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SRC kinase family inhibitor PP2 promotes DMSO-induced cardiac differentiation of P19 cells and inhibits proliferation ☆

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ABSTRACT

Background: It has been reported recently that PP2, a Src family kinase inhibitor, promotes selective cardiogenesis in embryonic stem cells. However, there is no other research proved pro-cardiogenic characteristic of PP2 so far. In this study, we explored the potential cardiogenic effect of PP2 on P19 cells differentiation.
Methods: P19-αMHC-EGFP cell line was established by transfecting P19 cells with αMHC-EGFP vector in order to evaluate cardiogenesis with EGFP. P19-αMHC-EGFP cells and P19 cells were induced to differentiate into cardiomyocytes with 1%DMSO, 5 μmol/L PP2, or both 1%DMSO and 5 μmol/L PP2. Differentiated cells from P19-αMHC-EGFP cells were then assessed under confocal microscope. Western-blot and RT-PCR were also performed to detect expression of cardiac troponin I and cardiac transcription factors respectively. In addition, the effects of PP2 on proliferation of P19 cells were further examined using Cell Counting Kit-8.
Results: EGFP positive cells were firstly detected on day 7 and PP2 alone cannot induce efficient cardiac differentiation of P19-αMHC-EGFP cells. However PP2 supplementation dramatically increases DMSO induced cardiac differentiation than DMSO alone. It was also found that PP2 inhibit proliferation of P19 cells in both a dose-dependent manner and a time-dependent manner.
Conclusion: PP2 alone cannot substitute DMSO to induce cardiac differentiation, however, PP2 supplementation drastically promotes DMSO-induced cardiac differentiation of P19 cells. The increased percentages of differentiated cardiac myocytes is partly resulting from cell proliferative inhibit effect of PP2 in undifferentiated P19 cells. P19-αMHC-EGFP cell line has the potential to be used for regenerative therapies in experimental models of heart repair.

1. Introduction

Murine P19 embryonal carcinoma (EC) cells were derived from a teratocarcinoma artificially induced in C3H/HeHa mice [1] and represent as one of the most widely studied pluripotent EC cell lines. Distinct from embryonic stem (ES) cells, P19 cells can be maintained in culture in an undifferentiated monolayer state without the need to supplement the medium with leukemia inhibitory factor (LIF) and a feeder-cell layer. Since the cells are capable of differentiating into a variety of cell types representative of all 3 germ layers when induced by chemical agents [2], P19 cell line now is a well-established system for studying cardiac, skeletal muscle and neural differentiation [3]. The formation of embryoid bodies (EB) in response to exposure to dimethyl sulfoxide (DMSO) is the main protocol that has been mainly used to induce the differentiation of P19 cells into cardiomyocytes [4]. In addition to DMSO, other factors such as 5-azacytidine [5], oxytocin [6,7], retinoic acid [8,9] and cardiogenol C [10] have been found to induce cardiac differentiation in P19 cells. However, the low efficiency of P19 cells to differentiate is a major limitation.

Recently, it has been reported that PP2, a Src family kinase inhibitor, dramatically promotes selective cardiogenesis during embryoid body adhesion when exposed to ES cells [11]. PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*] pyrimidine), was identified as a potent and selective inhibitor of the Src family of protein tyrosine kinases. Recent studies showed that PP2 efficiently inhibits the proliferation and growth of cervical cancer cell lines [12] and it was reported that PP2 stimulates hormonal differentiation of human trophoblast cells [13], thus promotes trophoblast cells differentiation [14]. However, so far, there are no studies that focus on the effects of PP2 on the cardiogenesis of P19 cell line, whether PP2 exposure inducing cardiac differentiation in P19 cells

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Table 1
Primers used for quantitative RT-PCR.

Gene name	Size (bp)	primers	Tm (°C)	cycles
GAPDH	450	5′-ACCACAGTCCATGCCATCAC-3′ 5′-TCCACCACCCTGTTGCTGTA-3′	60	26
GATA4	480	5′-CCAACTGCCAGACTACCAC-3′ 5′-GGACCAGGCTGTTCCAAGA-3′	58	32
NKX2.5	313	5′-TTCCAGAACCGTCGCTACAAGT-3′ 5′-AGTTCACGAAGTTGCTGTTGGC-3′	58	32
TBX5	641	5′-TGAACGTGAACTGTGGCTGAAG-3′ 5′-GGCTGTGGTTGGAGGTGACTT-3′	58	32
MEC2C	610	5′-CAGCACTGACATGGATAAGG-3′ 5′-CTGCCAGGTGGGATAAGAACG-3′	60	32

Tm, annealing temperature.

or not was needed to be elucidated. In our present study, we explored the potential cardiogenic effect of PP2 on P19 cells differentiation. We found that PP2 dramatically increased DMSO-induced cardiomyocytes differentiation of P19 cells, while PP2 alone cannot induce efficient cardiac differentiation.

2. Materials and methods

2.1. Cell culture and differentiation

P19 cells were grown in a 100-mm tissue culture grade dish under adherent conditions with α-minimal essential medium (Gibco, USA) supplemented with 10% fetal bovine serum (JRH Bioscience, USA), penicillin (100 U/ml), and streptomycin (100 mg/ml) (growth medium) and were maintained in a 5% CO2 atmosphere at 37 °C.

2.2. Establishment of pαMHC-EGFP expressing P19 sublines

The plasmid pαMHC-EGFP, which contains 5.5 k bp fragment α-myosin heavy chain promoter, driving the expression of the enhanced green, was kindly provided by Dr E. Kolossov [15]. After linearization, the plasmid was transfected into P19 cells with Fugene6 (Roche, Switzerland). Two days after transfection, 1000 μg/mL G418 (Roche, Switzerland) was added to the growth medium for selection of stable αMHC-EGFP expressing cells. Drug-resistant cells began to form small colonies after 2 weeks of G418 addition. Individual colonies were then isolated, propagated, and differentiated to cardiomyocytes. The neomycin-resistant clones showing bright fluorescence matching beating areas were further selected. In the present study we established a novel P19 cells derived clonal cell line P19-αMHC-EGFP cells harboring a cardiac α-myosin heavy chain promoter-driven enhanced green fluorescent protein gene to monitor the generation of cardiac cells under fluorescence microscopy.

2.3. Differentiation of P19-αMHC-EGFP cells

To induce differentiation, 0.25×10^6 cells were allowed to aggregate for 4 days in non-adhesive bacteriological grade Petri dishes (10-cm diameter) containing 10 ml of complete medium, with 1% DMSO (defined as group D), with 5 μM PP2 (Merk) (defined as group P), or with both 1% DMSO and 5 μM PP2 (defined as group PD), respectively. At day 2 of aggregation, the inducing culture medium was replenished. At day 4, aggregates were transferred to tissue culture grade vessels (6-cm diameter dishes or 24-well plates) and cultured in complete medium. 5 μM PP2 was maintained in group P and group PD after day 4.

2.4. Confocal laser scanning microscopy

At days 7–13 of differentiation, P19-αMHC-EGFP cells were washed with 4 °C pre-cooled PBS for three times. Cells were then observed under laser scanning confocal microscope (Zeiss LSM510, Germany). Randomly selected images were processed and measured by Image J (NIH, USA) software.

2.5. Western blot

P19 cells at different differentiating days were lysed in cell lysis buffer containing proteinase inhibitor cocktail (Sigma, USA), and the protein concentrations were determined with a bicinchoninic acid protein assay kit (Pierce Biotechnology, USA). Equal amounts of total protein (20 μg) were separated by electrophoresis on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels, transferred to a polyvinylidene difluoride membrane (Millipore, USA) and incubated with mouse monoclonal anti-cTnI antibody(clone 19C7, Abcam Cambridge, USA) or mouse monoclonal anti-β-actin antibody (A1978; Sigma, USA). The blots were next incubated with peroxidase-conjugated mouse IgG secondary antibodies (Amersham Pharmacia Biotech, USA). The protein bands were detected with an ECL system (Amersham Pharmacia Biotech, USA). Image J software was used for further quantitative analysis.

2.6. Real-time qRT-PCR

For quantitative real-time PCR (Q-PCR) expression analysis of P19 cells, total RNA was harvested from differentiating cells using TRIzol (Invitrogen, USA). The cDNAs were made from 2 mg of total RNAs using SuperScript III (Invitrogen, USA) and

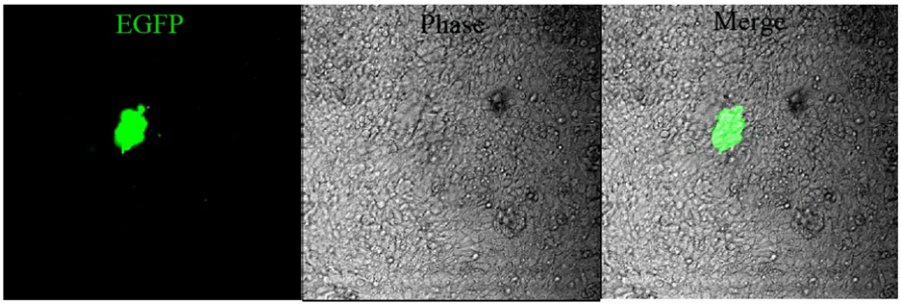

Fig. 1. Characterization of P19-αMHC-EGFP cell line. P19-αMHC-EGFP cells were induced to differentiate into cardiomyocytes with 0.5% DMSO. EGFP positive cells were firstly detected on day 7, these cells started to beat 1 day later.

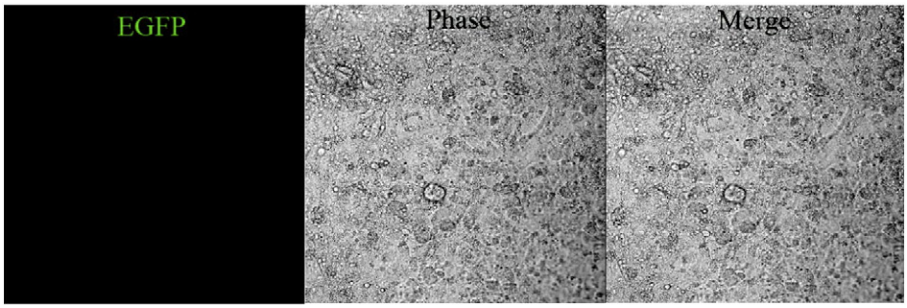

Fig. 2. PP2 alone cannot induce cardiac differentiation of P19-αMHC-EGFP cells. P19-αMHC-EGFP cells were induced to differentiate into cardiomyocytes with 5 μM PP2 alone. There are no EGFP positive cells in day 10.