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Topographical guidance of mouse neuronal cell on SiO_2 microtracks

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Abstract

Three different microtrack patterns were developed on SiO_2 layer deposited on a quartz wafer for the observation of topographical contact guidance of living neurons. The patterns were linear, dashed, and zigzag types. Mouse N2a neuroblastoma cells were cultured on the pattern and differentiated into neuronal cells after 4 days in the presence of dibutyryl cyclic AMP (dbcAMP) showing several neurites and growth cones. Among the three patterns, zigzag type pattern was the most effective for directional guiding of neuonal growth and motility. Two different types of growth cones – growth cone with relatively long filopodia and with large lamellipodia - were observed at the ends of axon shafts using a scanning electron microscope. Our research showed that continuous and complex pattern is more favorable for the stimulation of neuronal cell growth and motility, which will be helpful for the design and development of artificial tissues.

Keywords: Topographical guidance; Microtrack; N2a neuroblastoma; Axon shaft; Growth cone

1. Introduction

Neurite guidance, especially axonal guidance [1,2] is one of the key processes performed during embryogenesis or neural tissue engineering. Neural cells find their counterparts for signal delivery by the aid of expanded terminal structure called growth cone at the end of axon shaft. At this biological terminal, by the continuous stimuli and proper interpretation of the various environmental cues, several signaling molecules diffuse through the medium until they find consumers [2–5], and neural cells expand their own neurites longer and longer. The growth cone has some specific features consisted of filopodia and lamellipodia developed by F-actins and microtubules. Those cellular cytoskeletons change their spatial organization inside the growth cone continuously by the external cues [3]. Those external cues are mostly originated from the substratum over which cells adhere by a phenomenon known as contact guidance [4,5]. The interactions between cells and substrata always occur during development and natural or artificial regeneration of tissues, where those substrata provide chemical cues and surface specific topography.

The phenomena can be divided into two sorts—chemical guidance and physical guidance. While chemical guidance is based on biochemical point of view and includes properties like biocompatibility, cytotoxicity, biodegradability, etc, and affects on cellular behavior directly, physical guidance is closely related with the mechanical characteristics of the surface [6,7] over which anchorage-dependent cells adhere [8]. For example, cytoskeletal proteins including collagen, laminin, fibronectin, vitronectin, etc, in various extracellular matrices show biochemical guidance phenomena [9], while their innate fiber structure of various networks and meshes can induce physical guidance, though in nanometer scale [10]. These physical structures are quite complex and not always linear; however, only linear ridge/groove (R/G) pattern has been widely applied with different feature sizes for the study of physical contact guidance [8,11]. For example, in the stage of neuroblasts migration to the cortical plate in the development of forebrain, neurons in the ventricular zone follow the tracks created by radial glial cells. Here, the tracks directed to the cortical plate are one dimensional, how-

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ever, not straight and a lot of low-angle turns are included [12]. Therefore, to understand physical contact guidance, the effect of track features also should be considered. We had special interest about those track features. In this article, we prepared three different microtrack surface patterns for physical contact guidance using conventional photolithography technology. For the cellular adhesion on the surface, fibronectin was deposited on the patterned surfaces and murine N2a neuroblastoma cells were cultured on the patterned surfaces.

2. Experimental

2.1. Micropattern fabrication

We fabricated ridge-based three different microtrack patterns on the oxidized silicon layer deposited on a quartz substrate by the conventional photolithography method as shown in Fig. 1(a). For the convenience of direct optical observation, all materials for substrate and microstructure fabrication layer were chosen as transparent like glass or quartz. On 4 in. quartz wafer (500 μm in thickness), silicon dioxide layer was deposited with a thickness of 5 μm by plasma enhanced chemical vapor deposition (PECVD). For the photolithography of required patterns on the SiO_2 layer, a positive type of photoresist layer was deposited on the SiO_2 layer, and then the microstructure patterns were transferred on the photoresist from a photomask by UV exposure. Though both silicon dioxide and quartz are known as biocompatible, patterns were developed only over the silicon dioxide layer, not on quartz, because the etching processes like reactive ion etching (RIE) are better established for silicon dioxide. The height of ridges for all patterns was kept as the same about 4 μm. With this dimension, cells were grown only on the SiO_2 layer. Patterns 1 and 2 (Fig. 1(b and c)), frequently found from the literatures, have continuous and dashed ridges, while pattern 3 in Fig. 1(d) consists of zigzag feature. The main differences between those three patterns are that in the patterns 1 and 2, neurites can find only linear path following the tracks without any interruption; however, linear motility was periodically interrupted by tilted tracks for neurites in the pattern 3. Neurites should find the appropriate new paths using their growth cone for further motility. Actually, the track of radial glial cell for neural cell migration is not straight, rather similar with zigzag

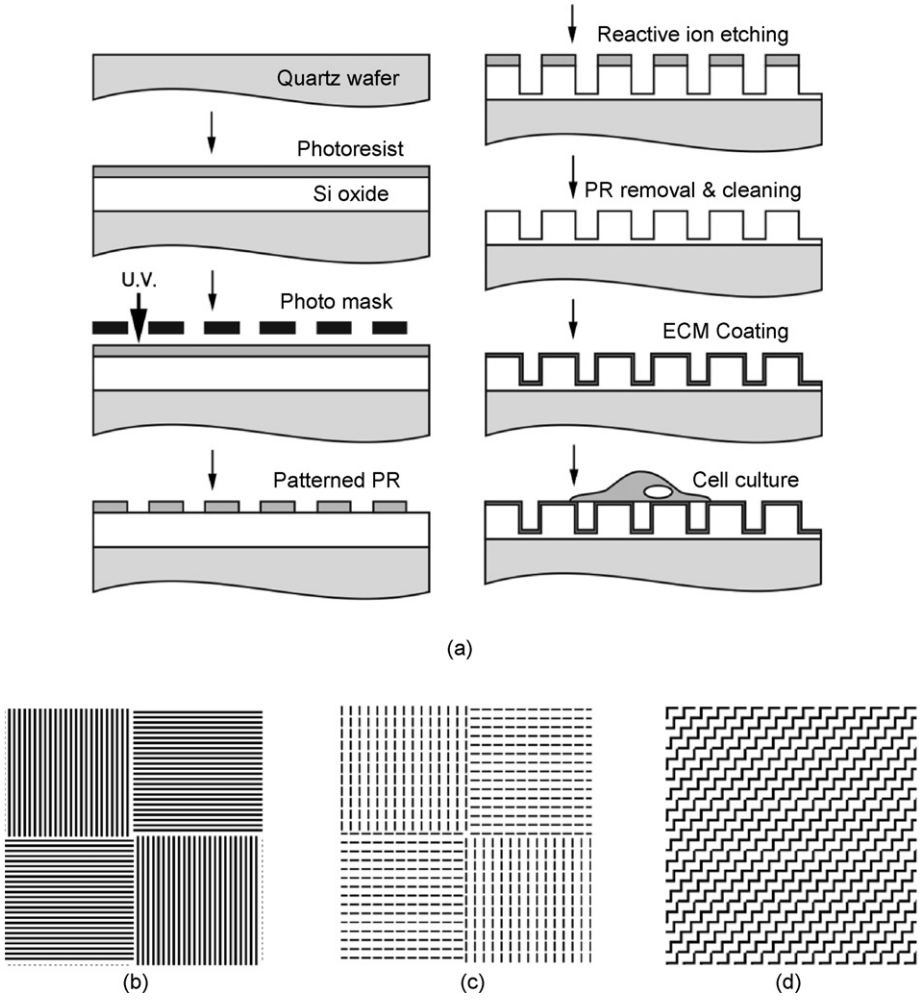

Fig. 1. Microtrack fabrication process on SiO_2/quartz using conventional photolithography technology. Each pattern has a size of 2 mm × 2 mm. The height of a ridge on SiO_2 was 4 μm. After the formation of microtrack, fibronectin was coated on the surface as an extracellular matrix. (a) Process flow, (b) linear-type pattern, (c) dashed-type pattern, and (d) zigzag-type pattern.

pattern [12]. After the pattern formation, the whole samples were cleaned with ethanol and deionized water.

2.2. Cell culture

The microtrack pattern formed wafer was cut into small samples of 14 mm × 14 mm size, which is enough for 60 mm petridish. Before cell culture, the entire patterns were cleaned using ultrasonicator and the entire surface of the substrate was with 10 μg/ml fibronectin for both reliable cell adhesion on the pattern surfaces and neuronal cell differentiation. N2a neuroblastoma cells were cultured on the patterns for 1 day, allowing them to attach firmly. Subsequently, the cells were induced to differentiate into neuronal cells by the addition of dbcAMP for 3 days.

2.3. Lipophillic dye staining and scanning electron microscopy

After 3 days of culture, the cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) to examine the outlines of neurites and axon clearly. They were then incubated in 50 μg/ml 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO, Invitrogen Co.) in ethanol for 1 h and subsequently fixed in 4% (w/v) paraformaldehyde in PBS for 20 min. They were further washed in PBS several times to remove the excess DiO dye molecules. By doing so, it was possible to count whole cell numbers in each area of pattern and the number of neurites in a cell. After the observation under a fluorescence microscope, the identical samples were also processed for scanning electron microscopy (SEM) to study the characteristic patterns of growing axon, neurites, extensions of filopodium and lamellipodium in detail.

3. Results and discussions

Fig. 2 shows pattern-specific adaptations of neuronal cells grown on three different patterns after the differentiation from N2a neuroblastoma cells. In Fig. 2(a), most of cells extended their neurites following the linear microtrack as repeatedly found in the literatures [5,7]. However, neurites of cells cultured on the dashed pattern in Fig. 2(b) did not show the one-dimensional pattern adaptation, rather each neurite finds new pathways between dashes during growth. Moreover, neurites of each cell were relatively short compared to those of cells on the other two patterns. Cells in zigzag type pattern in Fig. 2(c) shows the longest neurites among the three different surface patterns examined. To quantify the results, we counted whole cell numbers and cells with pattern specific longer neurites (>100 μm). In Fig. 3(a), zigzag pattern was favorable for cell adhesion and neurite growth like other two patterns. However, zigzag pattern was more favorable for neurite extension than the other two patterns. About twice of the cells showed longer neurites as shown in Fig. 2(b). Because filopodia were trying to find alternative pathways not only over the flat surface, rather on three dimension [13], we assumed that cell motility would be influenced by how much area is touched by filopodial tips. By this hypothesis, we calculated the sidewall areas of our three patterns within 100 μm around a cell body as shown in Fig. 4 and Table 1. The zigzag pattern provided more space for the contact of a filopodial tip. Dashed pattern provided slightly more space than linear pat-

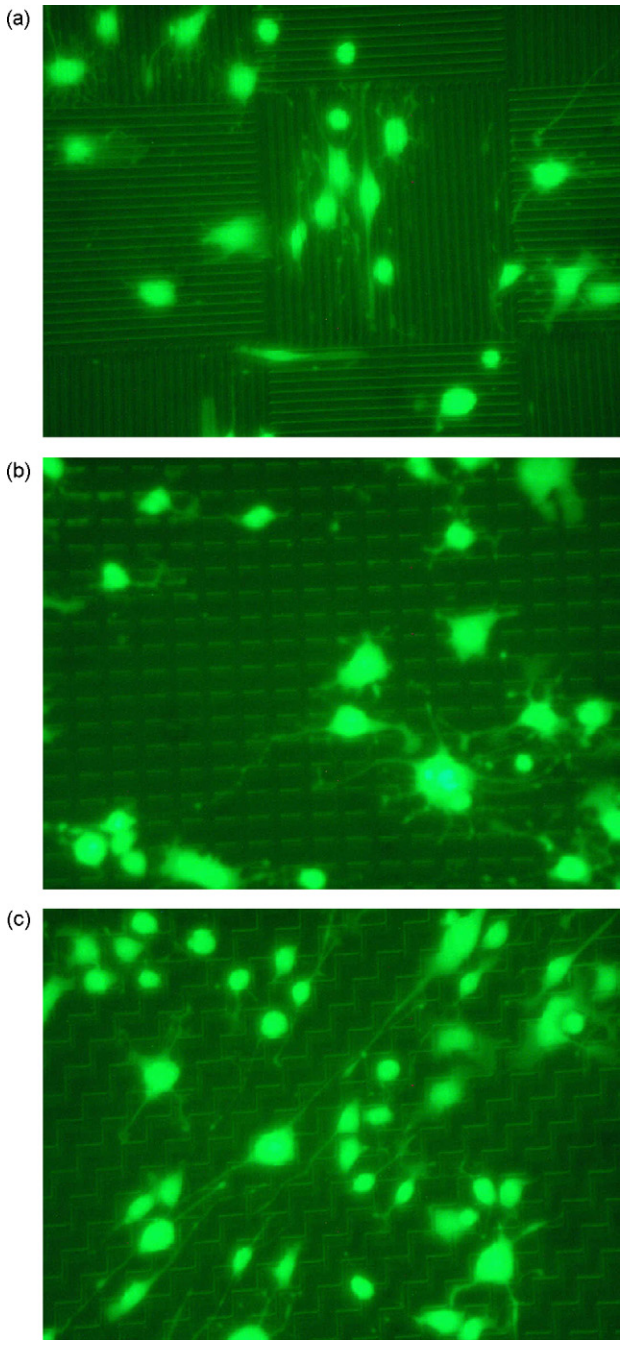

Fig. 2. Parts of optical microscopic images of stained neural cells cultured on three microtracks: (a) linear pattern, (b) dashed pattern, and (c) zigzag pattern.

Table 1
Measured sidewall areas supplied by three microtracks. Because all patterns have the same height (4 μm), each area is measured using only the length of each track

Pattern	1 (Linear)	2 (Dashed)	3 (Zigzag)
Area/4 μm (μm)	199.7	201.8	274.8

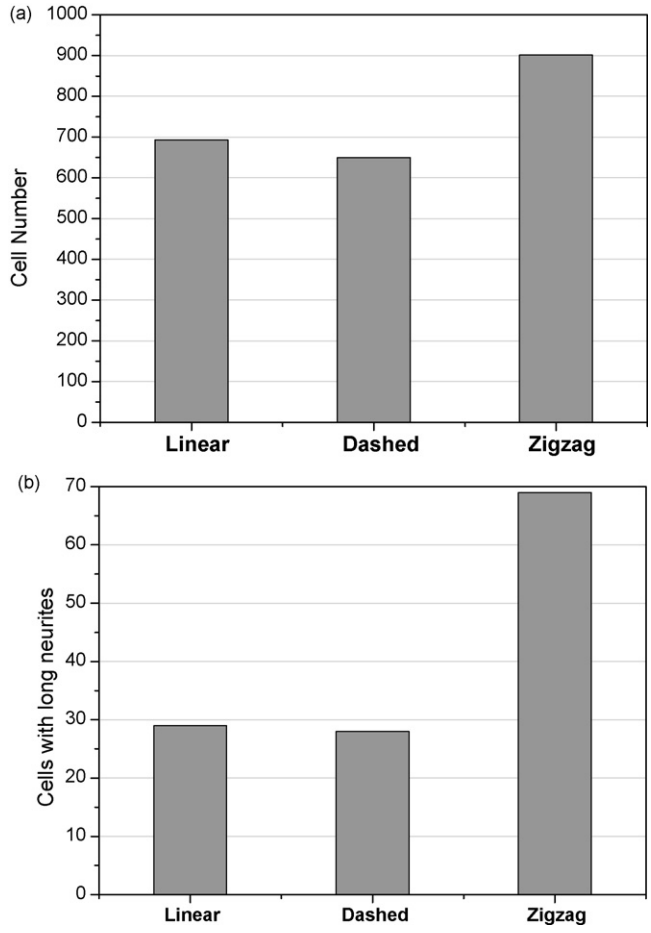

Fig. 3. Quantification of cellular adhesion and growth on microtracks: (a) whole cell number attached on three microtracks and (b) numbers of cells with neurites longer than 100 μm.

tern, however, the numbers of both whole cells and long neuritis in Fig. 3(a) and (b) were not quite different. Therefore, from the above results, it was suggested that complex but continuous pattern is relatively more effective for neurite outgrowth.

Two specific features of cell morphologies with long neurites in Fig. 5 are SEM images – finished by patching series of SEM images to each other – of neuronal cells cultured on the zigzag pattern. In the figures, a single axon shaft and a big growth cone at the end can be observed. The dimensions of axon shafts, filopodia and growth cones were measured in Table 2. As shown in the figures, filopodia around the growth cone in Fig. 5(a) were longer than those in Fig. 5(b), though the size of growth cone was smaller. In addition, short filopodia are shown in Fig. 5(b) around the axon shaft, but not in Fig. 5(a). Interestingly, those additional filopodia in Fig. 5(b) were directed to only one direction. In Fig. 5(a), a tiny part of growth cone was separated and left behind from the main body. As reported by Dent et al. [1], this phenomenon might be the "consolidation" during axonal outgrowth. This branching of growth cone is not found in Fig. 5(b).

Table 2
Measured dimensions of axon shafts, growth cones, and filopodia of two neural cells in Fig. 2

	Fig. 4(a)	Fig. 4(b)
Axon shaft thickness (μm)	0.2 – 0.4	0.55 – 0.58
Growth cone length (μm)	16 ± 2	38 ± 2
Filopodia length around growth cone (μm)	2.3 – 7.0	1.3 – 5.0

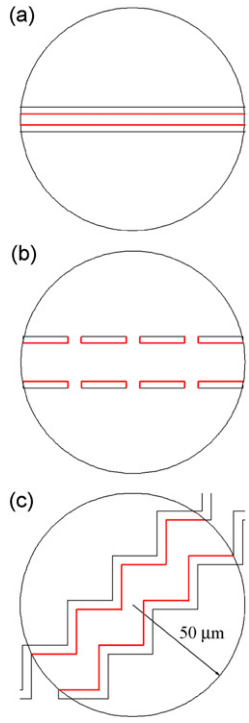

Fig. 4. Sidewall areas supplied for filopodia contact (red color) from three microtracks within a defined area with a diameter of 100 μm. Following the microtrack, filopodia from a cell will touch the sidewall continuously for pathfinding activity: (a) linear pattern, (b) dashed pattern, and (c) zigzig pattern.

As reported, the steering [14,15], turning and thereby pathfinding [16] of a growth cone are driven by filopodia protrusion [17] and retraction promoted by cytoskeleton assembly at the tip (actin polymerization) and retrograde flow (myosin activity) [13,18,19]. Those cellular behaviors on a surface generally consist of four stages: filopodial protrusion, filopodial adhesion, cellular traction, and tail retraction or de-adhesion. The cellular traction is normally accompanied by filopodial retraction or lamellipodial dilation [11]. The long filopodia in Fig. 5(a) might be in the step of filopodial protrusion, while the short one in Fig. 5(b) is in filopodial adhesion and lamellipodial dilation. Following the dilation of lamellipodia, focal adhesion will increase, and thereby neurite growth and motility will be restricted. Therefore, we can decide that the growth cone in Fig. 5(b) is approaching to permanent adhesion, while the growth cone in Fig. 5(a) is still under motility. Also, it can be estimated that the additional filopodia were not extruded during axonal extension, because filopodial withdrawal will follow the forward motion of the growth cone [20]. Therefore, the additional filopodia from the stem of axon shaft are developed during the adhesion of filopodia around growth cone. However, they are still tracking the surroundings for neurite branching and they found signals from the nearest pattern.

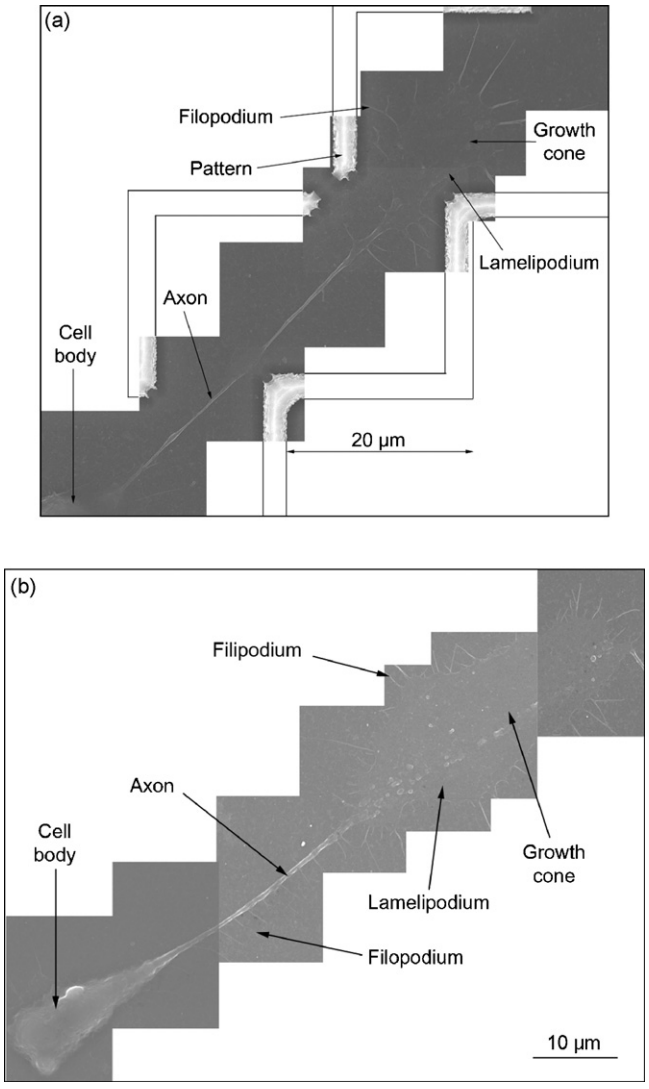

Fig. 5. Scanning electron microscopy images of two neural cells grown on zigzag pattern showing an axon shaft, a growth cone, and several filopodia: (a) A neural cell with small growth cone. Filopodia can be found only around the growth cone and (b) a neural cell with big growth cone. Additional filopodia can be found from the stem of axon shaft. The growth cone is even larger than its cell body.

After the massive work by Curtis and Wilkinson group [21] about the contact guidance in microscale, the research trend is rapidly shifted to nanoscale following the development of various nanoscale fabrication technologies [22]. In microscale, the vertical wall in ridge-groove pattern is recognized by cells because the height of the wall is in the range of single cell scale. However, the nanoscale step will be differently recognized by cells in molecular level. Therefore, we think contact guidance phenomenon in microscale is basically different topic from that in nanoscale. Further studies are required about contact guidance in microscale, which is under investigation.

4. Conclusion

To observe the effect of the microstructures of a biocompatible substratum on the morphological guidance of living cells, three different microtrack patterns: linear, dashed, and zigzag were developed on SiO_2 layer deposited on a quartz substrate. Mouse N2a neuroblastoma cells were cultured on the patterns and the morphological change including axon growth, filopodial protrusion, and growth cone extension during cellular differentiation of the cells into neuronal cells were observed. It was found that zigzag pattern was the most effective for neuronal cell adhesion and axonal outgrowth by direct cell counting. Neuronal cells on zigzag pattern showed two specific growth cone morphologies, small growth cone with relatively long filopodia and large one with short filopodia. It was estimated that the former one is still under motility, while the latter one is approaching to surface adhesion by the formation of several focal adhesion. In the future, we will investigate the formation of focal adhesion during the growth of growth cone guided by micro- and nanoscale surface structures.

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Biographies

Jin Woo Lee was born in 1966. He received the PhD degree in Materials Science from Seoul National University, Seoul, 1999. From 2000, he had worked in Max-Planck Institute for Microstructural Physics, and Walter-Schottky Institute of Technical University of Munich, Germany. From 2004, he had worked in Korea Institute of Science and Technology, Seoul, Korea. Currently, he is working in Korea University, Seoul, Korea. His main fields of interest include carbon nanotube based electronic devices, BioMEMS, nanobiotechnology and nanoimprint lithography.

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Sang Ho Lee received the BS and MS degrees from Korea University, Seoul, Korea in reproductive biology, 1978 and 1980, respectively, and then PhD in developmental biology from University of London, UK in 1989 and has worked in the MRC Experimental Embryology and Teratology Unit, St. George's Hospital Medical School, London, UK as a visiting scientist and non-clinical research scientist for 7 years. Currently, he is the professor of Department of Molecular and Cellular Biology, Korea University. His main interests are molecular and cellular mechanisms involved in early embryonic development and stem cell researches for cell replacement therapy.