Research Article

Optimized methods for rapidly dissecting spinal cords and harvesting spinal motor neurons with high survival and purity from rats at different embryonic stages

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Study design: Experimental study, protocol optimization.

Objectives: To investigate and compare the isolation of spinal motor neurons from embryonic rats at different embryonic stages, and develop optimized methods for rapidly dissecting spinal cords and harvesting spinal motor neurons with high survival and purity.

Setting: Guangdong Provincial Academy of Chinese Medical Sciences, Guangzhou, China.

Methods: Embryonic rats at different embryonic stages (12–18 days) were used to isolate spinal motor neurons. Their shape and corresponding dissection procedures, time needed and skills were compared. After dissecting and dissociating spinal cords, cells were randomly divided into immunopanning group and control group, in which antibodies to p75^{NTR} were used or not. After plating cells, different recipe were added at different stages in serum-free culture media. Morphological features of cells were observed during development. Immunofluorescence assay was performed to identify motor neurons and the proportion of motor neurons in both control and immunopanning group were evaluated and compared.

Results: We summarized the operation essentials for rapid isolation of spinal cords, as well as compared anatomical features and dissection procedures of embryos at different embryonic stages, which help us to better evaluate the developmental profile and isolate cells by adopting corresponding skills. Through the fast isolation procedure and optimized culture media, cells grow in good viability. Moreover, compared with control group, the purity of spinal motor neurons in the immunopanning group was significantly increased, reaching a proportion of over 95%.

Keywords: Embryos, Spinal cords, Spinal motor neurons, Immunopanning, Survival of motor neuron, High purity

Introduction

Neurons cultured *in vitro* are commonly used to study neuron development, signal transduction, neuronal network interactions and toxicology screening,^{1–5} which helps researchers better investigate the basic neurobiology under physiological, pathological or neurodegenerative conditions. In contrast to animal models or slice

recordings, where specific cells may be more or less influenced by extraneous variables, cell culture facilitates the evaluation of a given cell type by providing a controlled environment, thus making it a useful tool for studying the principle mechanisms in neuroscience.

Spinal motor neurons (SMNs) are neurons whose cell bodies are located in ventral horn of spinal cord and whose axon projects outside the spinal cord to directly or indirectly control muscles. Diseases like amyotrophic lateral sclerosis (ALS), primary lateral sclerosis (PLS) or progressive muscular atrophy (PMA), are characterized by progressive loss or degeneration of SMNs.^{6–8} Spinal cord injury, radiculopathy or peripheral nerve injury may affect SMNs, which lead to debilitating and even

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lethal paralysis. Progress in performing mechanism studies or drug screens about these diseases have been stymied by the inability to obtain sufficient quantities of motor neurons *in vitro*. Stem cell differentiation and transcriptional programming technology have been tried to overcome this challenge,^{9–13} and yet, these methods of stem cell differentiation to motor neurons were relatively inefficient (about 60–70% or even less), costly, and mostly require long time (usually 40–60 days, at least 10 days) before resulting cells exhibited electrophysiological characteristics of mature motor neurons. Hybrid cell-lines of motor neurons (NSC 34 and VSC 4.1) have been produced,^{14–17} but it is still questionable whether these cells authentically possess characteristics which may be vital for the development of neurodegeneration in motor neurons. A recent study has also demonstrated that NSC-34 motor neuron-like cells are unsuitable as experimental model for glutamate-mediated excitotoxicity, by evaluating the biochemical and electrophysiological processes. Therefore, the authors suggested that primary SMNs are more suitable to explore the pathogenesis of glutamate-mediated excitotoxicity at the cellular level, in ALS and other motor neuron diseases.¹⁸

How to gain sufficient SMNs from spinal cords to culture *in vitro* and investigate their function have always been challenging, for the difficulties in slowly dissecting spinal cords and complicated ways to purify cells. SMNs are highly susceptible to oxidative stress and other insults,^{15,19–22} which again imposes difficulties for the whole process and easily leads to low survival rate and limited quantities. Before the 1990s, some research about SMN isolation, purification and culture from chicken had succeeded,^{23–27} while similar research in mammals are far from enough. Although there have been some reports on the primary culture of SMNs from rats or mice, rapid and easy protocols have hardly been achieved, which makes it hard to do mechanism studies or drug screens by performing downstream analyses including protein, immunohistochemistry or RNA profiling systematically. For the isolation of rat SMNs, researchers tended to use 14-day-old embryos or newborn rats,^{23,28–32} but the reason is not very clear and the differences of SMNs derived from different-aged embryos had never been compared. Moreover, the different ways of purifying cells, ranging from density gradient centrifugation^{23,30,33–35} to fluorescence-activated cell sorting,^{26,28,29} make the process complicated, costly and time-consuming. Immunopanning strategy, first introduced for sub-population of central neurons,³⁶ has developed into a remarkable technique in cellular immunology for selecting cells which has affinity to specific antibody.^{37–39} Compared with other purifying

methods, immunopanning technique seems to be easier to perform, but more importantly, much blander for collected neurons, which ensures the cell viability.

In this study, our strategy was (i) to isolate SMNs from different-aged embryos, compare their differences and summarize the operative skills in order to optimize the dissection procedure; (ii) to adopt an effective and convenient purifying method by modifying the reported ones; (iii) to integrate the methods of SMNs culture these years and try some new steps, so that we can develop rapid, simple and improved methods for isolation and culture of highly purified SMNs.

Materials and methods

Animals

Sprague-Dawley (SD) rats were used in our experiments and housed in Experimental Animal Center approved by the Chinese Association for Assessment and Accreditation of Laboratory Animal Care. All of our experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Guangzhou University of Chinese Medicine. Pregnant SD rats at different stages of gestation, from 12–18 days, were used for our studies.

Dissection of spinal cords from rat embryos

The pregnant rat was anesthetized with 1% pentobarbital sodium (30 mg per kg) by intraperitoneal injection. After dissecting the lower abdomen (Fig. 1A1), the uteri containing embryos were removed to a sterile 100-mm Petri dish (Fig. 1A2) before all embryos were separated from foetal sac using ophthalmic scissors (Fig. 1B) and rapidly placed in a new 100-mm Petri dish containing ice-cold sterile Hank's balanced salt solution (HBSS, GIBCO Invitrogen, San Francisco, CA, USA). Each embryo was decapitated from occiput (Fig. 1C) and then placed backside up (Fig. 1D) so that its spine can be clearly exposed. Skin overlying the spinal cord was removed from cranial to caudal (Fig. 1E), following by removing the fascia of the spinal canal quickly by using forceps (Fig. 1F), so that the spinal cord can be lifted out easily (Fig. 1G) and then placed in ice-cold L-15 (GIBCO Invitrogen) (Fig. 1H). After the spinal cords of all embryos were dissected, vessels and meninges wrapped over the spinal cord need to be removed carefully (Fig. 1I) by using microsurgery forceps with the aid of stereomicroscope.

Dissociation of the spinal cord tissues

The spinal cords were then washed 2 times by HBSS and immediately cut into 1 mm slices or even smaller

Figure 1 Operating procedures and the skills during dissection of embryonic spinal cords (A) Dissect the lower abdomen and take the uteri out (B) Separate embryo from foetal sac (C) Decapitate the embryo from occiput, the yellow line shows the approximate level for decapitation (D) Place the embryo backside up so that its spine can be clearly exposed, dotted line shows the focus (E) Remove the skin overlying the spinal cord from cranial to caudal (F) Remove the fascia upon the spinal canal (G) gently lift out the spinal cord (H) spinal cords taken from all embryos (I) for each spinal cord, carefully strip the vessels and meninges (indicated by arrows) from cranial to caudal (J) the dissected spinal cord tissues.

using a pair of ophthalmic scissor and transferred a new dish with 10 ml 0.25% trypsin (GIBCO Invitrogen) and 0.4% DNase (Sigma-Aldrich) to incubate for 20 min at 37 °C. After digestion, DMEM F12 (GIBCO Invitrogen) with 10% FBS (GIBCO Invitrogen) was added to stop the enzyme activity. Then the tissues were gently triturated through a Gilson blue pipette tip and the suspension containing dissociated tissues was passed through a 100-mesh filter into a sterilized universal tube so that cells were dispersed into single-cell suspension. Cells were collected by centrifugation (at 1000 rpm for 10 min), re-suspended by Neurobasal medium (GIBCO Invitrogen) supplemented with 2% B27 (GIBCO Invitrogen), and plated on panning dishes. And the cells in control group were seeded on confocal Petri dishes without immunopanning.

Preparation of panning dishes and immunopanning

Panning dishes were incubated at 4°C with affinity-purified goat anti-mouse IgG (1:500 dilution, Jackson ImmunoResearch) in 7 ml of Tris-HCl buffer (pH 7.4) overnight the day before. Dishes were rinsed 3 times with PBS and then incubated with p75^{NTR} antibody

(1:500 dilution, Abcam, Cambridge, UK) at 37°C 2 hours later, p75^{NTR} antibody was removed and the dishes were washed 3 times by PBS before the cell suspension was added and allowed to bind for at least 1 hour (in our trials, the optimal time may be 2 hours).

Plating of purified cells

After immunopanning, the supernatant containing non-adherent cells were transferred to a new dish for further culture. Panning dishes were gently washed 3 times with HBSS to remove those loosely attached cells. Adherent cells (phase bright and quite big under the inverted phase-contrast microscope) were incubated with 0.25% trypsin solution for 1–2 min at 37°C to be detached from the panning dishes before DMEM F12 with 10% FBS was added to inactivate trypsin. Cells were then removed by gentle trituration, collected in a 15 ml tube, centrifuged at 1000 rpm for 12 min and re-suspended by plating media, whose components are Neurobasal medium supplemented with 2% B27 and 25 μM Glutamate (Sigma-Aldrich St. Louis, MO, USA). An aliquot (2–5 μl) was used for counting before cells were seeded in a proper density (10^4 – 10^5 /ml) on confocal Petri dishes, which

had been previously coated with poly-D-lysine (Sigma-Aldrich) (20 $\mu\text{g/ml}$, 30 min at RT).

Culture of purified cells

Twenty-four hours after plating, media was fully replaced by growth media, whose components are Neurobasal medium supplemented with 2% B27, 2 mM L-Glutamine (Sigma-Aldrich), 10 ng/ml Brain-derived neurotrophic factor (BDNF, Sigma-Aldrich) and 10 ng/ml Glial Cell Derived Neurotrophic Factor (GDNF, Sigma-Aldrich). Half medium was changed every three days.

Identification of motor neurons by immunofluorescence assay

Cells at 10 days were fixed in 4% paraformaldehyde (PFA, Sigma-Aldrich) for 15 min and then washed 3 times with 0.01 M PBS (GIBCO Invitrogen). Next, cells were permeabilized by using 0.2% Triton X-100 (in PBS, pH 7.4) and blocked in 5% goat serum for 1 hour at room temperature. Subsequently, the cells were incubated with primary antibodies overnight at 4°C. In the immunopanning groups, for the identification of spinal motor neurons, we chose mouse anti-SMI 32 (EMD Millipore, Billerica, MA, USA) and rabbit anti-CHAT (Abcam) as the primary antibodies. After incubation at 4°C overnight, cells were washed with PBS and incubated with Alexa fluor 488 conjugated donkey anti-mouse IgG (Invitrogen) and Cy3 conjugated sheep anti-rabbit secondary antibody (EMD Millipore) for 1 hour in dark. For nuclear counterstaining, 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) was used. Labeled cells were identified using Olympus FX-70 fluorescence microscope and Zeiss LSM 510 META laser scanning confocal microscope.

Calculation of the proportion of motor neurons in immunopanning groups and control groups (non-immunopanning groups)

Immunofluorescence assay was performed in the same way as mentioned above except the kind of primary antibodies. In order to identify both spinal motor neurons and astrocytes, we choose mouse anti-SMI 32 (EMD Millipore) and rabbit anti-GFAP (EMD Millipore) as the primary antibodies. After the immunostaining, cells were observed under fluorescence microscope to calculate the SMI-32 and GFAP immunopositive cell percentage. For each confocal Petri dish, at least 20 regions were randomly selected. Cell nuclei stained with DAPI showed blue. SMI-32 and GFAP positive cells (green and red respectively) were counted and the results were expressed as percentage of positive cells

relatively to the number of nuclei counted. For each experiment of cultured cells, more than 1000 cell nuclei were counted.

Reproducibility

To ensure the reproducibility of our methods, the culture procedures were carried out on 20 separate occasions. All data were obtained from at least three independent experiments and results were presented as Mean $\pm$ SD.

Results

Shape of embryos at different embryonic stages and the comparison of their dissection procedures, dissection time and skills

We dissected spinal cords of embryos at different stages to compare and determine their differences. It is clearly seen that embryos grew rapidly during development. Great changes took place in their size, structure and blood supply in a short time (every two days) (Fig. 2). The dissection of one single spinal cord took about 90–160 seconds from the time the embryo was killed to its placement in ice-cold L-15 (Table 1), and the total time of all spinal cords taken from a pregnant rat (containing 10–18 embryos) was about 20–45 min. We

Figure 2 Shape of embryos at different stages, and the spinal cords taken from embryos (A) In 12 d, the embryo is very tiny. When an embryo is 14–16 d, it would be twice or triple the size of that in 12 d. But when an embryo is 18 d, it grows more than five times bigger than before. Also, the blood supply gets better as embryos grow. (B) Spinal cords in 12 d embryos can't be dissected because their spinal cord tissues have not developed and look just like watery sacs. Spinal cord tissues taken from all embryos were placed in ice-cold L15. Those taken from E14 are too soft and easily hurt (indicated by yellow arrows). While in E18, the fused lamina become solid, so the process of opening the lamina may also break the spinal cord (indicated by yellow arrows).

Table 1 Size of embryos, the dissection time of their spinal cords and yield of motor neurons.

Embryonic stages (number)	12 d (47)	14 d (57)	16 d (64)	18 d (38)
Size of embryo	0.56±0.03	1.15±0.04	1.81±0.07	2.69±0.10
(body length and width)	0.15±0.02	0.41±0.03	0.78±0.06	1.10±0.05
(centimeters)				
Time it takes to dissect one spinal cord (seconds)	-	112 ± 6.9	93 ± 5.3	158 ± 7.2
Time it takes to strip vessels and meninges for each spinal cord (seconds)	-	19 ± 2.2	14 ± 2.5	20 ± 3.5
Yield of motor neurons	-	(5.76±1.46)	(2.51 ± 0.90)	(2.86 ± 0.79)
after immunopanning		×10 ⁵	×10 ⁵	×10 ⁴
(the average number				
for single spinal cord)				

didn't dissect the spinal cords in 12-day-old embryos because their spinal cord tissues had not developed and looked like watery sacs. In 14–16-day-old embryos, the lamina upon spinal cord has not developed and there is only an overlying layer of tough fascia that can be stripped away easily and quickly using forceps (Fig. 3A and Fig. 3B). Spinal cords in 17-day-old rat embryos (Fig. 3C), however, or newborn animals (rats and mice) can be stripped out without the aid of stereo-microscope, but the tissue at the back of neck has grown

too thick, and the lamina has fused. So it would take a longer time (Table 1) to expose the spinal cord in dissection, and could even destroy the spinal cord when opening the bony structure.

Morphological features of cells

When plating, the purified cells are plump and round. Almost all cells became adherent to the bottom of wells 2 hours after plating and even several already grew tiny neurites (Fig. 4A). Up until 24 hours, most cells have grown axons and exhibited a bright and ovoid cell body, with a ring-shaped halo, but all cells are solitary (Fig. 4B). Later, the growth of cells

Figure 3 Key differences of the spinal cord dissection procedures of embryos at different stages (A) In the 14 d embryo, skin and fascia overlying the spinal cord are very thin and soft (indicated by black arrow), which can be together removed easily. But the tissue adhesion (indicated by white arrow) makes it a little hard to lift out the spinal cord, so the most important thing to do is resolve adhesion to avoid damaging spinal cord. (B) In the 16 d embryo, there is only an overlying layer of toughness fascia (indicated by black arrow), we can prick the fascia from the edge (indicated by white arrow), then the spinal cord can be easily lifted out (indicated by yellow arrow). (C) In the 18 d embryo, the tissue back of neck has grown too thick (indicated by black arrow), and the lamina (vertebral plate) has fused (indicated by white arrow), so it needs greater efforts to remove fascia and open lamina before lifting out spinal cord (indicated by yellow arrows).

Figure 4 Phase contrast micrographs of purified cells obtained from 16 d embryonic rats. (A) 2 h after plating, almost all the cells had been adherent to the bottom and even several already grew tiny neurites; (B) Until 24 h, most cells had grown neurites and exhibited a bright and ovoid cell body, with a ring-shaped halo, but all cells were solitary; (C) 6 d in culture, cells grew with big body and high refraction, and formed obvious neurite networks; (D) 10 days in culture, cells grew more mature and showed vastly interconnected neurite networks. Scale bars represent 100 μm.

increased obviously after the first media replacement. Cells became larger, and their neurites grew longer and extended multiple branches. After 6 days in culture, cells grew mature with big body and high refraction, and form vastly interconnected neurite networks (Fig. 4C). In the long-time culture, cells were still alive after 14 days *in vitro*.

Identification of motor neurons

The CHAT and SMI-32 stainings showed cell bodies, dendrites and large axons. In 10th day, as signs for maturation and high differentiation, the motor neurons developed large cell bodies with long neurites, which formed networks throughout the culture wells and showed prominent arborization (Fig. 5).

The proportion of motor neurons in purified cells compared with the control group (non-immunopanning group)

In the control group, GFAP positive cells accounted for a proportion of 20–35%. While in the immunopanning group, there were little GFAP positive cells. (Fig. 6, Table 2) Moreover, we can find that there are also very little cells (less than 5%) immunostained negative for both SMI-32 and GFAP, indicating that there are other types of cells which may be interneurons, neurosensory cells or oligodendrocytes.

Discussion

We have shown the critical steps of dissecting spinal cords and developed a protocol, which allows rapid isolation of spinal cords, prospective purification of SMNs from rat embryos, established serum-free conditions that promote their survival *in vitro*. Our methods probably worked for several reasons including the choice of extended gestational age, the speedy removal of spinal cords, great increase of purity by immunopanning, viability enhancement through fast adhesion and the change of culture medium at different stages, cells surviving for at least 14 days. The purified cells display the characteristic morphology of motor neurons, but more importantly, stain positively for motor neuron markers with very high yield and purity. Furthermore, the operative skills of dissecting spinal cords might also be adopted to rapidly isolate other types of cells in spinal cord tissues.

Isolation procedure

The speed of neuron isolation is the key point of their survival and viabilities, for neurons are more sensitive and less tolerant to hypoxia. This would be even more demanding for motor neurons, which seem to be more vulnerable to oxidative stress compared to other

neurons because of their great demands on energy and high metabolic rates, which is due mainly to a high level of mitochondrial activity.^{15,19–22} There have been articles that describe methods for rapid isolation and culture of neurons from spinal cords.^{40,41} Their rapid dissection of spinal cords took about 10 min and 5 min respectively. In our trials, by exploring methods of spinal cord dissection, we have developed and summarized the operative skills to accelerate the speed of spinal cord dissection to about 2 min (Fig. 1, Table 1). Additionally, in order to maintain cell viability, the spinal cord should be placed in ice-cold buffer to reduce its metabolism during dissection.

Here we showed the critical steps in dissection procedure, as well as compared some differences in embryos at different stages (Fig. 2). We found that the body length of a 12-day-old rat embryo is only approximately 0.55 cm, whose tiny spinal cord has not developed and looks like watery sacs. When an embryo is 14–16 days old, it would be twice or triple the size of that in 12 days. But when an embryo is 18 days, it has grown more than five times bigger than before. More importantly, the difference of development structures (including the lamina, fascia and spinal cord tissues) in embryos at different stages determines the details in dissecting procedures (Fig. 3). In 13-day-old embryos, although spinal cords can be dissected as mentioned above, neurons are not differentiated and most cultured cells turn out to be neural stem cells. Previous studies once suggest that the initial cell numbers of motor neurons may be reduced from embryonic day 14 to postnatal day 3 down to half which called the physiological motor neuron cell death.⁴² Interestingly, we also found that purified cells derived from 18-day-old rat embryos were less than those from 14–16-day-old embryos. On the other hand, from the aspect of electro-neurophysiology, researchers once discovered that spontaneous rhythmic activity was first recorded when embryo grows to 13.5 days, when motor axons are migrating to and initiating contact with muscles,⁴³ indicating that the pivotal moment for motor neuron development may be around the 14th day. Our findings demonstrate that rat embryos at 14–16 days are a better choice for SMNs isolation, from the aspect of development, which are consistent with previous studies from aspect of apoptosis and electrophysiology. But through the protocol optimization, isolation and purification of spinal motor neurons from 14–18-day-old embryonic rats with high survival and purity can all be achieved.

During the tissue dissociation, trypsin or papain digesting technique can be used, but the enzyme concentration and its time must be noted. DNase was

Figure 5 Confocal images of cultured cells stained with CHAT (red) and SMI-32 (green) antibody for identification of motor neurons. DAPI stains the nuclei, and the CHAT and SMI-32 staining clearly defines the cell soma, dendrites and large axons. Scale bars represent 50 µm (top row) and 20 µm (bottom row) respectively.

required to avoid DNA wrapping over protein surface during dissociation. Cell viability is greatly influenced by trituration procedure.⁴⁴ Neurons are too vulnerable and cell loss may be caused during digestion and

trituration. Foaming should be avoided during trituration, for cells may be lysed at an air-liquid interface. It thereby needs very gentle trituration by a Gilson blue pipette tip.

Figure 6 Cells stained with the SMI-32 (green) and GFAP (red) antibody in immunopanning and control group (from 16 d embryonic rats). (A1, A2) SMI-32 immunopositive cells. (B1, B2) GFAP immunopositive cells. (C1, C2) DAPI stains the nuclei. (D1, D2) Merged photos depicting co-localization of the two antigens and the nuclei. (E) The percentage of SMI-32 and GFAP immunopositive cells in both immunopanning and control group were calculated. Scale bars represent 50 µm.

Table 2 The proportion of motor neurons and astrocytes in the immunopanning and control groups from rats of different embryonic stages.

Groups	Motor neurons	Astrocytes
14 d	(94.4 ± 7.9) %	(3.6 ± 7.3) %
Immunopanning group		
14 d	(61.3 ± 3.6) %	(19.7 ± 2.3) %
Control group		
16 d	(92.3 ± 3.2) %	(5.0 ± 2.6) %
Immunopanning group		
16 d	(52.0 ± 5.8) %	(25.3 ± 4.9) %
Control group		
18 d	(92.0 ± 6.7) %	(4.2 ± 7.0) %
Immunopanning group		
18 d	(43.5 ± 9.6) %	(34.9 ± 11.2) %
Control group		

The data show the percentage of motor neurons and astrocytes in immunopanning and control group under the culture conditions described in this paper. The data in control group can not entirely represent their original proportion in the spinal cord.

Actually, perfect dissection of spinal cords is the fundamental assurance for subsequent procedures. Great importance should be attached to stripping meninges (including pia mater) wrapped over spinal cords, as well as removing dorsal root ganglia. Cells would not be fully dissociated from spinal cords if meninges were not totally removed. At the same time, cell viability would be obviously weakened if fragments of meninges remained in culture system. Therefore, to some degree, the operative skills can greatly influence the yield and survival of cells. To make it better, after neutralizing trypsin digestion, cell suspension needs passing through a filter, so that possible residual fragments of meninges are removed and cells can be dispersed into single-cell suspension.

Purification of SMNs

Cell purification in primary neuron culture is critical, which provides powerful methods that enable the study of the intrinsic properties of a cell type and the interactions with other cell types. In our experiments, there are many types of cells in suspension, both neurons (motor neurons, interneurons and sensory neurons) and non-neurons (astrocytes, oligodendrocytes, fibrocytes and vascular cells). Many investigators have tried density gradient centrifugation or fluorescence-activated cell sorting to separate large motor neurons from the complex cellular environment of cell suspension,^{23,26,28–30,33–35} which have been invaluable methods for purifying SMNs during past decades. However, these methods are complicated, costly and require special apparatus. Here we adopted immunopanning, first introduced for sub-population of central neurons,³⁶ to specifically bond to desired cells, and

then to collect the panned cells. This procedure is simple, but more importantly, much blander for the vulnerable SMNs. The main three steps in immunopanning procedure are: (1) enzymatic preparation of a cell suspension, (2) panning this suspension over antibody-coated dishes, and (3) removing the purified cells from the final dish.^{30,32,37,38,45,46} Choosing appropriate antibodies are crucial for immunopanning. The secondary antibody used to coat panning dish should always be affinity purified, and the antibody diluents should not contain FBS or BSA which will interfere the binding sites. The first antibody (the one that actually binds to the cell surface antigen) should be a monoclonal supernatant whenever possible to ensure the specificity. Antibodies to p75^{NTR} were used to immunopan for SMNs because p75^{NTR} is highly expressed in motor neurons of embryonic and neonatal spinal cord, which have been adopted to isolate 15-day rat SMNs.^{30,32} On the basis of their studies, we tried immunopanning to purify SMNs in rat embryos at different stages and optimize the immunopanning time to be 2 hours. The comparison of SMNs proportion between immunopanning group and control group (non-immunopanning) (Fig. 6, Table 2) clearly revealed the effectiveness of immunopanning procedure in purifying SMNs.

Survival and development of sorted cells

For the isolated neurons, their development and maturation speed are closely related to attachment time and inoculation density. Neurons on the dishes pretreated by polylysine survive much better than those on unpretreated ones for faster adherence. Interestingly, previous studies once showed that sorted SMNs (from embryonic mice) did not adhere to plastic plates coated only with collagen or fibronectin, did attach to plates coated with polylysine or a combination of collagen and polylysine but failed to extend processes and did not appear to be viable after a 24-hour period.²⁸ But in our trials, cells can survive and grow very well on dishes pretreated with poly-D-lysine without adding fibronectin or laminin. We surmised this may be related to cell viability maintenance which owes to rapid isolation and mild purification procedure. Meanwhile, glutamate was added in culture media when plating, which helps cells better adhere to substrate, thus improving cell survival.

Appropriate inoculation density is critical for culture of SMNs. It will be very hard for cells to form interconnected neurites network if the origin density is too low. Whereas, if too crowded when plating, neurons will form neurites network earlier but cannot reach longer for

space limitation. The optimal plating density should be 2000–5000 per cm².

For the survival of motor neurons, researchers have reported that muscle extracts or conditioned media are essential for both the adhesion and the survival of cultured neurons, as well as stimulating their neurite outgrowth.^{28,47–51} Neurobasal/B27 media was used in our study because this medium was capable of not only providing nutritive maintenance but also decreasing the number of cultured non-neuronal cells and protecting cells against excitotoxicity.^{52,53} On this basis, different recipes were added at different culture stages in our trials. Studies have shown glutamate may play an important role in synapse formation and growth of motor neurons.⁵⁴ So within 24 hours, for better plating and higher survival, 25 μ M glutamate was added in culture media. Twenty-four hours later, media must be replaced to avoid glutamate toxicity and neurotrophic factors should be added for better development and maintenance of neurons. BDNF and GDNF have been proved to have the ability to promote the survival of early post-natal spinal motor neurons, reverse injured or degenerated neurons, as well as promote motor axon growth much effectively than other neurotrophic factors.^{42,55–60} Therefore, the neurotrophic factor cocktail containing GDNF, BDNF and L-Glutamine were added. In our culture system, all cells adherent to substrate within 2 hours and most cells started to grow axons within 24 hours. Obvious synapse and networks appeared as time went on when neurotrophic factor cocktail were added in culture media, and cells can grow at least 14 days with good viability (Fig. 4).

Identification of motor neurons

The three standard markers of motor neurons are the 75-kD low-affinity neurotrophin receptor (p75^{NTR}), choline acetyltransferase (ChAT) and 200-kD neurofilament heavy (SMI-32).^{61–63} Antibodies of p75^{NTR} have been used in the immunopanning procedure so later use CHAT and SMI-32 to identify motor neurons. Although both CHAT and SMI-32 got an excellent immunostaining of purified cells, it is obvious to see that SMI-32 stains neurite better than CHAT. Immunofluorescence staining of cultured cells suggested that CHAT and SMI-32 positive cells accounts for over 95%, indicating that spinal motor neurons were harvested and cultured with very high purity.

Conclusions

Our study provides methods to achieve efficient isolation and culture of SMNs with high survival, high

purity and good viability. On the basis of our study, it could be possible to develop research on SMNs in physiological or pathological states.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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