

ISOLATION OF SCHWANN CELL PRECURSORS FROM RODENTS

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Abstract

Schwann cell precursors are the first defined stage in the generation of Schwann cells from the neural crest, and represent the glial cell of embryonic nerves. Highly pure cultures of these cells can be obtained by enzymatic dissociation of nerves dissected from the limbs of 14 or 12 day old rat and mouse embryos, respectively. Since Schwann cell precursors, unlike Schwann cells, are acutely dependent on axonal signals for survival, they require addition of trophic factors, typically β neuregulin 1, for maintenance in cell culture. Under these conditions they convert to Schwann cells on schedule, within about four days.

1. Introduction

In rodent embryos, the sciatic nerve invades the hind limb between embryo day (E) 14/15 (rat) and E12/13 (mouse) (Fig. 1). In these early embryonic nerves axons, glia and connective tissue relate to each other in a way which is radically different from that seen in late perinatal or older nerves. These early nerves consist of tightly packed axons, mostly without individual glial ensheathment, and sparsely distributed, flattened glial cell processes (Fig.2). There is no significant extracellular space, matrix or basal lamina. The glial cell bodies lie among the axons inside the nerve or at the nerve surface. These cells represent Schwann cell precursors, the first stage of the Schwann cell lineage (1-4).

Schwann cell precursors are generated from neural crest cells and, in turn, generate the immature Schwann cells of perinatal nerves (Fig .3). Schwann cell precursors show multiple phenotypic differences from neural crest cells on the one hand and immature Schwann cells on the other. They differ from crest cells because they express glial differentiation genes, including myelin protein protein zero (Po), and other factors not expressed by crest cells, because they are found in association with axons in nerves, a characteristic glial feature, rather than migrating through extracellular matrix, and because they respond differently to survival, proliferation and differentiation signals (2, 3, 5). A large number of differences in molecular expression between

Schwann cell precursors and crest cells is also revealed in gene array analysis (6, 7). In turn, Schwann cell precursors and immature Schwann cells differ substantially in molecular expression, including up-regulation of S100 in immature Schwann cells (6, 7). Further, immature Schwann cells, but not Schwann cell precursors, relate to basal lamina and connective tissue containing blood vessels. But perhaps the most striking difference concerns survival regulation, since Schwann cell precursors are acutely dependent on the axon-associated survival signal β neuregulin1 (β NRG1) type III, while immature Schwann cells survive in culture without addition of survival factors due to secretion of autocrine survival signals (8, 9, 10). Autocrine survival mechanisms are not present in Schwann cell precursors. For this reason Schwann cell precursors are routinely cultured in medium containing β NRG1 (Fig. 4).

In late embryonic nerves, Schwann cells precursors convert to immature Schwann cells. But this is not their only developmental option, since they also give rise to endoneurial fibroblasts, melanocytes in the skin, parasympathetic neurons and tooth pulp cells (11-14). Schwann cell 1root ganglion (DRG) neurons and motoneurons (2, 4, 10)

2. Materials

For all procedures, use autoclaved, ultrapure (UP) water.

2.1 Enzyme cocktail for digestion of nerves

1. 10 X KREBS solution (without Ca^{++} and Mg^{++}): 14g NaCl, 0.7g KCl, 0.325g KH_2PO_4 . Make up to 200ml with UP water. Autoclave to sterilise. Store at 4-8 °C.
2. $\text{Ca}^{++}/\text{Mg}^{++}$ free medium: Mix 80 mL UP water, 10 ml 10X $\text{Ca}^{++}/\text{Mg}^{++}$ free 10X Krebs solution, 2ml 50% essential amino acid solution, 2.5 ml 7.5% NaHCO_3 , 0.5 ml 0.3% phenol red solution, 0.4 ml 50% glucose sterile. Filter mixture through 0.22 μm filter to sterilise and store at 4-8 °C.

3. Collagenase (Worthington Collagenase Type 2 LSO 041472)
4. Hyaluronidase (Type IV-S: From Bovine Testes, Sigma-H 3631)
5. Trypsin Inhibitor (Trypsin inhibitor from Glycine max (soybean) Sigma-T 6522).
4. Enzyme cocktail: Prepare 15 ml of Ca⁺⁺/Mg⁺⁺ free medium containing 2 mg/ml collagenase (see Note1), 1.2 mg/ml hyaluronidase and 0.3 mg/ml trypsin inhibitors. The solution will give 30x 600 µl aliquots. Filter mixture through 0.22 µm filter and aliquot into 1ml Eppendorf tubes. Snap freeze in liquid nitrogen and store at -20 °C.

2.2 Defined medium

1. DMEM/Ham's F-12 solution: Make 1:1 mixture of DMEM and Ham's F-12 plus Glutamax (Gibco life technologies 31331-028) and add penicillin/streptomycin (penicillin 100 IU/ml, streptomycin 100 IU/ml).
2. Transferrin stock solution (10 mg/ml): Weigh out 500 mg transferrin (transferrin, bovine Sigma T8027) powder and add to 50 ml 1xPBS, stir to dissolve, then filter sterilise using a 0.22 µm filter. Make 1 ml aliquots and snap freeze in liquid nitrogen and store at -20 °C.
3. Putrescine stock solution (1.6 mg/ml): Weigh out 80 mg of putrescine powder and add to 50 ml PBS, stir to dissolve, then filter sterilise using 0.22 µm filter. Make 1 ml aliquots, snap freeze in liquid nitrogen and store at -20 °C.
4. Bovine serum albumin (BSA) stock solution (3.5%): Prepare 35% BSA solution in PBS then dilute 1:10 in PBS. Make 1 ml aliquots then store at 4-8 °C.
5. T4 (L-Thyroxine) stock solution (400 µg/ml): Weigh out 50 mg of T4 powder, dissolve in 10 ml of 1 M NaOH to make a 5 mg/ml solution, then dilute this 1:12.5 in 1xPBS to make a 400 µg/ml stock solution. Make 100 µl aliquots, snap freeze in liquid nitrogen and store at -20 °C.

6. Progesterone stock solution (0.006 mg/ml): Weigh out 0.6 mg progesterone powder, dissolve in 10 ml of 100% analytic grade ethanol. Store at 4-8 °C; reseal stock tube with cling film after each use.

7 Insulin stock solution (5.7 mg/ml, 10^{-3} M): Add 2.5 ml of 10 mg/ml insulin solution (Sigma I9278-5ml) to 1.89 ml UP water to make a total of 4.39ml. Store both insulin solutions at 4-8 °C. Make up fresh insulin stock every 6 weeks.

8. Dexamethasone stock solution (0.05 mg/ml): Weigh out 0.5 mg dexamethasone (Sigma D4902, MW 392.46), dissolve in 10 ml 100% analytic grade ethanol. Keep stock at 4-8 °C; reseal with cling film after each use.

9. T3 (L-Thyronine) stock solution (0.101 mg/ml): Weigh out 1.01 mg T3 (3,3',5-Triiodo-L-thyronine sodium salt, Sigma T6397)and dissolve in 10 ml of 100% analytic grade ethanol, store at -20 °C. Do not aliquot. Store in 15 ml Falcon tube at -20 °C; reseal with cling film after each use.

10. Selenium stock solution (1.6 mg/ml): Weigh out 8 mg of sodium selenite (Sigma S5261) and dissolve in 5 ml of UP water, filter sterilise using a 0.22 µm filter, aliquot into 10 µl aliquots, snap freeze in liquid nitrogen and store at -20 °C.

10. Defined medium: To make 100 ml of defined medium, take 96 ml of 1:1 DMEM/Ham's F-12 solution and add stock solutions of each component as the following (final concentration of each component is shown in parenthesis): 1 ml transferrin (100 µg/ml), 1 ml putrescine (16 µg/ml), 1 ml BSA (0.035%), 100 µl T4 (400 ng/ml), 100 µl progesterone (60 ng/ml), 100 µl insulin (5.7 µg/ml), 77 µl dexamethasone (38 ng/ml), 10 µl T3 (10.1 ng/ml) and 10 µl selenium (0.16 ng/ml). (Meier et al., 1999). Store defined medium at 4-8 °C, and use within 2 weeks.

2.3 Defined medium containing βNRG1 for culture of Schwann cell precursors

Since Schwann cell precursors die without added β NRG1 (or FGF plus IGF, see reference 10), we routinely re-suspend the cells after centrifugation, and maintain them in culture, using defined medium supplemented with 10-20 ng/ml β NRG1.

1. PBS/1% BSA solution: Dilute 1 ml of 35% BSA prepared in PBS in 34 ml of PBS

2 High concentration β NRG1 stock solution (50 μ g/ml): Re-suspend lyophilized β NRG1 EGF domain (RD systems 396-HB-050) in 1 ml of PBS/1% BSA to make a 50 μ g/ml solution. Vortex thoroughly to ensure the entire pellet is re-suspended, make 200 μ l aliquots from this solution in 1.5 ml Eppendorf tubes, snap freeze in liquid nitrogen and store at -80°C (high-concentration stock).

3. Low-concentration β NRG1 stock solution (10 μ g/ml): Mix 800 μ l of PBS/1%BSA solution with one aliquot (200 μ l) of high-concentration 50 μ g/ml β NRG1 stock to obtain a 10 μ g/ml solution. Make 50 μ l and 10 μ l aliquots of this in 0.5 ml Eppendorf tubes, snap freeze in liquid nitrogen and store at -80°C (low-concentration stock).

4. Defined medium supplemented with β NRG1: Mix one 10 μ l aliquot of low-concentration (10 μ g/ml) β NRG1 stock with 10 ml of defined medium to give a final β NRG1 concentration of 10ng/ml.

2.4 Coating of coverslips and dishes for cell plating

1. Deep glass Petri dishes

2. 13 mm glass coverslips: Place approximately 100 coverslips in a deep glass Petri dish and bake in an oven for 4 hr at 140°C to sterilise. Use silica gel to keep them dry after baking unless they are to be coated immediately.

3. Tissue culture dishes

4. 1 mg/ml poly-L lysine prepared in UP water

5. 1 mg/ml laminin stock solution: Store at 80 °C until ready to use, then thaw slowly on ice over several hours, aliquot into 50 µl aliquots, snap freeze in liquid nitrogen, store aliquots at -20 °C.

2.5 Dissection of embryonic nerves and Schwann cell precursor preparation

1. Timed pregnant rats (embryonic day [E] 14.5) or mice (E12.5): Use female rats of mice aged optimally from 2 months to 5-6 months old. Males can be older than this. Count the day that a vaginal plug is observed at noon as E0.5.

2. Sylgard coated Petri dishes: Make up Sylgard 184 according to the manufacturer's instructions (10 parts elastomer to 1 part hardener) in a disposable plastic container with a disposable stirring stick. Pour it into several plastic 9cm petri dishes to approximately 0.5-1 cm depth, then allow to set at high temperature. When set the Sylgard will be transparent and embryos can be pinned to the surface of the dish using fine dissecting pins for dissection of the embryo nerve. The dishes should be sterilised with 100% analytic grade ethanol and washed with purified water and dried before use.

3. 9 mm plastic Petri dish (?) (is this size correct?)

4. L15 medium

5. 70% ethanol

6. Coarse scissors, sterilized using 70% ethanol

7. Forceps, sterilized using 70% ethanol

8. Finer forceps, sterilized using 70% ethanol

9. Fine iridectomy scissors, sterilized by 100% analytic grade ethanol

10. Fine tipped forceps (No. 5), sterilized by 100% analytic grade ethanol

11. Dissecting microscope with fibre optic lighting

12. Two 3 cm plastic Petri dishes
13. L15 medium supplemented with 5% horse serum (for mouse dissection) or 10% fetal calf serum (for rat dissection)
14. Defined medium supplemented with β NRG1.
15. PLL and Laminin coated coverslips or dishes (see Section 3.1)

2.6 Antibodies

1. S100 polyclonal rabbit antibody (DAKO)
2. p75NTR polyclonal rabbit antibody (Merck Millipore)
3. L1 ASCS4 mouse anti rat L1 antibody (Developmental Studies Hybridoma bank)
4. 324 rat anti mouse L1 antibody (Merck Millipore)
5. Alexa 488 or Cy3 secondary antibodies

3. Methods

3.1 Preparation of coated coverslips and dishes for cell plating

1. Poly-L-lysine coating of coverslips: Add 25ml of 1mg/ml poly-L-lysine in UP water to coverslips, seal with cling film (Nescofilm), put on a shaker for 24 hr, remove poly-L-lysine, wash with 6 changes of water over three days on the shaker, leave to air dry thoroughly by standing each coverslip on end around large sterile Petri dishes or in sterile mini-racks, store desiccated at room temperature in a sterile Petri dish sealed with cling film, and if possible do not to use until 24 hr after drying. They will keep for months.

2. Poly-L-lysine coating of dishes: Tissue culture dishes are treated with a 100 µg/ml solution of poly-L-lysine in UP water. Dilute 1 mg/ml solution 1:10 in UP water to make a 100 µg/ml solution. Treat dishes for 2 hr at room temperature, then remove the solution, and leave the dishes to dry without washing. Seal them with cling film and store desiccated at room temperature. They will keep for months.

3. Laminin coating of poly-L-lysine coated coverslips or dishes: Thaw an aliquot of stock laminin solution slowly (do not refreeze), dilute this stock solution to a final concentration of 20 µg/ml in DMEM or L15 medium in the case of coverslips and wells, and 10 µg/ml for tissue culture dishes. Do not adjust the pH of the solution as laminin binds better at an alkaline pH. Pipette the laminin solution on to the centre of a PLL-coated coverslip sitting in a 4 or 24 well tissue culture plate. We routinely use 10-20 µl for rat cells and 5-10 µl for mouse cells. For culturing larger cell number in a well without coverslip or in 3 cm tissue culture dishes, use 50-100 µl of laminin. Leave the solution on for at least 1hr at room temperature, and remove immediately prior to plating cells, do not allow to dry.

3.2 Embryo dissection and Schwann cell precursor preparation

Schwann cell precursors are prepared essentially according to the methods described previously (1, 8, 15).

1. Sacrifice pregnant female rat at E14.5 or mouse at E12.5 using an approved procedure.

2. After killing the pregnant animal, pin it out on a cork dissecting board covered with aluminium foil. Spray the fur with 70% ethanol to sterilise, open up the abdominal cavity with appropriate size sterile scissors and gently remove the uterine sac with embryos which will be clearly visible. Place this sac in a 9 mm plastic Petri dish containing L15 medium. Place the lid on the Petri dish and keep it on ice.

3. Using fine scissors carefully open up the sac to liberate one embryo to establish correct dating.

This is easily judged by observing the digits of front and hind limbs. Excellent illustrations are available at: https://embryology.med.unsw.edu.au/embryology/index.php/Rat_Development. This website shows both rat and mouse embryos. Comparable illustrations are also in reference 16.

4. Having established that the embryos are of the correct age (~E14.5 rat, ~E12.5 mouse), remove an embryo for dissection. Remove only one embryo for dissection at a time, and leave the others in the embryonic sac on ice. Remove the embryo from the amniotic sac with fine forceps and iridectomy scissors. Decapitate and pin the embryo out with the ventral side down using three metal pins on a Sylgard coated Petri dish that has been sterilised with 100% analytic grade ethanol and then washed with UP water and briefly with L15 medium (Fig.5).

5. The sciatic nerves are removed from the hind limb using a dissecting microscope and fine (No 5) dissecting forceps. The forceps need to be “as new”, namely completely straight and without scratches. First, the skin needs to be removed from the hind leg and a little way up the back. L15 medium can be added to the dish as needed to prevent drying. There is a quick and perhaps surprisingly easy way to liberate the nerves (not a single nerve at this developmental stage but a delicate plexus) from the leg. To do this, insert the number 5 forceps at the juncture of the developing limb and the torso (Fig.5) holding the forceps at about 45° angle relative to the surface of the lab bench. Push the forceps towards, but not quite to, the midline (spine) of the embryo. Now close the forceps firmly and pull out the nerves, which, with a little practice, come out intact as a single entity, a small plexus of nerves. To decrease the risk of the nerves slipping out of the forceps as they are pulled out of the leg, it is helpful to lower the forceps a little (decrease the 45° angle) after the forceps are closed but before they are pulled out. (see Note 2)

6. Place the dissected nerve in 3 cm plastic Petri dish containing L15 medium, which is kept on ice, until sciatic nerves from all embryos have been collected. When the nerve is released from the forceps into the medium, it is sometimes obvious that tissue fragments adhere to the nerve. They

should be gently cleaned away. It is important to prevent the nerves from reaching the surface of the medium, where they can be pulled apart by the surface tension. The best way of doing this is to entwine each nerve, after cleaning if this is needed, with the nerve(s) already there, seeking to form one ball of tangled nerves. As a rough guide, one litter should give 100,000 cells after dissociation.

7. For enzymatic dissociation, use forceps to transfer the nerves to 600 μ l of enzyme cocktail in a 3cm Petri dish, and place it in 5% CO₂, 95% air incubator at 37 °C for 1 hr. Tilt the dish so that nerves are fully immersed. At the end of the incubation time remove the dish from the incubator and place in a tissue culture hood.

8. Triturate 5-15 times through a 1 ml blue Eppendorf-type pipette tip and then 10- 15 times through a yellow 200 μ l pipette tip. If the cells are not fully dissociated, re-incubate for a further 10-15 min before being triturating a further 10 times with a blue 200 μ l pipette tip.

9. Transfer the cell suspension to a 15 ml Falcon centrifuge tube and make the volume up to 10 ml with L15/5% HS (mouse) or L15/10% FCS (rat), in order to dilute out the enzymes. Centrifuge for 10 min at 162.4g in a Hereaus Primo R Centrifuge with swinging bucket rotor or equivalent.

10. Carefully remove the medium, leaving the pellet behind. Re-suspend the cell pellet gently with trituration (yellow pipette tip) in 0.5-1 ml of defined medium typically supplemented with β NRG1.

11. Count the cells in a hemacytometer or other counting device. Adjust to the desired cell concentration using the re-suspension medium. The number and concentration of cells to be plated can obviously be varied. For plating on coverslips we routinely use 2000 cells per 10-20 μ l for rat cells, and 2000 cells per 5-10 μ l for mouse cells.

12. Plate the cells on PLL and laminin coated glass coverslips in wells in a 4 or 24 well tissue culture plate (or directly on PLL and laminin-coated tissue culture well or on a 3 cm tissue culture dish) using a volume of cell suspension equal to the volume of the laminin solution used to laminin-coat the coverslip, well or tissue culture dish. (see Section 3.1.3).

13. After 1.5 hr for mouse cells and 2.5 hr for rat cells, top up each well with 400 μ l of the same medium used for re-suspension and plating. Schwann cell precursors do not express autocrine survival loops and, unlike Schwann cells, they die by apoptosis within 24 hr in vitro, unless the medium is supplemented with survival factors (1). By far the most potent of these is (β NRG1) (15), although the combination of FGFs and IGF2 also supports precursor survival (17). For routine culturing, the defined medium we use for resuspension, plating and top-up contains 10-20ng/ml of β NRG1 EGF domain (Fig. 4). Schwann cell precursor purity can be monitored by immunolabeling cultures with L1 antibodies or alternatively antibodies to p75NTR which label a slightly higher percentage of cells (15) (See Note 1).

3.3. Schwann cell precursor survival assay

The survival assay described here is essentially that described previously (1, 8, 17) (see also 9).

1. Schwann cell precursors are plated in drops on coverslips as described above. The choice of culture media will depend on the question under investigation (see 3.1.13 above). After 3 hr at 37 $^{\circ}$ C and 5% CO₂, one set of coverslips is fixed, without top-up, for immunolabeling with L1 or p75NTR antibodies (see Note 3). The number of immunolabeled cells on these coverslips (typically about 95% of total cells present) is counted. This number is interpreted as the number of cells that plated successfully and is used as a reference point for the quantification of survival at later time points.

2. Sister coverslips are topped up to 400 μ l with appropriate medium and cultured for the desired length of time, typically 24 hr. The cells are then fixed for immunolabeling with L1 or p75NTR antibodies and stained with Hoechst dye, and the number of surviving Schwann cell precursors counted. Survival percentage is the number of cells present at a certain time point, such as 24 hr, as a percentage of the number of cells that had plated successfully at 3 hr. (see Note 4)

4. Notes

1. The concentration can vary between 2 and 4mg/ml depending on the batch but is typically used at 2mg/ml final concentration.

2. Note that using this method, the leg tissue is not dissected away to visualize the nerves. Rather, it relies on inserting the forceps into the leg in the correct location so that the nerves come to lie between the two tips of the forceps before they are closed. This is not as difficult as it may sound. Practising on one litter (~10 embryos) should be sufficient to achieve a good success rate. Although the method is described here for the hind leg, with practice it can be used equally to obtain brachial plexus nerves from the forelimb. As an alternative to this approach it is possible to dissect the nerves out under visual guidance. In our experience this achievable, but more difficult than the method described above.

3. Schwann cell precursor purity can be monitored by immunolabeling cultures with L1 antibodies (ASCS4 anti L1 antibody or 324 anti L1 antibody for rat or mouse respectively), or alternatively antibodies to p75NTR which labels a slightly higher percentage of cells (15). Rat precursor cultures are about 95% pure at 3 hr after plating when labelled with antibodies to p75NTR (1, 15). Mouse precursor cultures are $\geq 93\%$ pure when labelled with L1 antibodies after 20 hr in culture in the presence of β NRG1 (8, 15).

4. In contrast to Schwann cells (9), where cells that die during the culture period stay attached to the coverslips, Schwann cell precursors that die during the culture period detach from the coverslips and float into the medium, from which they can be collected and shown to have ladder DNA indicative of apoptotic death (1,16). Thus $\geq 98\%$ of the cells present at 24 hr under various culture conditions are living cells. It follows that the number of L1 or p75NTR positive cells present on the coverslips at any given time point accurately reflects the number of cells that have survived up to that point.

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Figure legends

Figure 1

Nerves in the mouse hindlimb visualized using TuJ1 antibodies and immunohistochemistry. The immunostaining was carried out on whole embryos which were subsequently sectioned transversely in the region of the hindlimbs. Note that nerves appear essentially confined to the trunk at E11, whereas at E12 they are present in the upper and middle part of the limb. Arrows point to DRG. x20. (Reproduced from reference 8).

Figure 2

The ultrastructure of embryonic nerves showing Schwann cell precursors, axons and connective tissue. An electron microscopic image of a transverse section of a nerve in the hindlimb of a rat embryo at embryonic day (E) 14. Schwann cell precursors branch among the axons inside the nerve and are also found in close apposition to axons at the nerve surface (vertical arrows). One precursor cell is undergoing mitosis (horizontal arrow). Extracellular connective tissue space (blue), which contains mesenchymal cells, surrounds the nerve but is essentially absent from the nerve itself. These nerves are also free of blood vessels and the axons are of smaller and more uniform diameter than those seen in mature nerves. Magnification, x2000. (Reproduced with modifications from reference 2).

Figure 3

The Schwann cell precursor in a developmental context. The diagram shows the Schwann cell precursor and key differentiation states in the lineage, including immature, myelin and Remak cells, and repair Schwann generated after injury. Also shown are developmental options for Schwann cell

precursors and the precursor functions of promoting nerve fasciculation and the survival of developing neurons. Dates (E) refer to mouse development.

Figure 4

Developmental changes in the morphology and cellular relationships between Schwann cell precursors and Schwann cells. The cells were dissociated from either E14 or newborn rat nerves. They were immunolabeled with p75 NTR antibodies and photographed with fluorescence optics (lower panels) and phase contrast (upper panels) 20 hr after plating; the cells were maintained in neuron conditioned medium. Radical changes in shape accompany precursor to Schwann cell conversion. The precursors are flattened, whereas the immature Schwann cells from newborn nerves are bi- or tri-polar and have tapered processes that often arise relatively abruptly from a rounded perinuclear area. Magnification, 1065x. (Reproduced from reference 1).

Figure 5

Diagram showing a rodent embryo, pinned on a Sylgard surface with three dissection pins. The appropriate position of the forceps for removing the sciatic nerve without visual guidance is indicated.

Fig. 1

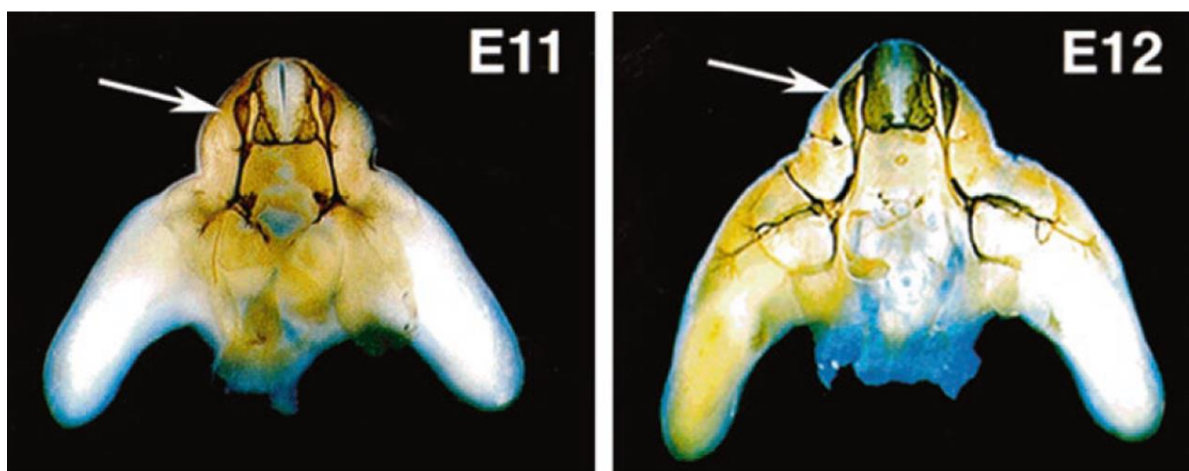


Fig.4

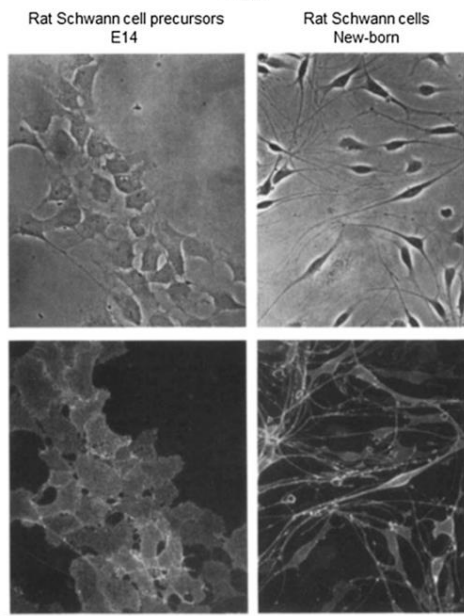


Fig. 5

