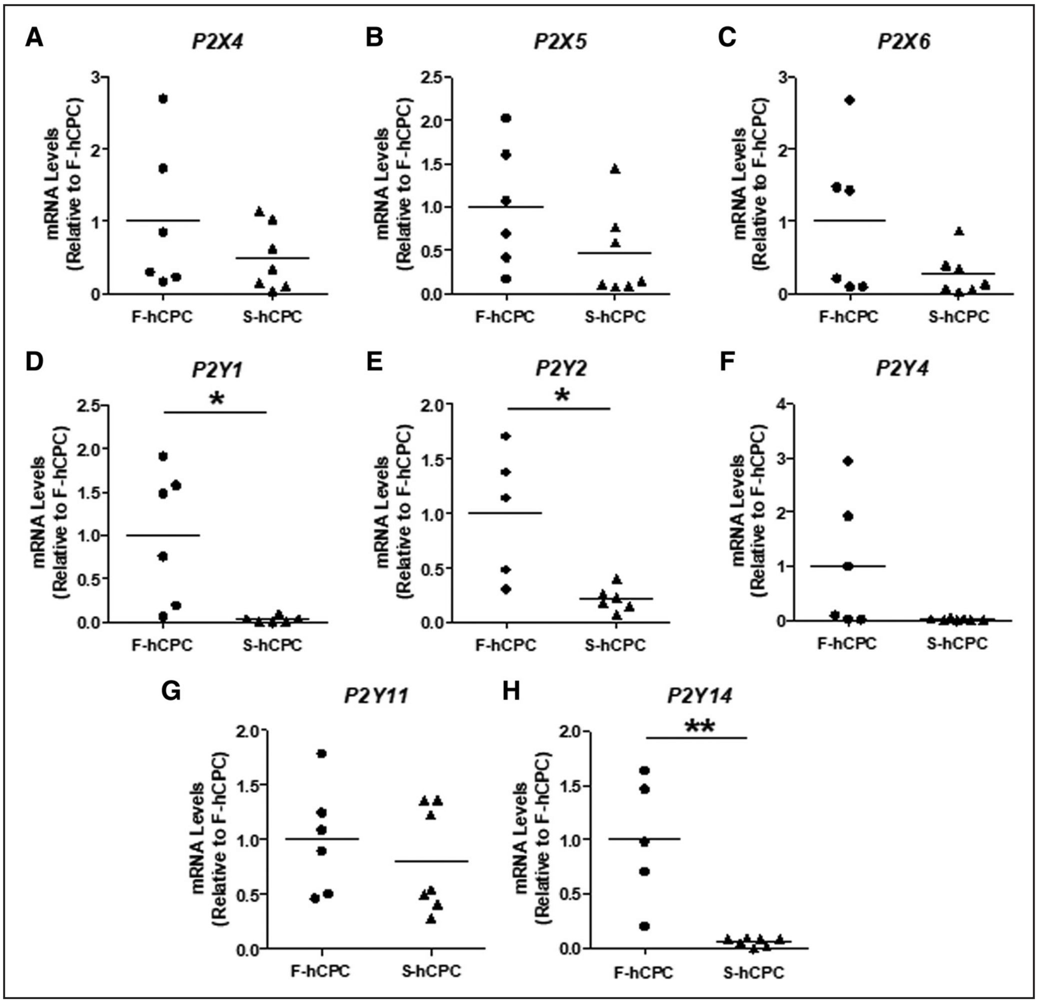

Figure 2. Differential P2 receptor subset expression in fast- and slow-growing cardiac progenitor cells (CPCs). Expression of (**A**) *P2X4*, (**B**) *P2X5*, (**C**) *P2X6*, (**D**) *P2Y1*, (**E**) *P2Y2*, (**F**) *P2Y4*, (**G**) *P2Y11*, and (**H**) *P2Y14* receptor mRNA by reverse transcriptase–polymerase 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.

experimental models[13–15,23,24] and in stem cells from diverse origins.[18,25] Higher $P2Y_2R$ mRNA expression levels corresponded with faster hCPC growth rates indicated by shorter doubling times (R=0.7101; P=0.0369; Online Figure IA). $P2Y_2R$ 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 $P2Y_2R$ Overexpression

hCPC proliferation and migration potential was improved by increasing $P2Y_2R$ levels. hCPCs were infected with lentiviral particles encoding $P2Y_2R$ 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 $P2Y_2R$ in hCPC-Y2 (3.52±0.95-fold change; Online Figure IIB), as well as by immunoblotting showing expression of $P2Y_2R$-mGFP fused construct (Online Figure IIC). Expression of GFP alone did not alter hCPC proliferation (Online Figure IID). $P2Y_2R$ 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). $P2Y_2R$ 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).

$P2Y_2R$ 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 Gαq protein-coupled receptors,[19] upstream regulatory extracellular signals are still largely unknown. Overexpression of the Gαq protein-coupled $P2Y_2R$ 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

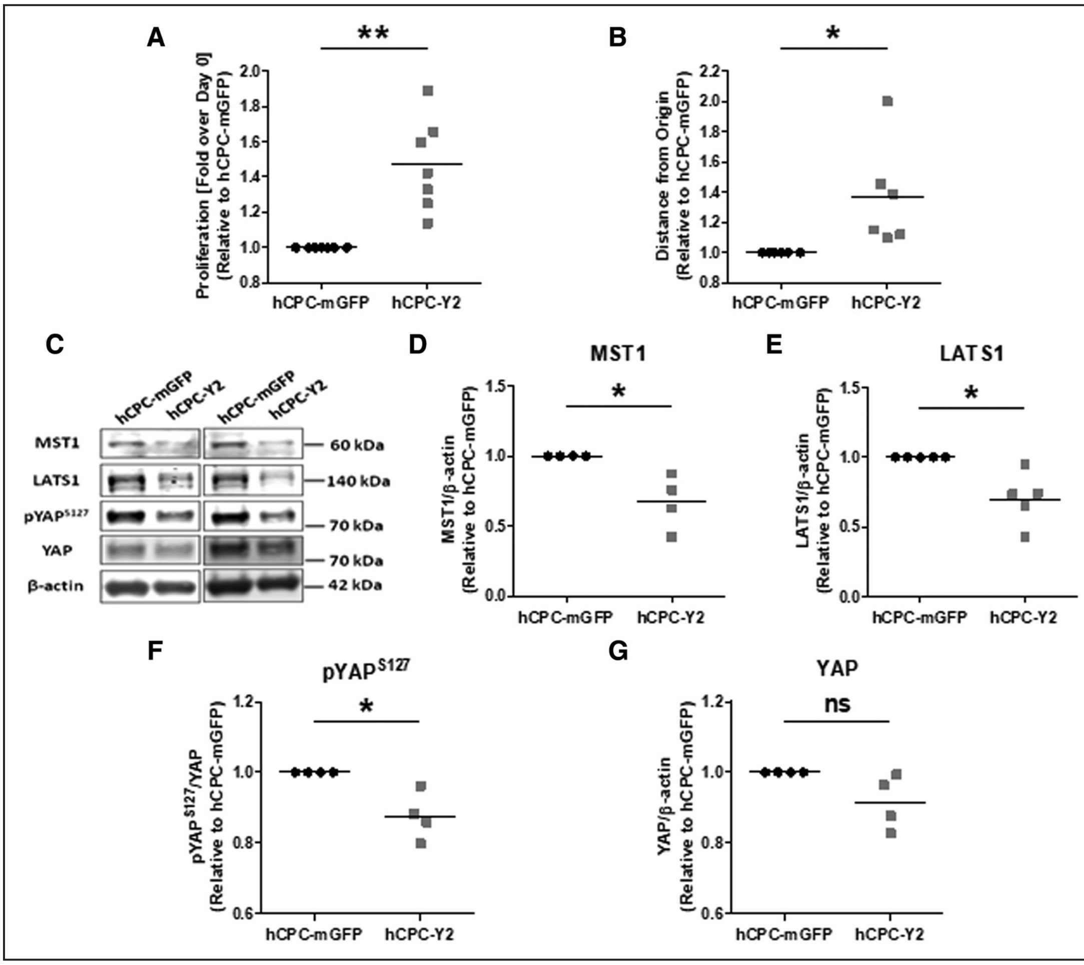

Figure 3. Enhancing cardiac progenitor cell (CPC) proliferation, migration, and yes-associated protein (YAP) activation by P2Y$_2$R overexpression. **A**, Proliferation (n=7) and (**B**) migration (n=6) analysis showing that human CPC (hCPC)-Y2 exhibit enhanced proliferative and migratory capabilities 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 β-actin (loading control). **P*<0.05 and ***P*<0.01 indicate significant difference from hCPC-mGFP as measured by paired 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 S^{127} residue (0.86±0.0097-fold change; *P*=0.005; Figure 3C through 3F). 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 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; Online Figure VI). P2Y$_2$R inhibition using the selective antagonist AR-C 118925XX impaired UTP-mediated calcium transients in a dose-dependent manner (0.1 μM, 0.71±0.095-fold change; 1 μM, 0.44±0.14-fold change; 10 μM, 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 growth factor reduced Matrigel as shown by increased distance travelled from origin (from 47 326±2029 to 67 145±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: 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 or scrambled shRNA as a control. Transduction

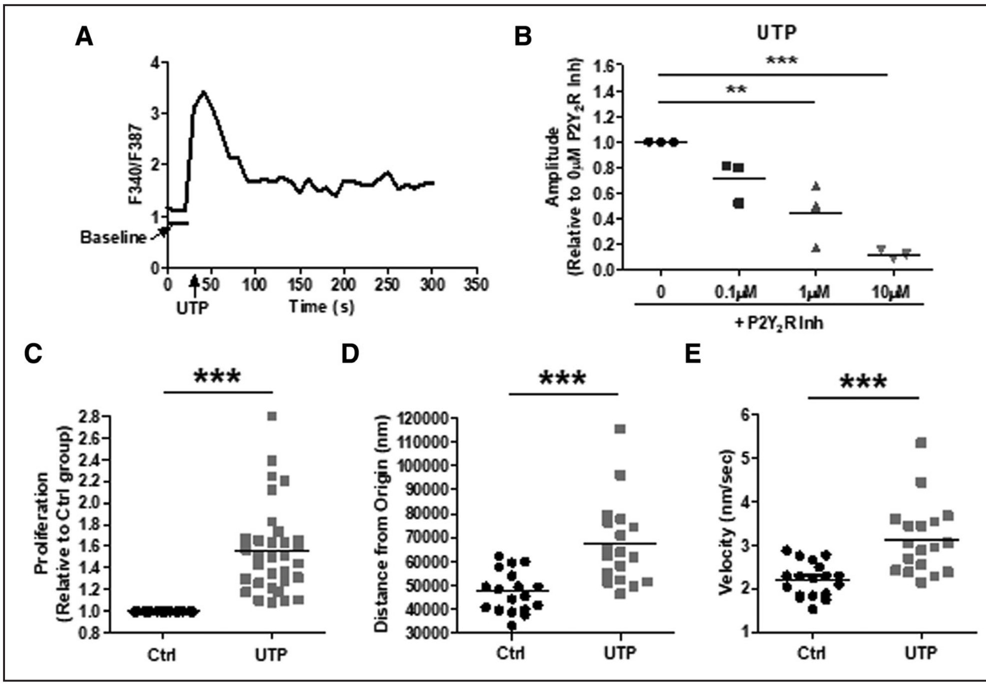

Figure 4. Cardiac progenitor cells (CPC) activation by the $P2Y_2R$ agonist UTP. A, Intracellular calcium $[Ca^{2+}]_i$ transient in human CPCs (hCPCs) in response to stimulation with UTP (100 μM). $[Ca^{2+}]_i$ transients were measured using the calcium-dependent fluorescent dye Fura-2 AM (4 μM). Data are represented as the ratio of fluorescence intensities at 340-nm excitation (F340) [Ca^{2+}-bound Fura-2] and 387-nm (F387) excitation [Ca^{2+}-free Fura-2]. **B**, $P2Y_2R$ is the primary mediator of UTP-induced $[Ca^{2+}]_i$ transients as indicated by a decrease in $[Ca^{2+}]_i$ amplitude after hCPC treatment with selective $P2Y_2R$ inhibitor AR-C 118925XX (0.1, 1, and 10 μM) for 2 h. Data are represented relative to 0 μM $P2Y_2R$ inhibitor. ***P*<0.01 and ****P*<0.001 indicate significant difference from 0-μM $P2Y_2R$ inhibitor as measured by 1-way ANOVA followed by Dunnett post hoc test. **C**, UTP induces hCPC proliferation as assessed by CyQuant assay on hCPCs treated with or without UTP (100 μM) for 24 h (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 growth factor reduced Matrigel as assessed by measuring the distance traveled from origin and (**E**) cell velocity 6 h after UTP (100 μM) 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.

efficiency was assessed by flow cytometry for percentage of GFP^+ cells (68.23±8.09% for hCPCs using lentivirus encoding $P2Y_2R$ shRNA and 59±10.48% for hCPCs using scrambled shRNA; Online Figure VIIIA). $P2Y_2R$ knockdown was confirmed by quantitative reverse transcriptase–polymerase chain reaction showing reduced $P2Y_2R$ mRNA levels (0.51±0.046-fold change; Online Figure VIIIB). $P2Y_2R$ 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 $P2Y_2R$.

UTP Prompts YAP Activation and Nuclear Localization

The role of $P2Y_2R$ in regulating Hippo signaling was validated via assessment of LATS1 kinase and YAP activity resulting from $P2Y_2R$ 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 S^{127} residue (5 minutes, 0.69±0.028-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 ($pYAP^{S127}$) was only detected in the cytoplasmic fraction and excluded from the nucleus (Online Figure IX). Collectively, these findings support $P2Y_2R$-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 $P2Y_2R$[35–38] and YAP signaling pathways.[39–42]

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;

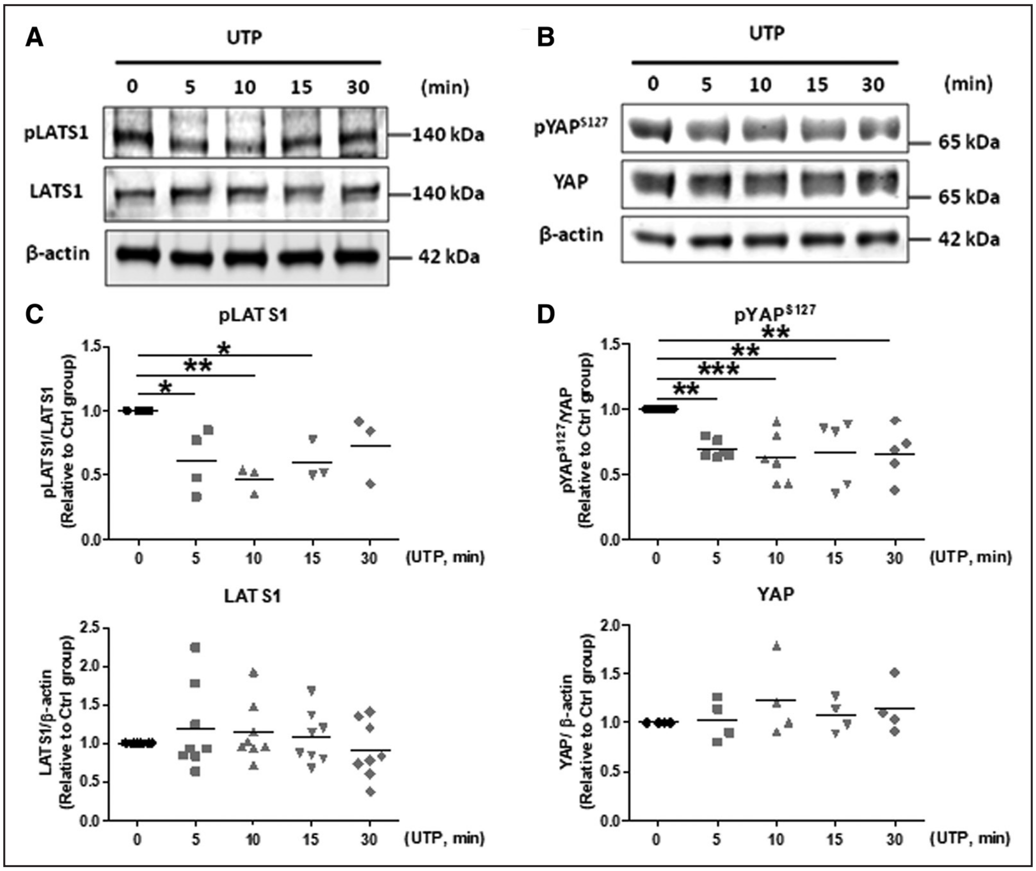

Figure 5. UTP inhibits LATS1 and activates YAP (yes-associated protein) in cardiac progenitor cells (CPCs). Human CPC immunoblotting analysis (**A** and **B**) and corresponding quantification (**C** and **D**) showing reduced phosphorylation of LATS1 (indicating inhibition) and YAP^{S127} (indicating 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.

$P<0.05$), *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 through 7G). 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 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 migration (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,[43] which could be addressed by identifying molecular components regulating these critical phenotypic characteristics of hCPCs. Findings in this study point to P2Y$_2$R as an important regulator of hCPC proliferation and migration and delineate underlying mechanisms. P2Y$_2$R was significantly downregulated in S-hCPCs compared with fast growers (F-hCPCs). Augmenting P2Y$_2$R levels or P2Y$_2$R stimulation with UTP in hCPCs efficiently improved proliferation and migration potential. P2Y$_2$R-induced responses involved downstream activation of YAP signaling, introducing a novel component into the P2Y$_2$R 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

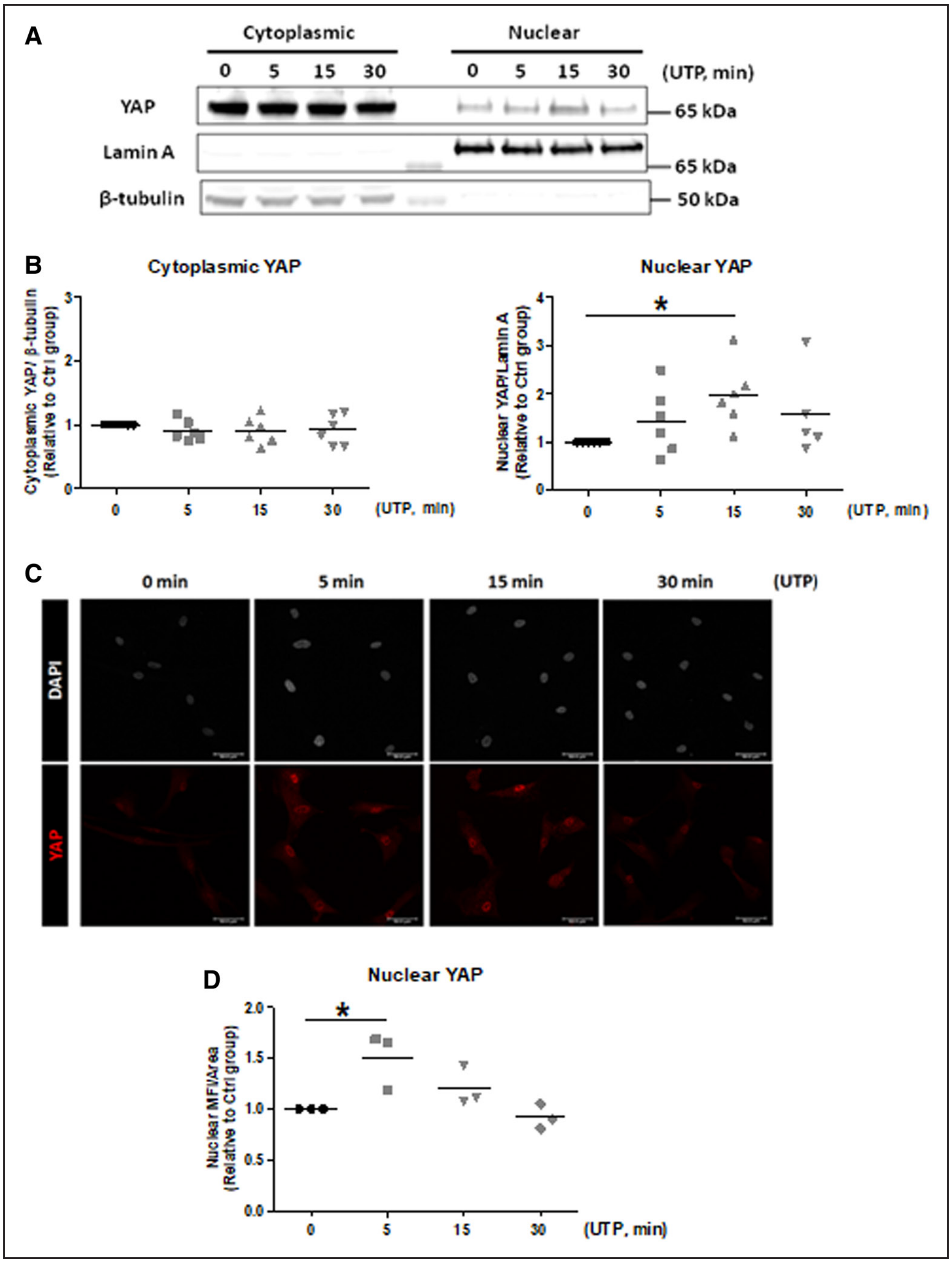

Figure 6. UTP enhances YAP (yes-associated protein) nuclear localization in cardiac progenitor cells (CPCs). Cytoplasmic and nuclear extracts of human CPCs (hCPCs) on immunoblot (**A**) with corresponding quantification (**B**) shows increased nuclear levels of YAP 15 min after UTP (100 μM) stimulation. 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 quantification showing increased nuclear levels of YAP 5 min after UTP (100 μM) treatment (n=3). YAP nuclear signal intensity was normalized to nuclear area. DAPI nuclear stain, white; YAP, red. Scale bar=50 μm. Data are represented relative to 0 min (no UTP treatment). *$P<0.05$ indicates significant difference from 0 min as measured by 1-way ANOVA followed by Dunnett post hoc test.

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 patients with HF (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 proproliferative in various experimental models, including hepatocytes,[15,44] corneal endothelial cells,[45]

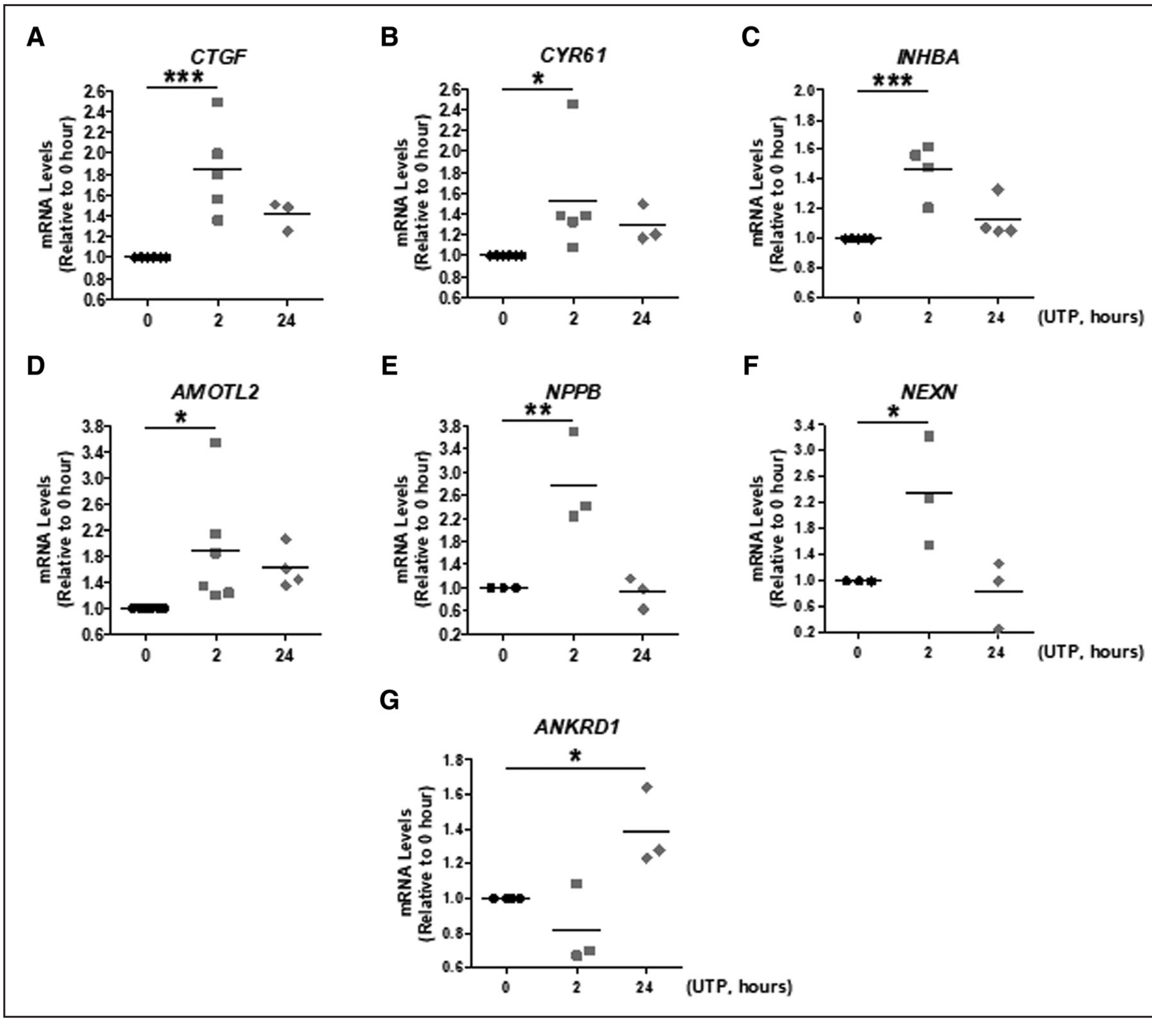

Figure 7. UTP enhances expression of YAP (yes-associated protein) target genes in cardiac progenitor cells. Enhanced mRNA expression of YAP canonical target genes by reverse transcriptase–polymerase chain reaction analysis for (**A**) *CTGF*, (**B**) *INHBA*, (**C**) *CYR61*, (**D**) *AMOTL2*, (**E**) *NPPB*, (**F**) *NEXN*, and (**G**) *ANKRD1* after UTP (100 μM) treatment. mRNA levels of *CTGF*, *INHBA*, *CYR61*, *AMOTL2*, *NPPB*, and *NEXN* peaked 2 h after UTP stimulation, whereas increase in *ANKRD1* 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.

and pancreatic duct epithelial cells.[46] In addition, P2Y$_2$R-induced migration in hCPCs is consistent with promigratory responses of P2Y$_2$R in fibroblasts,[24] salivary[14] and corneal epithelial cells.[13] Whether ex vivo manipulation of hCPCs by UTP preconditioning or P2Y$_2$R overexpression improves transplanted hCPC homing, expansion, and overall reparative potential for injured myocardium remains to be assessed.

P2Y$_2$R was implicated in mediating enhanced proliferation and differentiation after preconditioning of atrial-derived hCPCs with ATP for 30 minutes in vitro and in vivo,[25] but involvement of P2Y$_2$R 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.[19,47] Gαq/11- and Gαi/o-coupled signals induce YAP activity, whereas Gαs-coupled signals repress YAP.[20] Concordantly, our data show Gαq protein-coupled P2Y$_2$R 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$_2$R, 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[26–30] where YAP inhibition was proposed as a potential therapeutic target to halt tumorigenesis.

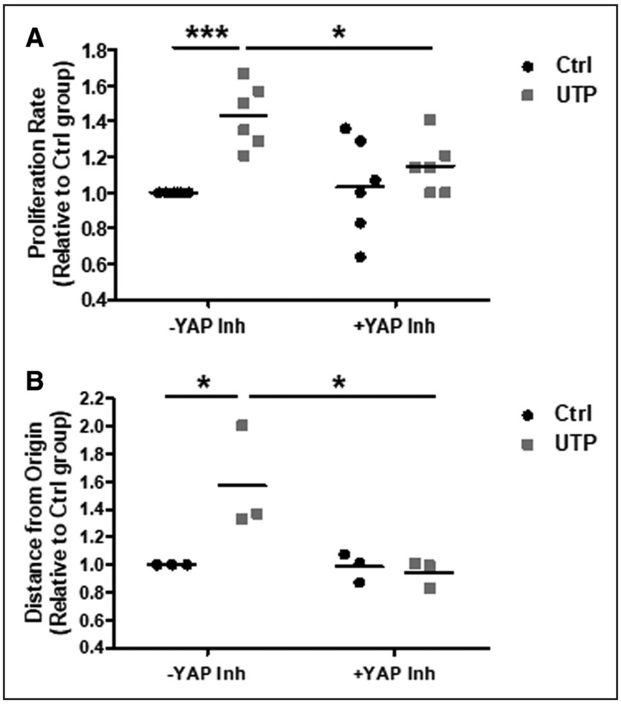

Figure 8. UTP-induced cardiac progenitor cell (CPC) proliferation and migration are dependent on YAP (yes-associated protein) activation. Human CPC (hCPC) proliferation (n=6; **A**) and migration (n=3; **B**) analysis showing that UTP-mediated responses require YAP activation as assessed by stimulating hCPCs with UTP (100 μM) after treatment with or without YAP selective inhibitor verteporfin (100 nM) for 1 h. Cell proliferation and migration were measured as described in Figure 4. *$P<0.05$ indicates significant difference between UTP groups treated vs nontreated with YAP inhibitor as measured by 2-way ANOVA followed by Bonferroni post hoc test.

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 with F-hCPCs (Figure 2D). $P2Y_1R$ regulates regenerative responses in several tissues. $P2Y_1R$ mediates chondrocyte proliferation and cartilage repair in osteoarthritis,[49] neuronal fiber outgrowth in organotypic brain slice cocultures,[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 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,[53] chemotaxis of human hematopoietic stem cells,[54] and human neutrophils.[55] In addition, $P2Y_{14}R$ enhances mouse hematopoietic stem cell 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 proregenerative 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 proregenerative 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 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 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. $P2Y_2R$ 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.[21] 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.

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Circulation Research

JOURNAL OF THE AMERICAN HEART ASSOCIATION

$P2Y_2$ Nucleotide Receptor Prompts Human Cardiac Progenitor Cell Activation by Modulating Hippo Signaling

Farid G. Khalafalla, Steven Greene, Hashim Khan, Kelli Ilves, Megan M. Monsanto, Roberto Alvarez, Jr, Monica Chavarria, Jonathan Nguyen, Benjamin Norman, Walter P. Dembitsky and Mark A. Sussman

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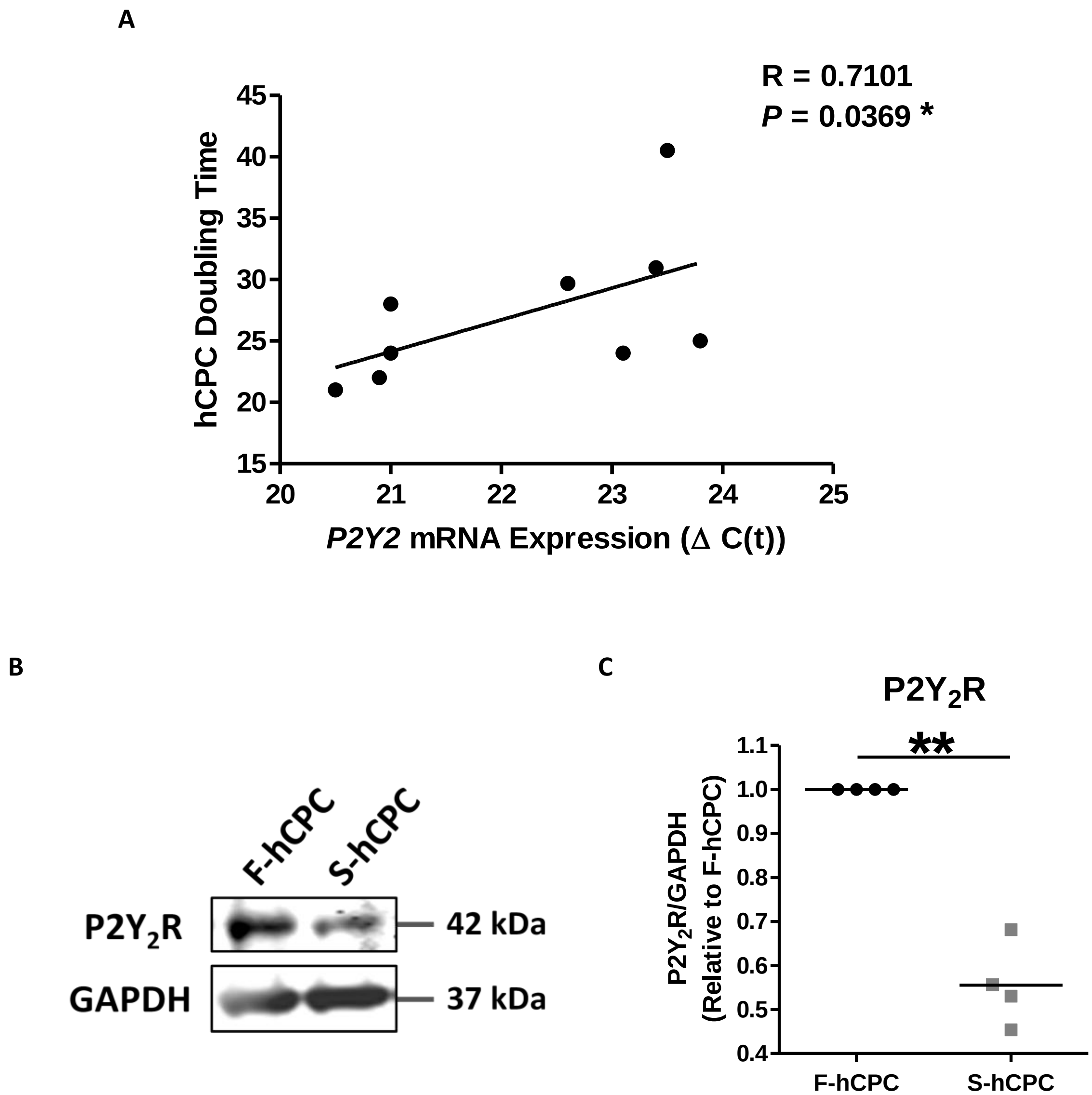
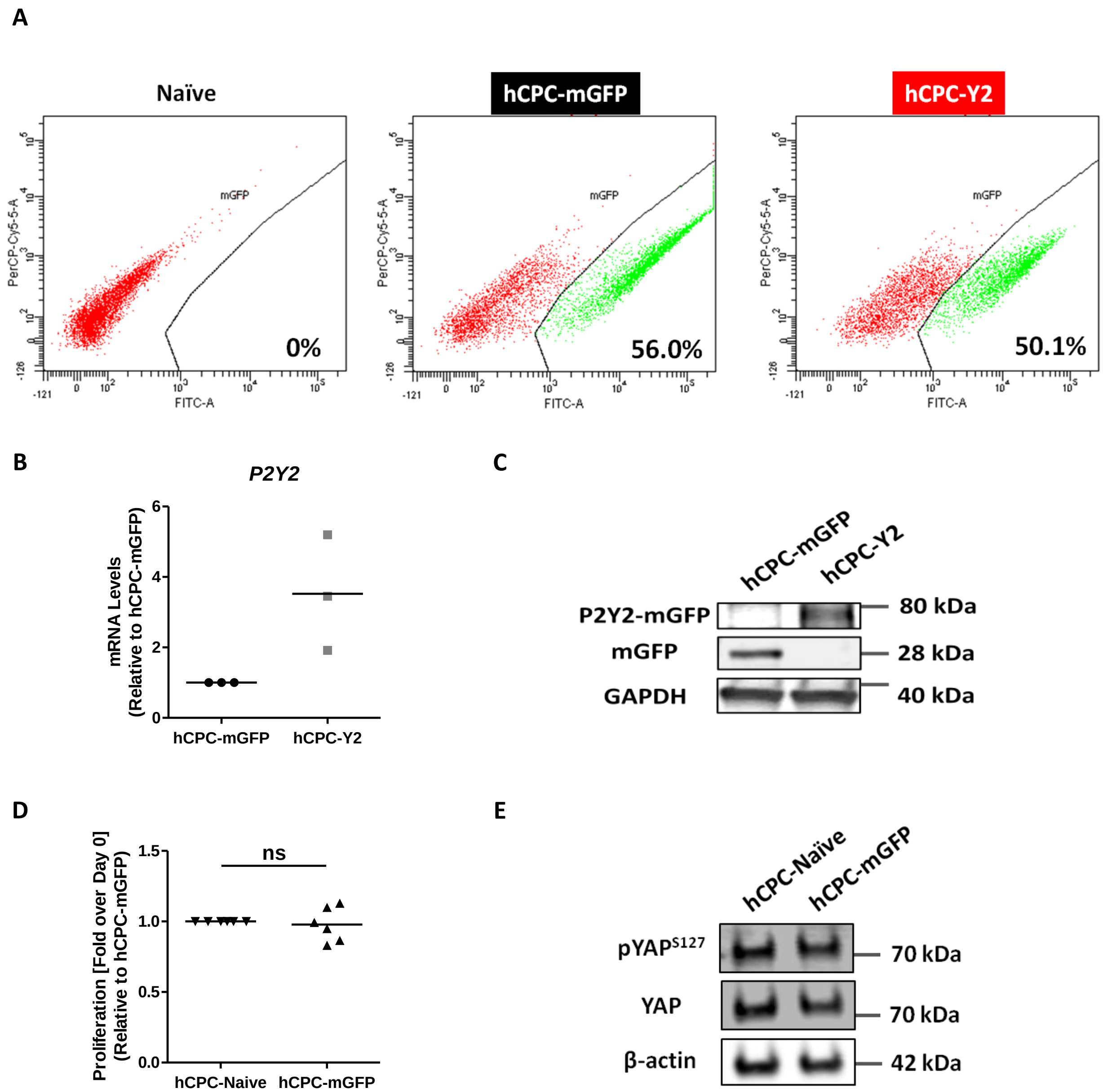
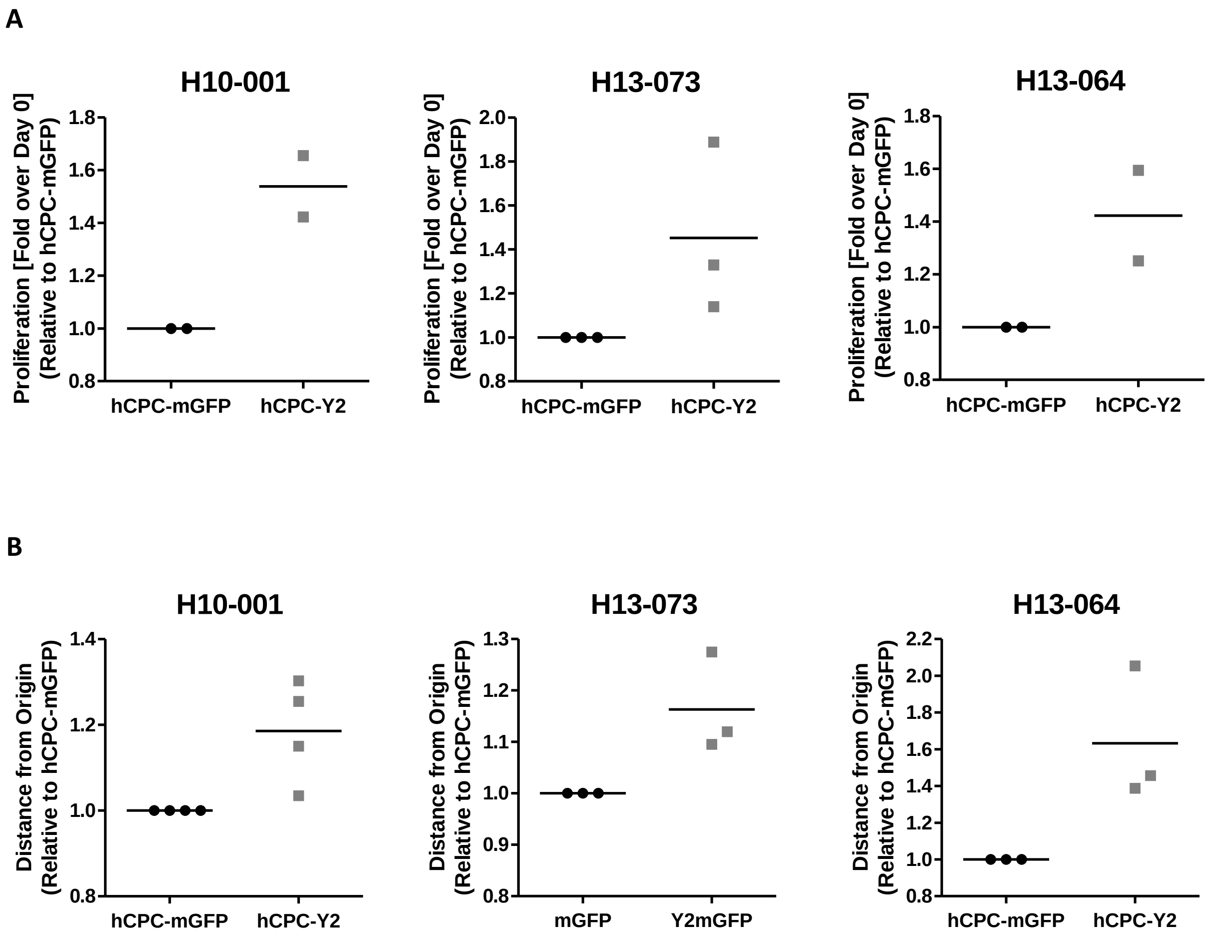
Supplemental Material



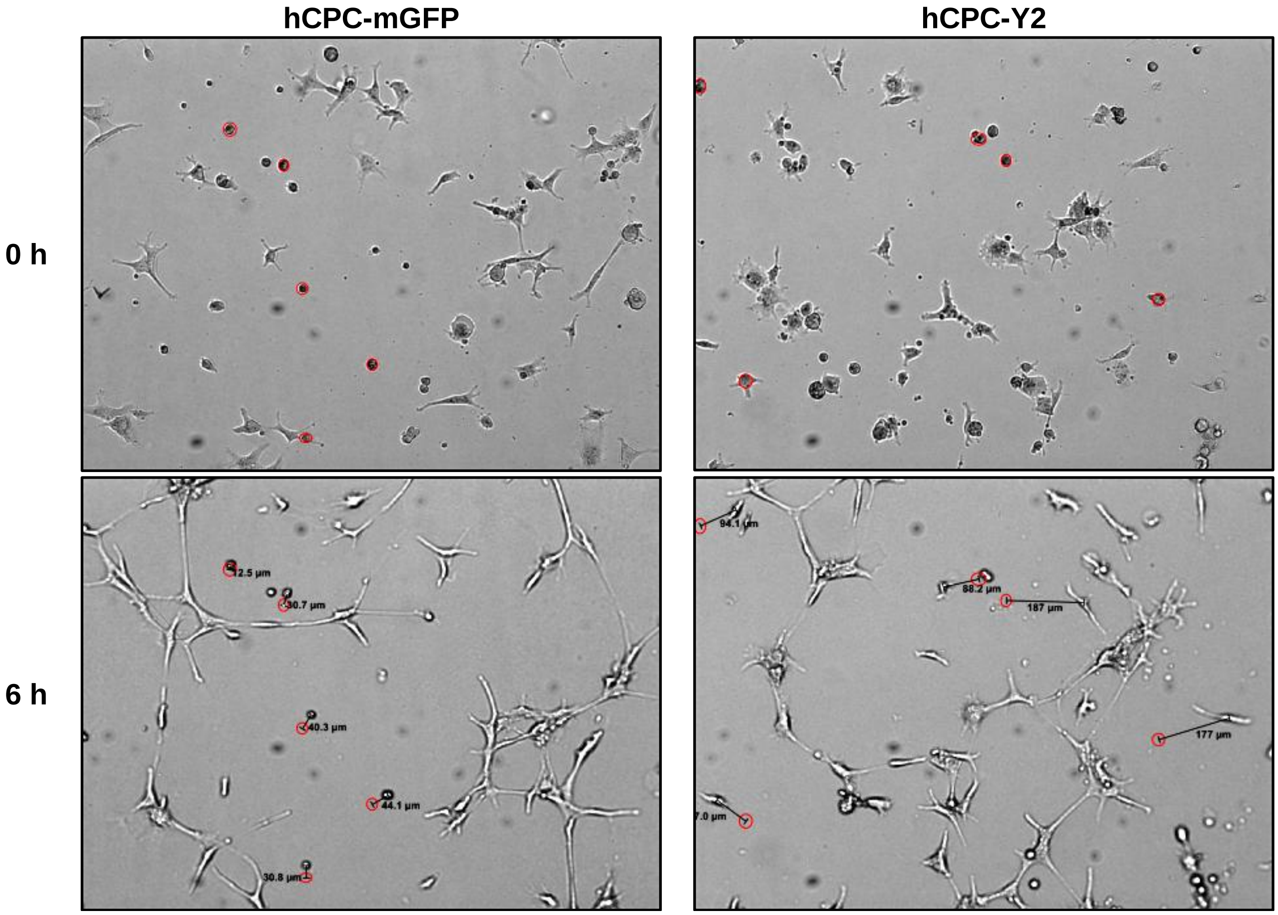
Online Figure I: $P2Y_2R$ expression correlates with hCPC doubling time.
(A) $P2Y_2R$ mRNA expression in hCPCs derived from multiple heart failure patients plotted against hCPC doubling time showing that low doubling time (fast growth kinetics) corresponds to low cycle number (high $P2Y_2R$ mRNA expression 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 $P2Y_2R$ 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.



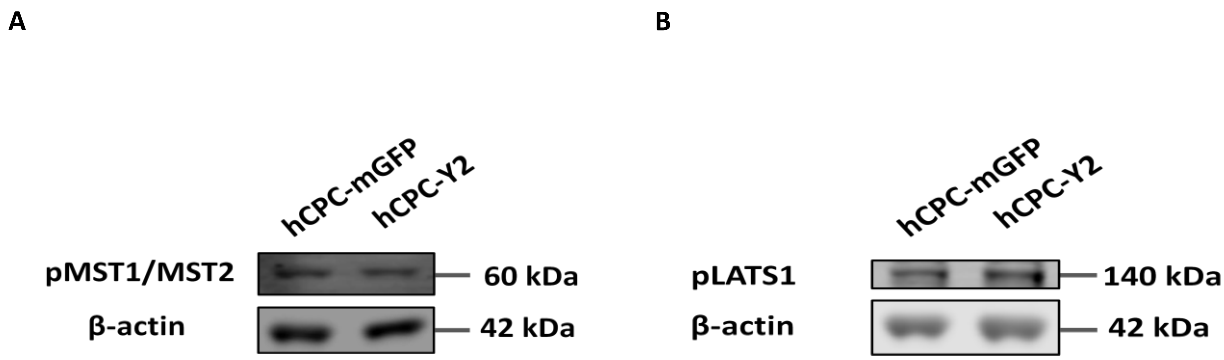
Online Figure II: Lentiviral-mediated overexpression of P2Y$_2$R-mGFP in CPCs.
(A) Transduction efficiency of hCPCs with lentiviral particles encoding for P2Y$_2$R and mGFP (hCPC-Y2) or mGFP alone (hCPC-mGFP) by flow cytometry analysis with percentage of GFP$^+$ cells (56% for hCPC-mGFP and 50.1% for hCPC-Y2). **(B)** Elevated P2Y$_2$R 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 P2Y$_2$R-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.



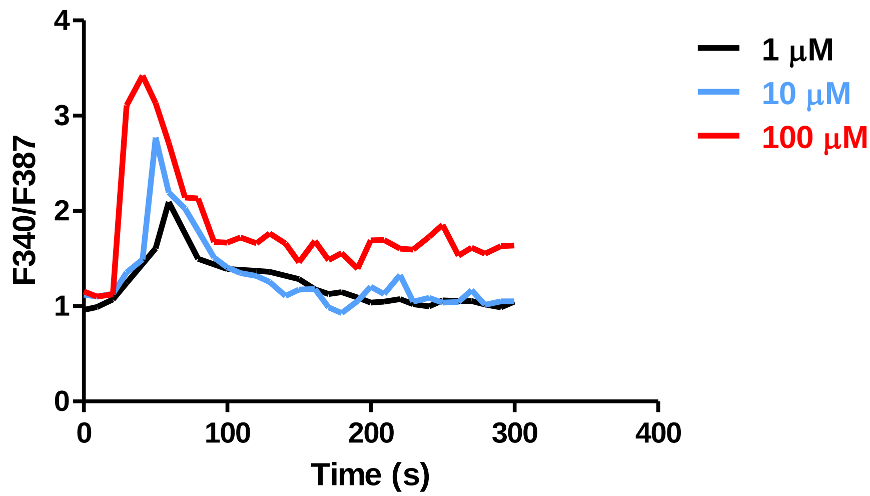
Online Figure III: Enhancing proliferation and migration of individual hCPC lines by $P2Y_2R$ overexpression.
Enhanced proliferation (n=2-3/line) **(A)** and migration (n=2-3/line) **(B)** of 3 representative hCPC lines by $P2Y_2R$ overexpression. Cell proliferation and migration were assessed as described in Figure 3.



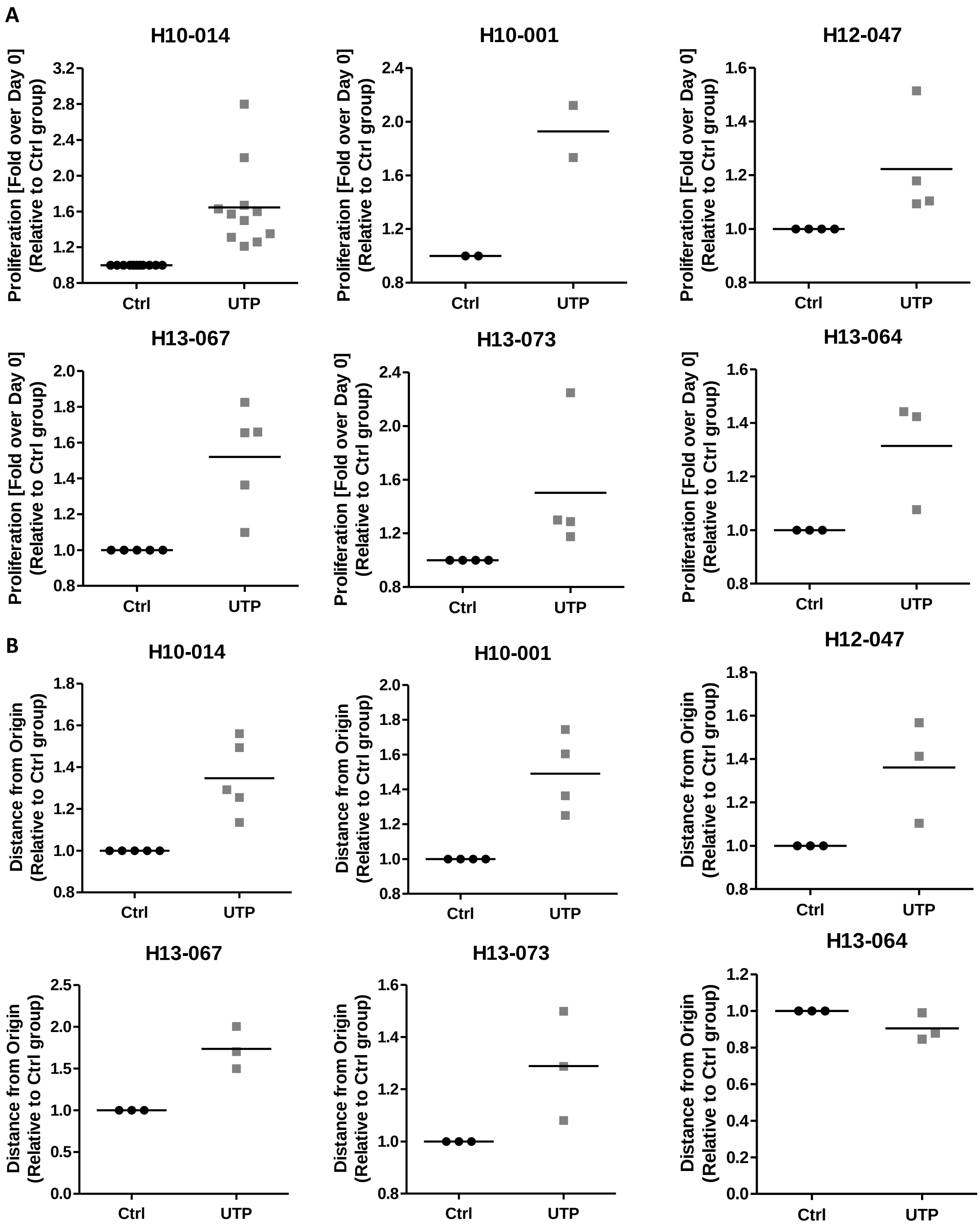
Online Figure IV: Improving hCPC migration by $P2Y_2R$ overexpression.
Representative images from time-lapse live cell imaging showing enhanced migration of hCPCs overexpressing $P2Y_2R$ (hCPC-Y2) on GFR matrigel as assessed by increased distance travelled by single cells from origin (denoted by red circle).



Online Figure V: MST1/2 or LATS1 phosphorylation is not impacted by $P2Y_2R$ overexpression.
(A, B) Immnuoblot analysis showing no difference in levels of phosphorylated MST1/MST2 or phosphorylated LATS1 in hCPCs overexpressing $P2Y_2R$.

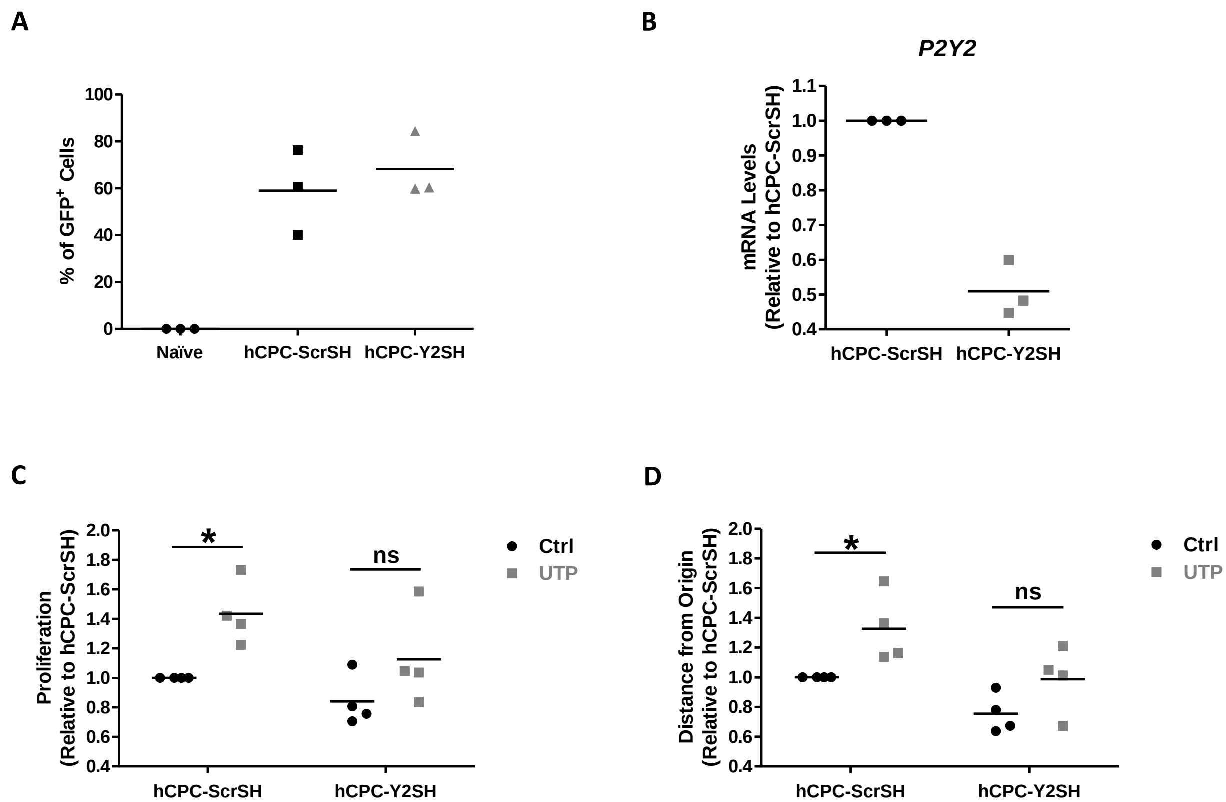


Online Figure VI: Functional response to $P2Y_2R$ agonist UTP in hCPCs.
A dose response of $P2Y_2R$ agonist UTP (1, 10 and 100µM) showing increase in intracellular calcium $[Ca^{2+}]_i$ levels, indicated by increased amplitude of $[Ca^{2+}]_i$ transient, in a dose-dependent manner.

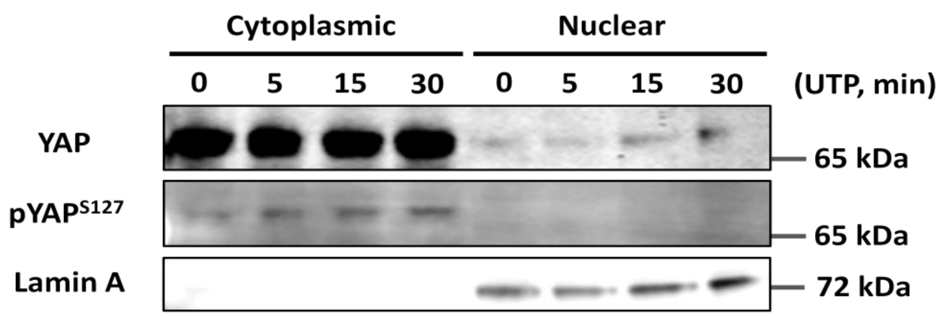


Online Figure VII: Enhancing proliferation and migration of individual hCPC lines by $P2Y_2R$ agonist UTP.

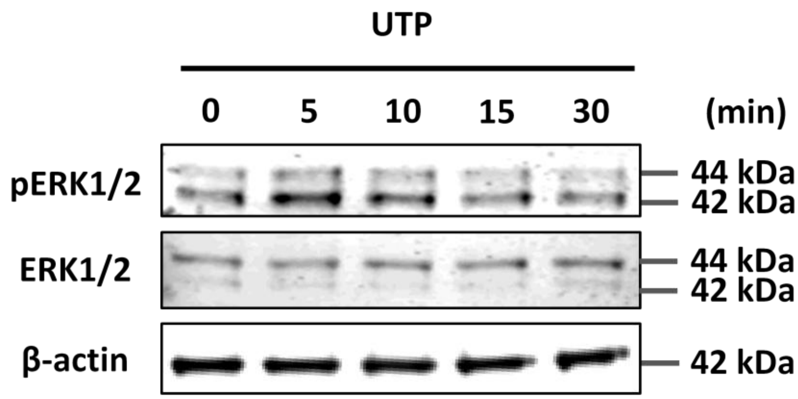
Enhanced proliferation (n=2-11/line) **(A)** and migration (n=3-5/line) **(B)** of 6 hCPC lines in response to UTP treatment (100µM). Cell proliferation and migration were assessed as described in Figure 3.



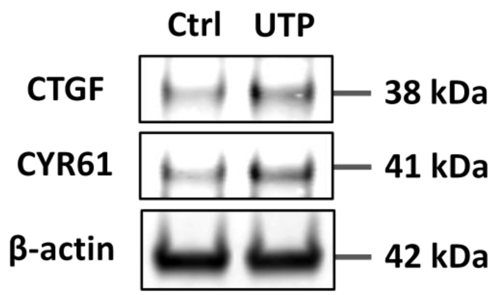
Online Figure VIII: UTP-induced hCPC proliferation is primarily mediated by $P2Y_2R$.
(A) shRNA-mediated $P2Y_2R$ knockdown using lentiviral particles encoding for $P2Y_2R$ shRNA (hCPC-Y2SH) or scrambled shRNA (hCPC-ScrSH). Transduction efficiency was 59% for hCPC-ScrSH and 68% for hCPC-Y2SH as assessed by flow cytometry analysis for percentage of GFP^+ cells. **(B)** Lower $P2Y_2R$ mRNA levels by qRT-PCR analysis in hCPC-Y2SH compared to control hCPC-ScrSH confirming $P2Y_2R$ knockdown. **(C, D)** Proliferation and migration assays showing impairment of UTP-induced responses in hCPC-Y2SH (n=4) validating that UTP acts primarily via $P2Y_2R$. 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.



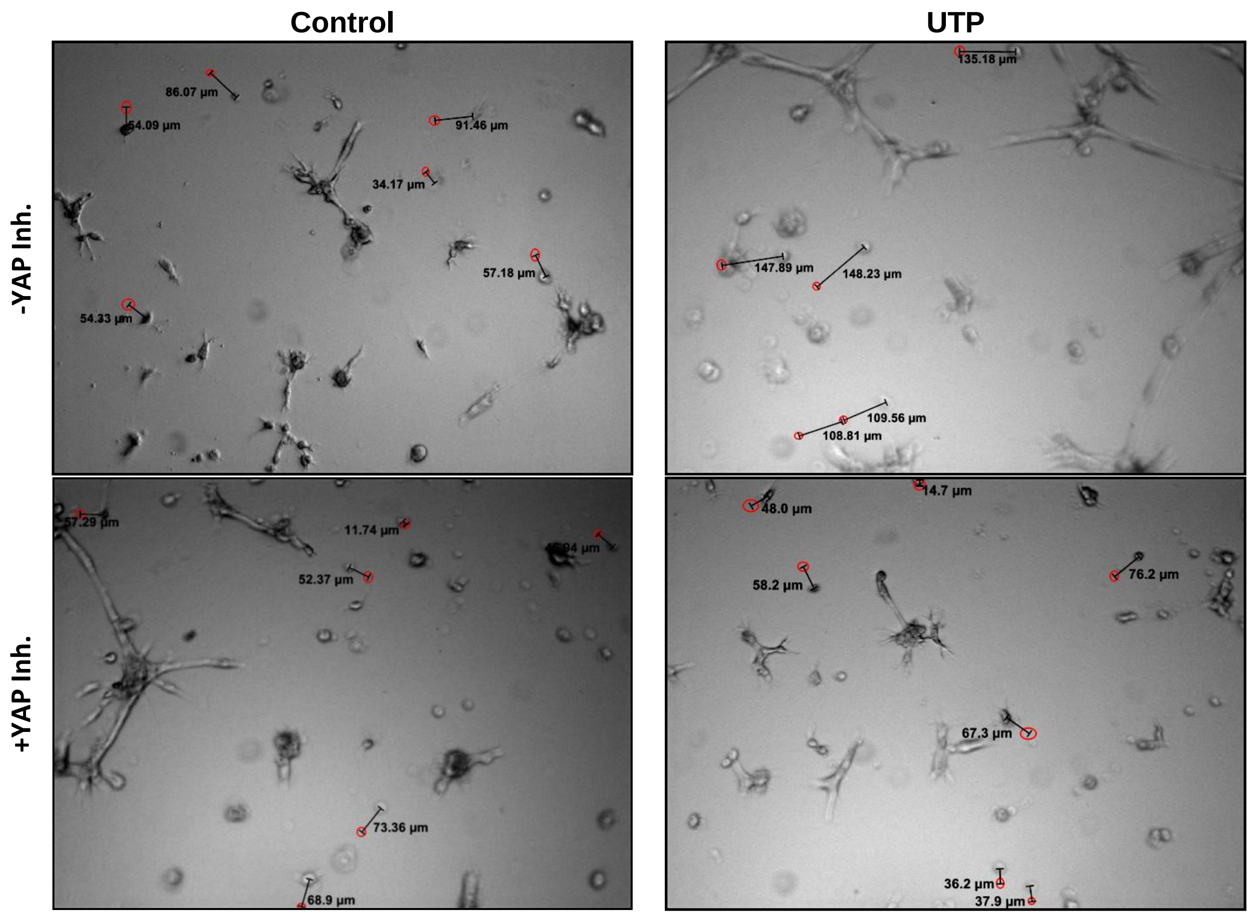
Online Figure IX: Exclusion of inactive pYAP^{S127} from the nuclear fraction of hCPCs.
Representative immnuoblot showing exclusion of the inactive phosphorylated form of YAP from the nuclear extracts of hCPCs. Nuclear protein Lamin A was used as additional control to validate the purity of nuclear fraction.



Online Figure X: Induction of ERK1/2 activation by UTP treatment.
Representative immnuoblot 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 immnuoblot 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

Target	FWD Primer Sequence	REV Primer Sequence	REF
P2X4	GATACCAGCTCAGGAGGAAAAC	GCATCATAAATGCACGACTTGAG	1
P2X5	GGCATTCCTGATGGCGCGTG	GGCACCAGGCAAAGATCTCAC	1
P2X6	AGCACTGCCGCTATGAACCAC	AGTGAGGCCAGCAGCCAGAG	1
P2Y1	CTTGGTGCTGATTCTGGGCTG	GCTCGGGAGAGTCTCCTTCTG	2
P2Y2	CGGTGGACTTAGCTCTGAGG	GCCTCCAGATGGGTCTATGA	3
P2Y4	CGCTGCCCACCCTCATCTAC	CCGAGTGGTGTCATGGCACAG	2
P2Y11	GAGGCCTGCATCAAGTGTCTG	ACGTTGAGCACCCGCATGATG	2
P2Y14	CGCAACATATTCAGCATCGTGT	CAAAGTATCTGTGCTTTCAAGT	4
CTGF	CAAGGGCCTCTTCTGTGACT	ACGTGCACTGGTACTTGCAG	5
INHBA	CCTCGGAGATCATCACGTTTG	GGCGGATGGTGACTTTGGT	6
CYR61	GAGTGGGTCTGTGACGAGGAT	GGTTGTATAGGATGCGAGGCT	7
ANKRD1	AAGCAGGAGGATCTGAAGACACTT	GTTGTTTCTCGCTTTTCCACTGT	8
AMOTL2	GACAGCCTTCTGGGGTGCAGCAGTA	GCTCAGAGTCCTGAAGCACCACCTCCT	
NPPB	GCTTTGGGAGGAAGATGGAC	GCAGCCAGGACTTCCTCTTA	9
NEXN	TGGAGAAACAAGAATTTGAACAAC	TGCTCAATCCAAAGGTTTCA	9
18S	CGAGCCGCCTGGATACC	CATGGCCTCAGTTCCGAAAA	10

Online Table II. List of antibodies

Antibody	Vendor	Catalog Number	Immunoblotting Dilution	Slides Dilution
$P2Y_2R$	Abcam	ab168535	2.5μg/mL	-
mGFP	Origene	TA150124	1:500	-
GAPDH	SICGEN	AB0067-200	1:2000	-
$pMST1^{T183}$/ $MST2^{T180}$	CST	3681	1:500	-
MST1	CST	3682	1:500	-
$pLATS1^{S909}$	CST	9157	1:500	-
LATS1	CST	3477	1:500	-
$pYAP^{S127}$	CST	13008	1:500	-
YAP	CST	12395	1:500	1:100
CTGF	R&D	MAB9190-100	2μg/mL	-
CYR61	CST	14479	1:500	-
β-actin	Santa Cruz Biotechnology	sc-81178	1:500	-
Lamin A	Sigma-Aldrich	L1293	1:500	-
β-tubulin	CST	2146	1:500	-
DAPI	Sigma-Aldrich	D9542	-	1:10,000

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