

Materials required:

1. Source human TERA2-derived embryonal carcinoma (EC) stem cells. For TERA2.cl.SP12 cells see Przyborski Stem Cells 2001, v19, p500 and Stewart et al. 2003, v21, p248. NTERA2.cl.D1 lineage is available from the American Type Culture Collection (ATCC), Manassas VC, US.
2. Growth media for EC stem cells: Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) foetal calf serum, 2mM Lglutamine, 100 µg/ml penicillin and 10 µg/ml streptomycin.
3. Stock solutions of synthetic retinoid ec23[®] prepared to a concentration of 10mM in DMSO.
4. General cell culture consumables (culture flasks, serological pipettes, etc.), reagents (phosphate buffered saline (PBS, calcium-free), trypsin EDTA solution, poly-D-lysine, laminin, cytosine arabinosine, fluorodeoxyuridine, uridine, coverslips) and access to equipment for conventional cell culture to expand cell populations (laminar flow hoods, CO₂ incubators, etc.).

Protocol I: Induction of stem cell differentiation

1. Expand stock populations of cultured EC stem cells under standard laboratory conditions. Passage stock cultures using acid-washed glass beads. For optimal culture of TERA2-derived EC stem cells, maintain cell populations at high confluency and split at a ratio of no more than 1:3 (see **Figure 1**). Note: these cells will spontaneously differentiate if their seeding density and confluency become too low.
2. Treat confluent cultures of EC stem with 0.25% trypsin / 2mM EDTA in PBS for 2-3 min at 37° to produce a suspension of single cells. Wash cells with growth medium and determine cell number using a hemocytometer.
3. Seed EC stem cells at 2×10^4 cells per cm² (equivalent of 1.5×10^6 cells per T75 tissue culture flask) in growth medium containing 1µM ec23.
4. Refresh culture medium containing retinoid every 3-4 days.

Note: a single dose of retinoid is sufficient to induce cell differentiation. Its persistent inclusion for the first 1-2 weeks appears to maximize yield of neural derivatives.

5. Differentiating cultures can be maintained for up to 6-8 weeks. After 14-21 days, large numbers of neurons will become visible. See **Figures 2-3**.
6. Follow Protocol II below to isolate and purify neurons from retinoid treated cultures of human EC stem cells.

Reinnervate Ltd

NETPark Incubator
Thomas Wright Way
Sedgefield
Co. Durham
TS21 3FD
UNITED KINGDOM

Tel: +44 (0) 1740 625 266

Fax: +44 (0) 1740 625 251

Email: info@reinnervate.com

Web: <http://reinnervate.com/>

Protocol II: Purification of Neurons from Retinoid Induced Cultures

This method was developed to isolate populations of neurons from retinoid-induced cultures of human EC stem cells. See Stewart et al. *Stem Cells*, v21, p248 for further information.

1. After 21 days exposure to retinoid, completely dissociate the cultures using 0.25% trypsin / 2mM EDTA in PBS for 10 min at 37°C. Wash cells in growth medium and re-suspend.
2. Split the suspensions of differentiated cells 1:4 and seed into fresh tissue culture flasks containing growth medium without retinoid.
3. After 3-4 days, briefly expose cultured to 0.1% trypsin / 2mM EDTA in PBS for 2 min.
4. Physically dislodge cells loosely attached to the surface of confluent cultures by a lateral motion provided by 5 short, sharp blows with the palm of the hand to the side of the horizontal tissue culture flask.
5. Collect cells displaced by enzymatic / mechanical disruption.
6. Wash the cells in growth medium and seed onto tissue culture plastic or poly-D-lysine/laminin (10µg/ml) coated 16mm diameter glass coverslips at a density of $\sim 2 \times 10^5$ cells/cm².
7. At this stage, maintain cultures in either one of two ways:
 - a. To enhance the purity of neuronal cultures and reduce the proliferation of non-neuronal cell types, add mitotic inhibitors to the media as follows: 1µM cytosine arabiosine (for the first 10 days only); 10µM fluorodeoxyuridine; 10µM uridine;
 - b. To promote limited proliferation of astrocytes, reduce the concentrations of mitotic inhibitors accordingly: 0.1µM cytosine arabiosine (for the first 10 days only); 3µM fluorodeoxyuridine; 5µM uridine.
8. After a further 2-3 weeks, populations TERA2.cl.SP12-derived neurons (see **Figure 4**) and astrocytes are established. Astrocytes adhere well to the tissue culture surface whereas neurons are less well attached and are easily removed by gentle enzymatic and mechanical dissociation as described above.

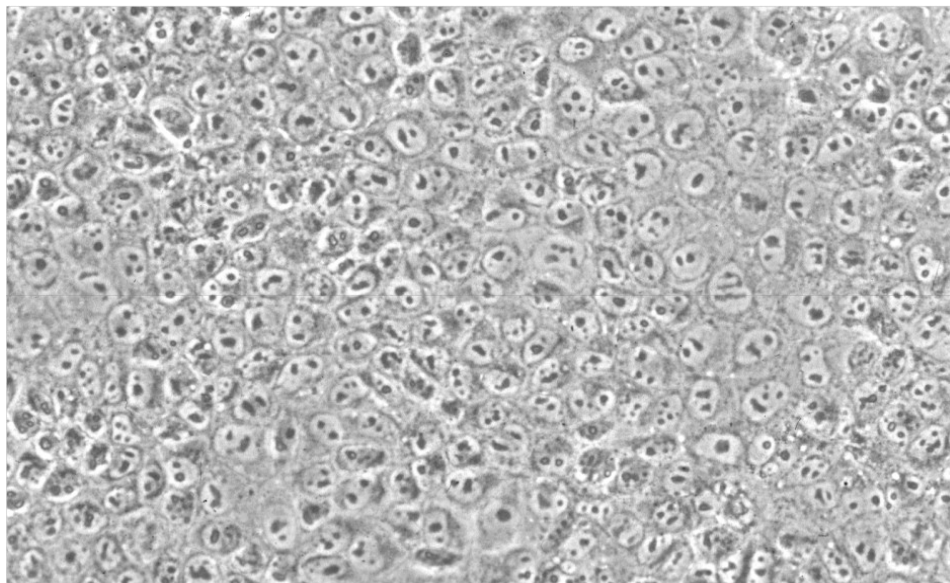


Figure 1: Phase contrast micrograph of human TERA2.cl.SP12 EC stem cells maintained at optimal confluency.

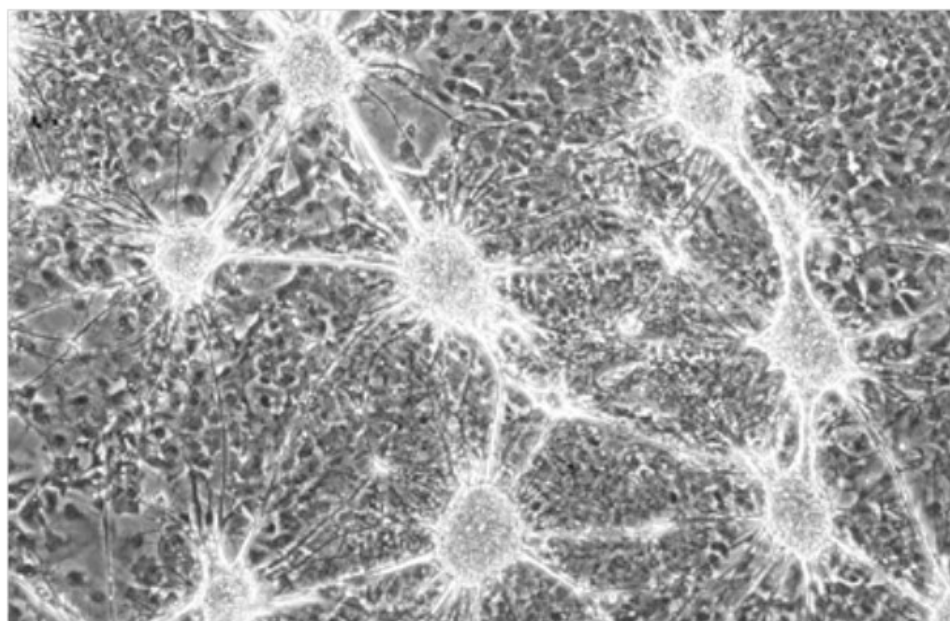


Figure 2: Phase contrast micrograph of human TERA2.cl.SP12 EC cells treated with retinoid for 28 days. Note phase bright aggregations of neurons linked to each other by bundles of axons.

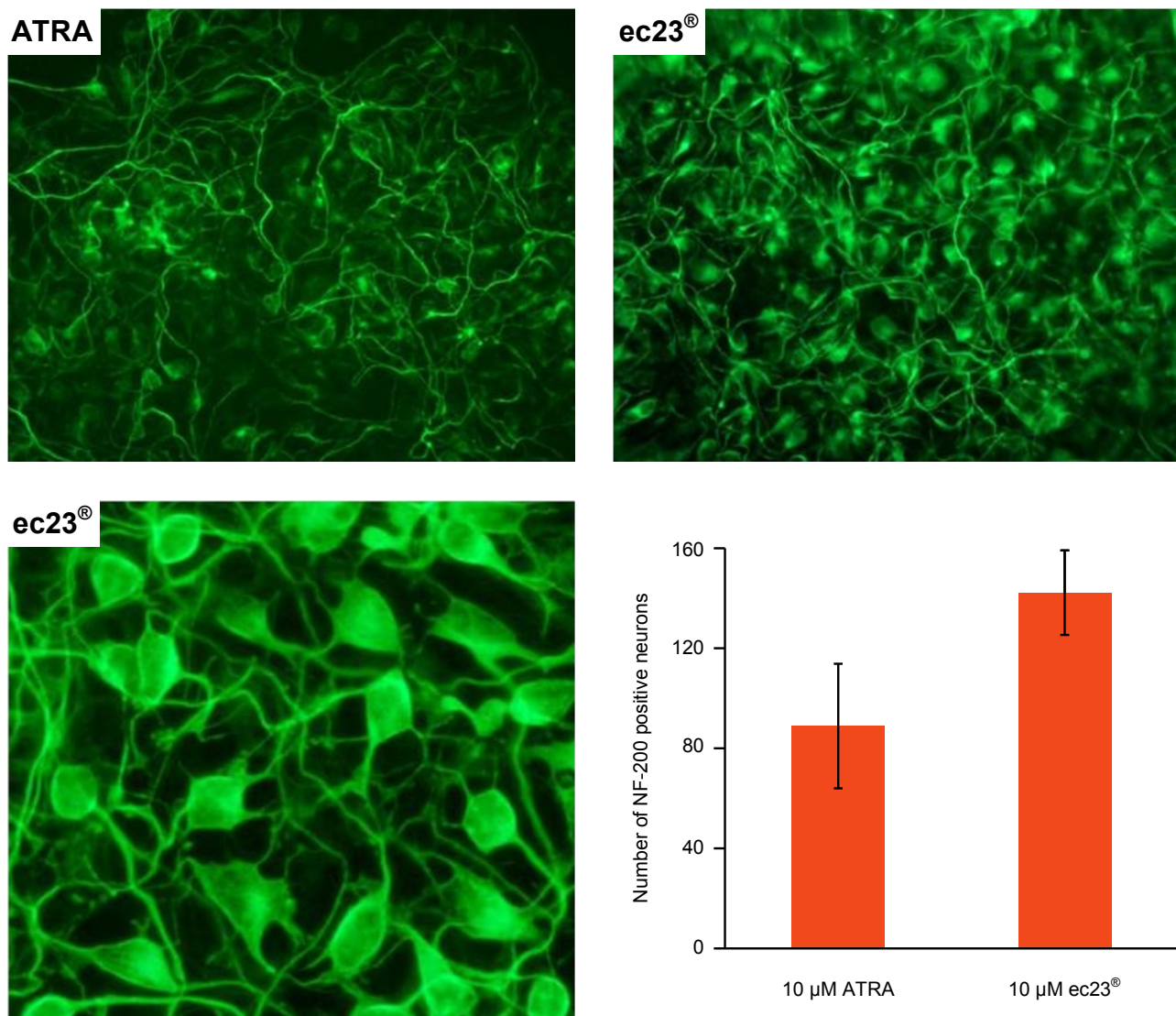


Figure 3: Human TERA2.cl.SP12 EC cells exposed to either ec23® or ATRA form populations of terminally differentiated neurons after 21 days. Immunocytochemical staining for neurofilament-200 was performed on differentiated cultures and the number of positive cells quantified. Results show that ec23® is a more potent inducer of neurogenesis than ATRA. In direct comparison to ATRA, synthetic retinoid ec23® induces larger numbers of neural cells with far less variability and with the added advantage of compound stability. See Christie et al. 2008, Org Biomol Chem, v6, p3497.

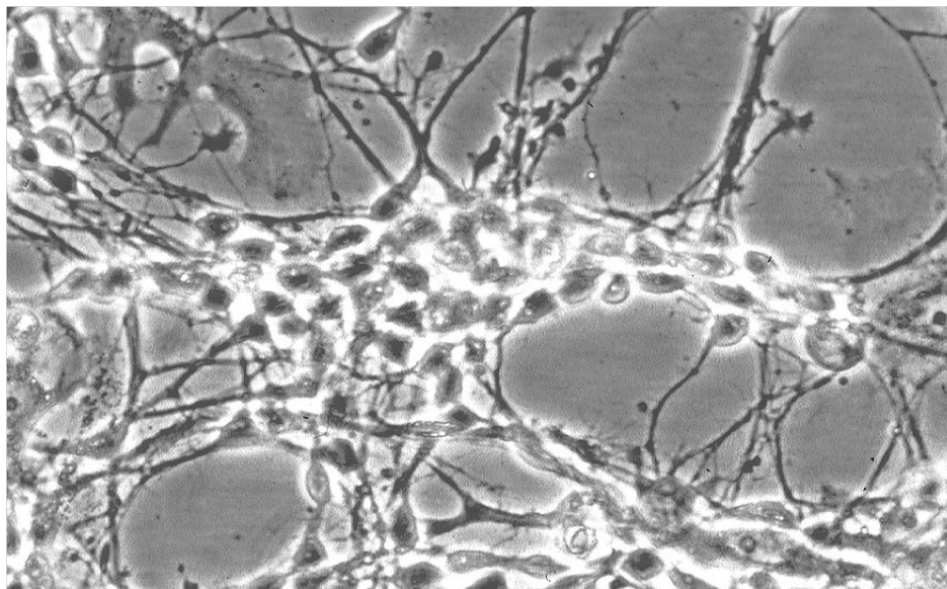


Figure 4: Phase contrast micrograph of purified neurons derived from human TERA2.cl.SP12 EC cells treated with retinoid for 21 days.

Consult the following papers for further information on handling these cells and their differentiation into neural derivatives:

- Christie V.B., Barnard, J.H., Bridgens, C.E., Batsanov, A.S., Cartmell, E.B., Collings, J.C., Maltman, D.J., Marder, T.B., Redfern, C.P.F., Whiting, A.P., Przyborski, S.A. (2008). Synthesis and evaluation of synthetic retinoid derivatives as inducers of stem cell differentiation. *Organic and Biomolecular Chemistry*, 6, 3497-3507.
- Przyborski, S.A., Christie, V.B., Hayman, M.W., Stewart, R. & Horrocks, G.M. (2004). Human embryonal carcinoma cells: models of human embryonic development in man. *Stem Cells and Development*, 13, 400-408.
- Stewart R., Christie V. & Przyborski S.A. (2003). Manipulation of human pluripotent embryonal carcinoma stem cells and the development of neural subtypes. *Stem Cells*, 21, 248-256.
- Horrocks G.M., Lauder L., Stewart R. & Przyborski S.A. (2003). Formation of neurospheres from human embryonal carcinoma stem cells. *Biochemical and Biophysical Research Communications*, 304, 411-416.
- Przyborski S.A. (2001). Isolation of human embryonal carcinoma stem cells by immuno-magnetic sorting. *Stem Cells*, 19, 500-504.
- Przyborski S.A., Morton I.E., Wood A. & Andrews P.W. (2000). Developmental regulation of neurogenesis in the pluripotent human embryonal carcinoma cell line NTERA2. *European Journal of*