REVISED STAP CELL PROTOCOL. 09.03.14:

Below is a revised protocol to improve the chances of successfully creating pluripotent STAP stem cells from mature somatic cells, not dependent on the source of cells. The protocol has been revised to reflect improved techniques. As we learn more about the STAP cells, it is likely that new stresses will be identified and that nutrients and chemicals will be developed which are much more effective at creating STAP cells. This protocol has been modified from what was originally published (and then retracted), and also from the previous protocol that we posted online on this site, for the following reasons:

1. We made a significant mistake in our original declaration that the protocol was "easy" to repeat. This was our belief at the time, but it turned out to be incorrect. Many of the steps described appear to be a function of the technique of the individual investigator. Consequently, the revised protocol below should increase the likelihood of success. It combines the use of ATP as a supplemental source of energy with two very effective stresses to achieve the desired result; that is, creation of pluripotent STAP cells.

2. In reviewing our original approach, and the rationale exposing the cells to a low pH, it was recalled that we had originally experimented with the addition of ATP as an external energy source in hope of improving the viability of the cells and the spheres generated. At that time, it was noted that the addition of ATP not only resulted in better sphere formation, but was also associated with a marked decrease in the pH of the solution to which the mature cells were exposed. Consequently, we started to focus on the effect of low pH alone as a stress to induce the creation of STAP cells, without evaluating other potential beneficial effects of ATP. In recent months, our lab decided to re explore the utility of a low pH solution containing ATP in generating STAP cells. We found that while pH alone resulted in the generation of STAP cells, the use of a low pH solution containing ATP, dramatically increased the efficacy of this conversion. **When this acidic ATP solution was used in combination with mechanical trituration of mature cells, the results were even more profound**. Consequently, our protocol has been now modified as described below to utilize these findings to increase the efficacy of generating STAP cells. The revised protocol seems to be much more effective for generating STAP cells in our lab, regardless of the cell type being studied. It combines the addition of ATP as a supplemental energy source, with elements of the most effective approaches originally published and later retracted in the article, (Obokata et. al., Stimulus triggered fate conversion of somatic cells into pluripotency. Nature 505. 641-647, 2014).

It is very important that each step be performed precisely as described. The protocol will vary slightly if you are starting with tissue rather than a cell suspension. It is especially important to triturate the cell suspension in the acidic ATP solution for a minimum of 30 minutes, until the suspension can be easily triturated up and down the reduced bore pipettes of the smallest orifices. We first describe the protocol when starting with a suspension of cells, and then describe additional steps necessary when starting with a soft tissue.

Generating STAP cells from a suspension of mature somatic cells:

A1. First, make a stock solution of ATP, 200 mM, (which will be added to HBSS in the next step), by adding 110.22 mg of ATP powder (Adenosine 5[1] Triphosphate Disodium Salt Hydrate - Sigma

A2383) to each 1 mL of water (MilliQ water). The pH of the resulting ATP solution solution is about 3.0.

A2. Place 5 mL of HBSS Hanks Balanced Saline Solution (HBSS Ca+Mg+ Free: Gibco 14170-112) into a 15 mL tube. Place a clean pH sensor into the HBSS. Titrate in the ATP stock solution, drop by drop, into the HBSS until the desired pH of 5.0 is obtained (roughly a few hundred microliters). Mix the solution regularly to ensure that the measurement is accurate. The final acidic HBSS solution is has an ATP concentration of about 3-5 mM. This will be used below in Step A5.

A3. Add the live somatic cells to be treated, as a cell suspension to a centrifuge tube, and then centrifuge at 1200 rpm for 5 minutes. Note: Trypsin-EDTA, 0.05 % (Gibco: 25300-054) can be added to the tissue culture dish containing cells, for 3-5 minutes, to release adherent cells to be added to the centrifuge tube.

A4. Aspirate the supernatant down to the cell pellet.

A5. Resuspend the resulting pellet at a concentration of 1 million cells/ml in the low pH, Hanks Balanced Saline Solution with ATP, (made above in Step 1A) in a 50 ml tube. Note: We recommend working with a volume of 2-3ml of the cell suspension in a 50ml tube.

A6. Precoat a standard 9" glass pipette with media (so the cells do not stick to the pipette - we use: (Fisher brand 9" Disposable Pasteur Pipettes: 13-678-20D). Triturate the cell suspension in and out of the pipette for 5 minutes to dissociate cell aggregates and any associated debris. This can be done with a fair amount of force.

A7. As a final extremely important step in the trituration process, make two fire polished pipettes with very small orifices as follows: Heat the standard 9" glass pipette over a Bunsen burner and then pull and stretch the distal (melting) end of the pipette, until the lumen collapses and the tip breaks off, leaving a closed, pointed glass tip. Wait until the pipette cools, and then break off the closed distal tip until a very small lumen is now identifiable. Repeat this process with the second pipette, but break the tip off a little more proximally, creating a slightly larger distal lumen. The larger lumen should be about 100-150 microns in diameter, while the other pipette should have a smaller lumen of about 50-70 microns. Now triturate the cell suspension through the pipette with the larger lumen for 10 minutes. Follow this with trituration through the pipette having the smaller lumen (50-70 microns) for an additional 15 minutes. Continue to triturate the suspension until it passes easily up and down the fire polished pipette of the smaller bore. This is a very important step. Do not skip this step, or take a shortcut. Again, remember to precoat a each pipette with media. Also, during trituration, try to avoid aspirating air and creating bubbles or foam in the cell suspension.

A8. Add normal HBSS (containing no ATP) to the suspension to a total volume of 20ml, centrifuge at 1200rpm for 5 minutes, and then aspirate the supernatant.

A9. Resuspend the resulting pellet in 5ml of what we term “sphere media” (DMEM/F12 with 1% Antibiotic and 2 % B27 Gibco 12587-010) and place at a concentration of 10^5 cells/ml, within a non-adherent tissue culture dish in the presence of the following supplements: b-FGF (20ng/ml), EGF (20ng/ml), heparin (0.2%, Stem Cell Technologies 07980).

LIF (1000U) should be added if the cells are murine). Note: Supplements such as bFGF, EGF and heparin may not be necessary.
Important: After the cells are placed in tissue culture dishes, they should be gently pipetted using a 5ml pipette, twice/day for 2 minutes, for the first week, to discourage them from attaching to the bottom of the dishes. This is important to generate good sphere formation. Add sphere media containing the supplements described every other day. (Add 1ml/day to a 10cm culture dish, or 0.5ml/day to a 6cm dish.)

Generating STAP cells when starting with soft tissues that contain many RBCs.

B1. Place the excised, washed sterile organ tissue into an 60mm petri dish containing 50-500µl of collagenase, depending on the size of the tissue. Add a sufficient volume of the collagenase to wet the entire tissue.
Note:
Different types of collagenase or other enzymes are better for digestion of different organ tissues. (The spleen may not need to be exposed to any digestive enzymes.) B2. Mince and scrape the tissue for 10 minutes using scalpels and scissors to increase surface area that is exposed to the collagenase, until the tissue appears to become gelatinous in consistency.

B3. Add additional collagenase to the dish to make the total volume = 0.5ml, and place in an incubator/shaker for 30 minutes at 37°C at 90rpm.

B4. Add 1.5ml of the low pH HBSS/ATP solution (see step A2) to the dish (yielding a total volume of 2.0ml) and then aspirate the entire contents via a 5ml pipette and place into a 50ml tube.

B5. Now proceed to triturate as previously described above (step A6-A7) when starting with mature somatic cells.

B6. After trituration is completed (through step A5 when using a culture dish of mature somatic cells), add 3ml of HBSS, yielding a volume of 5ml, to the 15ml tube and then slowly add 5ml of Lympholyte to the bottom of the tube to create a good bilayer.
Note: It is important to add this as described to create a bilayer and avoid mixing of the two solutions.

B7. Centrifuge this tube at 1500g for 10 min. Rotate the tube 180° and recentrifuge at 1500g for an additional 10 min. This will cause the erythrocytes to form a pellet at the bottom of the tube.

B8. Using a standard 9” glass pipette, aspirate the cell suspensions layer between HBSS and Lympholyte and place in a new 50 ml tube.

B9. Add HBSS to the suspension to a total volume of 20ml of HBSS and then thoroughly mix the suspension by pipetting via a 5ml pipette for 1 minute.

B10. Centrifuge the solution at 1,200rpm for 5 minutes and aspirate the supernatant.

B11. To finish the process, proceed with Steps A5 – A9, above.

The above protocol seems to be the most effective protocol “du jour” based on our most recent studies. In time, as more is learned about the STAP cell phenomenon, we are certain that the approach will continue to be modified to effectively utilize new stresses and chemical environments to create pluripotent STAP stem cells from mature somatic cells.

We have developed this most recent protocol to address concerns that to date, to our knowledge, other groups have been able to generate STAP cells using our previously published or posted protocols. While we are confident that the original protocols published, will work if performed with meticulous attention to detail, we have tried to develop a protocol based on new information, that should be much more effective in demonstrating a phenomenon in which we have absolute confidence.

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