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REVIEW

Application of Rho-associated protein kinase (ROCK) inhibitor to human pluripotent stem cells

Hiroshi Kurosawa*

Division of Medicine and Engineering Science, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, 4-3-11 Takeda, Kofu, Yamanashi 400-8511, Japan

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Susceptibility of human pluripotent stem cells (hPSCs), such as human iPS and embryonic stem (ES) cells, to single-cell dissociation has been a large obstacle to develop the efficient manipulation techniques required for stem cell research. When hPSCs are completely dissociated into single cells, programmed cell death (apoptosis) is immediately induced. A specific inhibitor of Rho-associated protein kinase (ROCK inhibitor), Y-27632, is of particular interest as a useful reagent that allows hPSCs to escape the dissociation-induced apoptosis. ROCK inhibitor has been used in a variety of applications associated with cell dissociation in the process of stem cell research, such as passaging, expansion, cryopreservation, gene transfer, differentiation induction, and cell sorting, suggesting that it may be a crucial reagent for the handling of hPSCs. This article reviews the current applications of ROCK inhibitors to stem cell research from the viewpoint of quality control of hPSCs.

[**Key words:** Y-27632; ROCK inhibitor; Embryonic stem cells; Dissociation; Adhesion]

Human pluripotent stem cells (hPSCs), human induced pluripotent stem (hiPS) and human embryonic stem (hES) cells have the potential to be an important source of virtually any cell type for basic research, drug development, and clinical cell therapies. hES cells are pluripotent cells derived from the inner cell mass of human embryos at the preimplantation stage (1), whereas hiPS cells are generated from adult human dermal fibroblasts by the transduction of defined transcription factors to induce reprogramming. Once established, hiPS cells are similar to hES cells in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, telomerase activities, *in vitro* differentiation, and teratoma formation (2). However, these hPSCs are technically much harder to culture (or handle) than mouse embryonic stem (ES) cells due to their slow growth rate, insensitivity to leukemia inhibitory factor (LIF), and dissociation-induced apoptosis (1,3). Mouse ES cells typically grow as compact and domed colonies that are insensitive to single-cell dissociation. hPSCs grow as monolayer colonies that are larger than mouse ES colonies and have epithelial structure. When the epithelial structure of the colony is disrupted by cell dissociation, programmed cell death (apoptosis) is induced (4). Although cell dissociation causes massive cell death, it is a usual procedure involved in various manipulations in the process of stem cell research, such as passaging, expansion, cryopreservation, gene transfer, differentiation induction, and cell sorting. To circumvent the problem of the dissociation-induced apoptosis in hPSCs, some manipulations must be performed using only partially dissociated colonies, or clumps of cells. Using clumps, however, does not assure reproducibility of the manipulation due to the heterogeneous size and indeterminate form of the clumps. To improve the efficiency of these manipulations, the colonies must be dissociated into single cells. Thus, the vulnerability of hPSCs during single-cell dissociation is a large obstacle to developing the efficient manipulation techniques required for stem cell research.

Recently, it was reported that a specific inhibitor of Rho-associated protein kinase (ROCK inhibitor) could promote the survival of dissociated hES cells (4). ROCK inhibitor, a small-molecule chemical compound, has now been recognized as a useful tool in the cultivation of both hES cells and hiPS cells. Recent evidence suggests that the use of ROCK inhibitor can improve the efficiency and reproducibility of manipulations in stem cell research. This article reviews the applications of ROCK inhibitor to stem cell research from the viewpoint of quality control of hPSCs.

ROCK INHIBITOR

ROCK inhibitor is a general term for inhibitors of Rho-associated coiled coil protein kinase (Rho kinase; ROCK). ROCK was initially discovered as a downstream target of the small GTP-binding protein Rho (5–8), which regulates cellular growth, adhesion, migration, metabolism, and apoptosis through control of the actin cytoskeletal assembly and cell contraction (9). Consequently, ROCK mediates membrane blebbing, enhances actin–myosin contraction, and activates caspase signaling cascades and cellular apoptosis.

* Tel.: +81 0 55 220 8535; fax: +81 0 55 220 8525.
E-mail address: kurohiro@yamanashi.ac.jp.

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Several chemical compounds, such as Y-27632 (a pyridine derivative as shown in Fig. 1) (10,11) and HA-1077 (Fasudil) (12), are known as ROCK inhibitors, and are widely used in many biological systems such as cultured cells, isolated tissues, and animal models. Y-27632 [(+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexane carboxamide dihydrochloride monohydrate ($C_{14}H_{21}N_3O \cdot 2HCl \cdot H_2O$, molecular weight 338.3)] (Fig. 1), a pyridine derivative, is soluble in distilled water and its aqueous solution is stable at room temperature for at least 4 weeks. When Y-27632 is added to culture medium, it is taken up by the cells in a time- and temperature-dependent manner (11). Therefore, Y-27632 is quite suitable for *in vitro* applications in isolated tissues and cultured cells. The concentration of Y-27632 most commonly used for the treatment is 10 μM. Prior to its application in the culture of hPSCs, Y-27632 was used *in vitro* in studies of many biological systems (13), including inhibition of Rho-mediated stress fiber formation and cell transformation in NIH 3T3 fibroblasts, inhibition of formation of stress fibers and induction of shape change in Swiss 3T3 fibroblasts, and inhibition of Rho-induced formation of stress fibers and focal adhesions in HeLa cells. Moreover, it was reported that Y-27632 enhanced the *in vitro* survival of mouse ES cells-derived neural precursors (14), mouse intestinal stem cells (15), and human keratinocytes (16).

INCREASING SURVIVAL RATE AND PLATING EFFICIENCY OF DISSOCIATED hPSCs

A peculiar feature of hPSCs is their susceptibility to dissociation-induced apoptosis. Watanabe et al. (4) conducted the first application of ROCK inhibitor to control dissociation-induced apoptosis in hES cells. Y-27632 treatment increased colony formation in dissociated hES cells and facilitated the selective subcloning of hES cells after gene transfer. At that time, the mechanism of action of Y-27632 was unclear, but it was demonstrated that the use of Y-27632 enabled the growth of dissociated hES cells. More recently, Y-27632 has been used in various routine manipulations in stem cell research (Fig. 2). For example, Y-27632 has been applied to improve cell recovery of hES cells after fluorescence-activated cell sorting (FACS) (17). When applied to FACS, hES cells must be dissociated into single cells, therefore the cell recovery after sorting will be low due to the dissociation-induced apoptosis. But when Y-27632 was added to the plating medium at final concentration of 10 μM, the survival rate of post-sorted hES cells was improved. Additionally, Y-27632 has been useful in the generation of hiPS cells by the introduction of reprogramming factors (18–20). Park et al. (18) added Y-27632 to recipient plates when reprogramming of hES cell-derived fetal fibroblasts, and found that Y-27632 enhanced survival and clonogenicity of post-infected cells. In other cases, Y-27632 was used to increase the seeding efficiency of hiPS cell colonies picked from transfected fibroblast cultures for the initial colony expansion (19,20). It was found that the use of Y-27632 promoted the induction efficiency of hiPS cells and improved the maintenance of established hiPS cell lines.

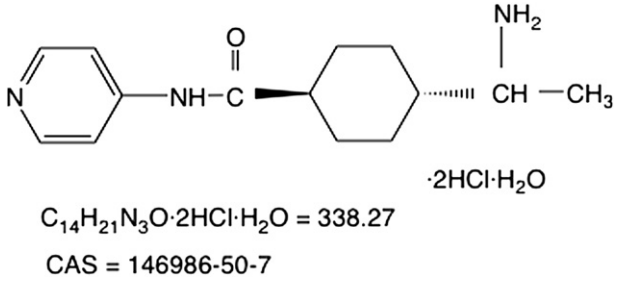

FIG. 1. Chemical structure of Y-27632. [(*R*)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide dihydrochloride monohydrate].

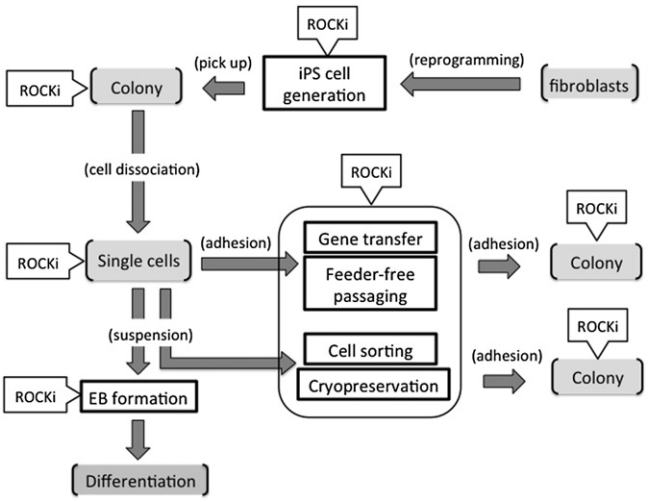

FIG. 2. Application of Y-27632 to manipulations in stem cell research using hES cells and hiPS cells. ROCK inhibitor, ROCKi.

Cell dissociation is the most common manipulation in stem cell research, therefore, the use of Y-27632 will improve the yield of viable single cells and facilitate handling of dissociated hPSCs.

IMPROVING EFFICIENCY OF CRYOPRESERVATION OF hPSCs

The slow-freezing and rapid-thawing method using dimethylsulfoxide (DMSO) is most commonly employed as a cryopreservation technique. This procedure is efficient for mouse ES cells, but it is inappropriate for hES cells because of the low survival rate. Therefore, a fast-freezing protocol (vitrification method) using a high concentration of cryoprotectant is usually employed for the cryopreservation of hPSCs (21). In standard cryopreservation protocols, hPSCs are cryopreserved as small clumps to avoid dissociation-induced apoptosis. However, vitrified hPSCs still suffer from high levels of cell death and do not passage well as single cells.

Martin-Ibañez et al. (22) examined the effect of Y-27632 on the cryopreservation of dissociated hES cells in the slow-freezing and rapid-thawing method. In brief, dissociated hES cells were frozen in the freezing medium, and then stored in liquid nitrogen. After cryopreservation, hES cells were rapidly thawed and resuspended in post-thawing medium. The addition of Y-27632 (10 μM) to both the freezing medium and post-thawing medium exhibited an additive effect to enhance the efficiency of colony formation. Treatment with Y-27632 during cryopreservation significantly increased the survival rate and cell adhesion of freeze-thawed dissociated hES cells. Li et al. (23) have also reported that Y-27632 increased the post-thaw survival rate of hES cells in the slow-freezing and rapid-thawing method. Y-27632-treated freeze-thawed hES cells retained morphology, stable karyotype, expression of cell surface markers, and the potential to differentiate into derivatives of all three germ layers. They also demonstrated that Y-27632 treatment is effective in serum/feeder-free conditions (24). Claassen et al. (25) examined whether use of Y-27632 could also improve the recovery from frozen stocks and passaged hiPS cells. The addition of 10 μM Y-27632 to culture medium in plates with Matrigel and a growth-inactivated mouse embryonic fibroblast (MEF) feeder layer improved the recovery and growth of freeze-thawed dissociated hiPS cells. These results suggest that Y-27632 seems to have a dual role of preventing apoptosis and increasing cell adhesion.

Cryopreservation is a labor-intensive process in stem cell research, during which the cells are exposed to severe condition.