Assessment of the developmental totipotency of neural cells in the cerebral cortex of mouse embryo by nuclear transfer

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When neural cells were collected from the entire cerebral cortex of developing mouse fetuses (15.5–17.5 days postcoitum) and their nuclei were transferred into enucleated oocytes, 5.5% of the reconstructed oocytes developed into normal offspring. This success rate was the highest among all previous mouse cloning experiments that used somatic cells. Forty-four percent of live embryos at 10.5 days postcoitum were morphologically normal when premature and early-postmitotic neural cells from the ventricular side of the cortex were used. In contrast, the majority (95%) of embryos were morphologically abnormal (including structural abnormalities in the neural tube) when postmitotic-differentiated neurons from the pial side of the cortex were used for cloning. Whereas 4.3% of embryos cloned with ventricular-side cells developed into healthy offspring, only 0.5% of those cloned with differentiated neurons in the pial side did so. These facts seem to suggest that the nuclei of neural cells in advanced stages of differentiation had lost their developmental totipotency. The underlying mechanism for this developmental limitation could be somatic DNA rearrangements in differentiating neural cells.

Animal cloning has been achieved for many years by transferring the nucleus of a somatic embryonic or fetal cell into an enucleated oocyte (1). Successful cloning by using adult somatic cells first was reported in the sheep (2), then in mice (3), cattle (4–8), goats (9), and pigs (10, 11). The cloning technique is a very powerful tool to analyze the developmental potentials and genomic status of various somatic cell nuclei.

Site-specific DNA rearrangement in developing lymphocytes of the immune system is largely responsible for generating the highly diverse array of immunoglobulins and T cell receptors (12). Whereas neurons do not proliferate, there are superficial similarities between the immune system and the central nervous system. Extreme complexity, the capacity for memory, and extensive apoptosis during development are examples. These similarities have led to the hypothesis that the central nervous system and the immune system use similar somatic DNA rearrangement strategies during their development (13, 14). The rearrangement activating gene, RAG-1, in the immune system also was detected in the central nervous system (15). Furthermore, knockout mice lacking DNA-repair enzymes DNA ligase IV and XRCC4 failed to repair DNA double-stranded breaks, causing defects in the immune system, and had gross cell death along neural differentiation in the embryonic cortex (16). It has been suggested recently that DNA rearrangement may play a role in neural cell development (17).

Previously, we generated embryonic stem cells by transfer of fetal neuronal cell nuclei into enucleated mouse oocytes (18). To further examine developmental totipotency of differentiated neurons, we produced cloned mice from undifferentiated and differentiated neurons of the embryonic cerebral cortex. The use of the animal cloning technique by nuclear transfer technology may lead us to obtain important information on neural cell differentiation.

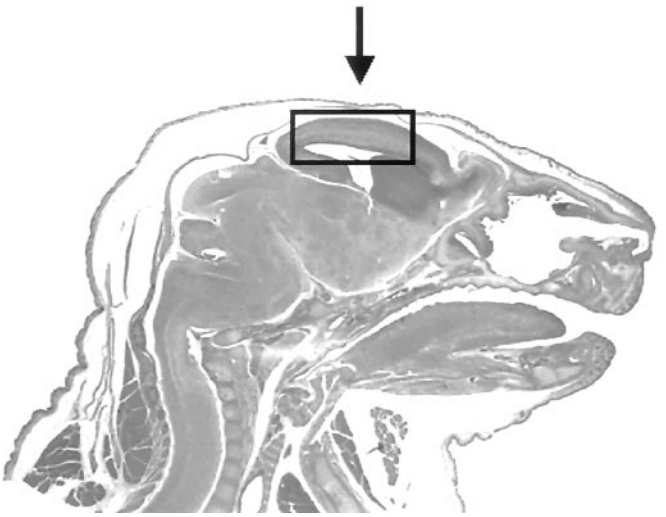

Fig. 1. Semisagittal section (hematoxylin/eosin-stained) of the head of 17.5-dpc mouse embryo, showing an example of the area (arrowed rectangle) from which cerebral neural cells were collected.

Materials and Methods

Preparation of Donor Cells. Neural cells were collected from B6D2F1 (C57BL/6 × DBA/2) fetuses at 15.5–17.5 day postcoitum (dpc) after natural mating of parent animals. The telencephalic region was removed from each fetus and put in Ca^{2+} and Mg^{2+}-free PBS. The meninges were dissected and removed. The cerebral cortex was dissected away from the rest of the telencephalon by using a fine razor blade. In the first series of experiments, a piece (about 0.5 mm in length along anterior–posterior and about 0.25 mm in width along the dorsolateral

Abbreviations: dpc, days postcoitum; P zone, pial-side region; V zone, ventricle-side region.

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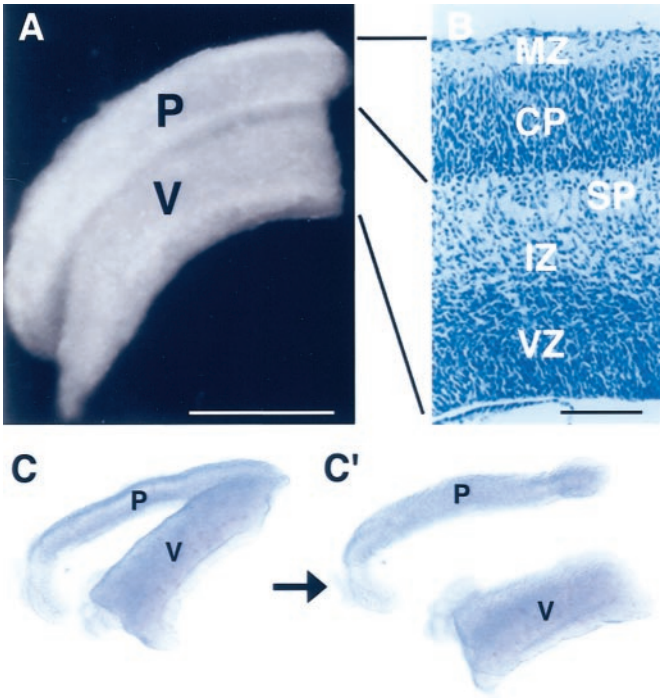

Fig. 2. Embryonic cerebral cortex. During the development of the telencephalon, the cerebral wall can be subdivided into four transient embryonic zones from the ventricular surface to the pial surface: the ventricular zone (VZ), where cell division occurs; the intermediate zone (IZ); the cortical plate (CP), in which postmigratory neurons accumulate to form the cellular layers of the adult cortex; and the marginal zone (MZ). The IZ is a complex structure that contains a variety of cellular elements, including postmitotic neurons migrating on route to the CP and radial glial cells. A subdivision of the IZ is the subplate (SP), a region located just below the CP. (*A*) Embryonic cerebral cortex with the P zone (P) and the V zone. (Bar = 0.1 mm.) (*B*) Nissl-stained section of cerebral cortex at 15.5 dpc. The P zone comprises MZ and CP. Most cells in the CP are differentiated neurons. The V zone comprises SP, IZ, and VZ in which premature neural cells exist. (Bar = 0.5 mm.) (*C* and *C′*) P- and V zones separated along the SP.

axes) of the dorsolateral cortex (Figs. 1 and 2*A*) was used. In the second series of experiments, the cortex was dissected into 3–5 thin slices (about 0.15 mm in thickness), and each was cut further into two fragments, the ventricle-side region (V zone) and the pial-side region (P zone). For cloning, the entire cortex, P zone, and V zone were placed separately in PBS supplemented with 1 mM EDTA (PBS-EDTA) and then pipetted repeatedly to disperse the cells. After washing with PBS-EDTA by centrifugation for 3 min at 250 × *g* at 4°C, cell suspensions for cloning were prepared.

Characterization of Neural Cell Types. Isolated fragments of V zone and P zone were dissociated separately and stained with the primary antibodies anti-nestin (mouse mAb RE6–96) (19) and anti-Hu (Oregon University; mouse mAb 16A11) (20). The secondary staining was done with FITC-conjugated goat anti-rabbit IgG (Vector Laboratories) and Cy3-conjugated goat anti-mouse IgG (Chemicon). Staining patterns were examined with a Nikon inverted microscope.

Nuclear Transfer and Oocyte Activation. Enucleation of the oocytes and transfer of donor cell nuclei into the oocytes were performed by using the Honolulu technique (3). The inner diameter of the injection pipette was ≈6 μm at its tip. Enucleated oocytes injected with donor cell nuclei were kept in CZB medium (3) for 2 h before they were activated by a 6-h treatment with Ca^{2+}-free CZB medium containing 10 mM $SrCl_2$ and 5 $\mu g \cdot ml^{-1}$ cytochalasin B. Sr was to activate reconstructed oocytes, and cytochalasin B was to retain all chromosomes of donor cell by preventing the loss of donor cell chromosomes into the pseudopolar body during oocyte activation (3).

Embryo Transfer. After 72 h of culture, embryos that had developed into the morula or blastocyst stages were transferred into the oviducts of day 1 pseudopregnant CD-1 surrogate females mated during the previous night with vasectomized males of the same strain. Some females were euthanized at 10.5 dpc to examine the developmental status of embryos. Some other females were euthanized at 19.5 dpc, and live-term fetuses obtained by Cesarean section were raised by lactating foster CD-1 mothers.

Histological Analyses of Midterm Fetuses. Cloned fetuses at 10.5 dpc were fixed with 4% paraformaldehyde, dehydrated in graded ethanols, and embedded in Epon 812, and 2-μm sections were stained with 0.1% toluidine blue and 1% borax at 60°C for 2–10 min.

Results

We first used neural cells collected from the entire cerebral cortex of 15.5-dpc fetuses. The activation rate of reconstructed oocytes was high (91–96%), and 30–35% of them developed into morulae/blastocysts *in vitro*. When transferred to surrogate mothers and examined at 10.5 dpc, four of nine transferred embryos were developing (Table 1, experiment a). One of them was a normal-sized fetus, and the other three were smaller than normal (data not shown). When fetuses were allowed to develop to term, five live pups were obtained by Cesarean section (Table 1, experiment b). Their body and placental weights at birth were 1.73 ± 0.10 g and 0.21 ± 0.06 g, respectively (n = 5). These pups developed into fertile adults (two females and three males).

The cerebral cortex of developing fetuses at 15.5–17.5 dpc contains neural cells in different stages of differentiation (Fig. 2 *A* and *B*). Premature neural cells proliferate in the ventricular side and migrate toward cortical layers in the developing cerebral cortex. We separated the cortex by a subplate into two zones, the V zone and the P zone (Fig. 2 *C* and *C′*). It is known that the V zone (mixture of the ventricular zone and the intermediate zone) contains mitotic and premature neural cells, whereas the cortical plate (most of the P zone) contains postmitotic differentiated neurons (21). When we analyzed the cells from the V zone, more than 99% of them were nestin-positive. Nestin is an intermediate filament expressed by immature neural progenitor cells (22). The

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Table 1. Mouse cloning by using neural cells from the entire cerebral cortex of 15.5-dpc fetuses

Experiment	No. of reconstructed oocytes	No. (%) of activated oocytes	No. (%)* of morulae/blast embryos	No. of transferred embryos	No. (%)* of fetuses at 10.5 dpc	No. (%)* of normal, live-born pups
a	30	29 (96.3)	9 (30.0)	9	4 (13.3)	—
b	91	83 (91.2)	32 (35.5)	32	—	5 (5.5)

*Percentages of fetuses/pups developed from all reconstructed oocytes.

Table 2. Mouse cloning by using nuclei of neural cells collected from V and P zones of fetal cerebral cortex

Donor cell	No. of reconstructed oocytes	No. (%) of activated oocytes	No. (%)* of morulae/blastocysts	No. of embryos transferred	No. (%)* of fetuses at 10.5 dpc	
					Total	Normal
V zone (premature or early-differentiated)	69	68 (98.6)	26 (37.7)	26	9 (13.0)	4 (5.8)
P zone (differentiated)	412	386 (93.7)	113 (27.4)	113	21 (5.1)	1 (0.2)

*Percentage of fetuses developed from all reconstructed oocytes.

V zone cells were a mixture of premature and early-differentiated neurons (19). In the P zone, 96% of the cells were Hu-positive. Hu is an RNA-binding protein (23). It appears just after the final cell division and, therefore, is a marker of postmitotic differentiated neurons (24). This finding indicated that almost all of the cells collected from P zone were differentiated postmitotic neurons. Cajal–Rezius cells were also in the P zone, but they were less than 1% in population.

In the second series of experiments, we separately used nuclei from V zone and P zone cells to compare their developmental potentials. The majority (94–99%) of enucleated oocytes that received the nuclei of V zone and P zone cells were activated, and 27% and 38% developed to morulae/blastocysts, respectively (Table 2). When transferred to surrogate mothers and examined at 10.5 dpc, nine (35%) of 26 transferred embryos of V zone group and 21 (19%) of 113 embryos of P zone group were developing as fetuses, respectively (Table 2). Whereas half of the fetuses cloned with nuclei of V zone cells were normal in size and morphology (Fig. 3, V zone 1), the majority of the fetuses cloned by nuclei of P zone cells were abnormal (Fig. 3, P zones 1–4). The number of normal fetuses at 10.5 dpc with the P zone nuclei was statistically lower than that of normal fetuses with V zone nuclei ($P < 0.05$ by Fisher's exact probability test). Morphological abnormalities were more severe in the fetuses from P zone cells than in those from V zone cells. Small body size, small heads, and enlarged pericardiac cavity were features of the abnormalities. Histological examination of 10.5-dpc, normal-sized fetuses cloned with V zone cells revealed that fetuses had neural tubes with normal morphology (Fig. 4 *A–D*). Fetuses cloned with P zone cells, on the other hand, commonly had neural abnormalities such as undulated neural tubes, protrusions of neural epithelium into the cerebral lumen at the fourth ventricle, and collapsed lumen of the spinal cord (Fig. 4 *E–H*). The cells in the abnormal neural tube were loosely compacted (Fig. 4*F*).

In the last series of experiments, cloned embryos were allowed to develop to term fetuses (Table 3). Five (4%) of 42 embryos cloned with nuclei of V zone cells developed to term fetuses. Mean body and placenta weights of these fetuses were 1.62 ± 0.12 g and 0.20 ± 0.07 g, respectively. Five pups, born by Cesarean section, were raised by foster mothers. They developed into mature fertile adults (all males). Fetal development of the embryos cloned with P zone neural cells was far inferior to that of the embryos cloned with V zone cells. Only two of 42 transferred embryos reached term. One was much smaller than the other. The body and placenta of the larger one were 1.53 g and 0.36 g, respectively. This pup (male) developed into a fertile adult. The smaller one (body and placenta weight: 0.95 g and 0.17 g, respectively) looked normal at the time of Cesarean section, but died within several hours. The number of pups developed into adults was statistically different between V zone and P zone groups ($P < 0.05$ by Fisher's exact probability test).

Discussion

We report here that as high as 6% of enucleated mouse oocytes injected with nuclei of fetal neural cells develop into fertile offspring (Table 1). This cloning success rate (the proportion of live offspring developed from all reconstructed oocytes) was considerably higher than the rates previously reported for mouse cloning when using cumulus cells (1.2–1.3%) (3), tail-tip cells (0.5%) (25), and Sertoli cells (average, 1.9%) (26). The cloning efficiency when using mouse embryonic stem cells (2.4–3.0%) (27–29) was intermediate. Somatic genomes of embryonic neural cells may be more readily reprogrammable to the zygotic state than those of most other types of cells. One may argue that the cloned offspring we obtained (Tables 1–3 and Fig. 3) all devel-

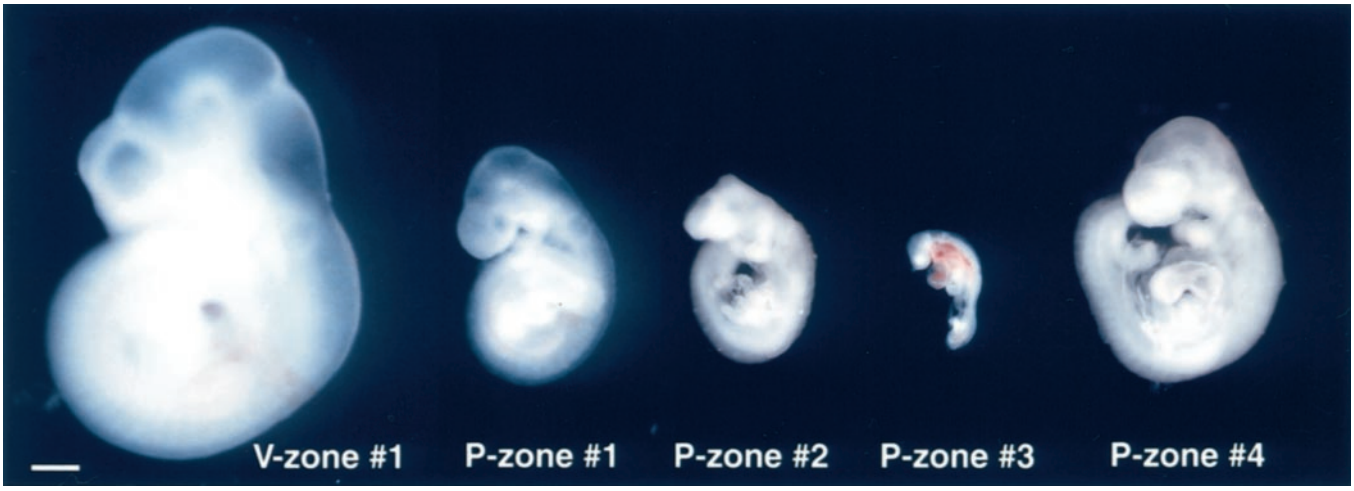

Fig. 3. Fetuses, at 10.5 dpc, cloned with neural cell nuclei. V zone no. 1, a fetus cloned with a premature, postmitotic neural cell in the V zone. P zones 1–4, fetuses cloned with postmitotic differentiated neural cells in the P zone, showing various morphological abnormalities. (Bar = 0.5 mm.)

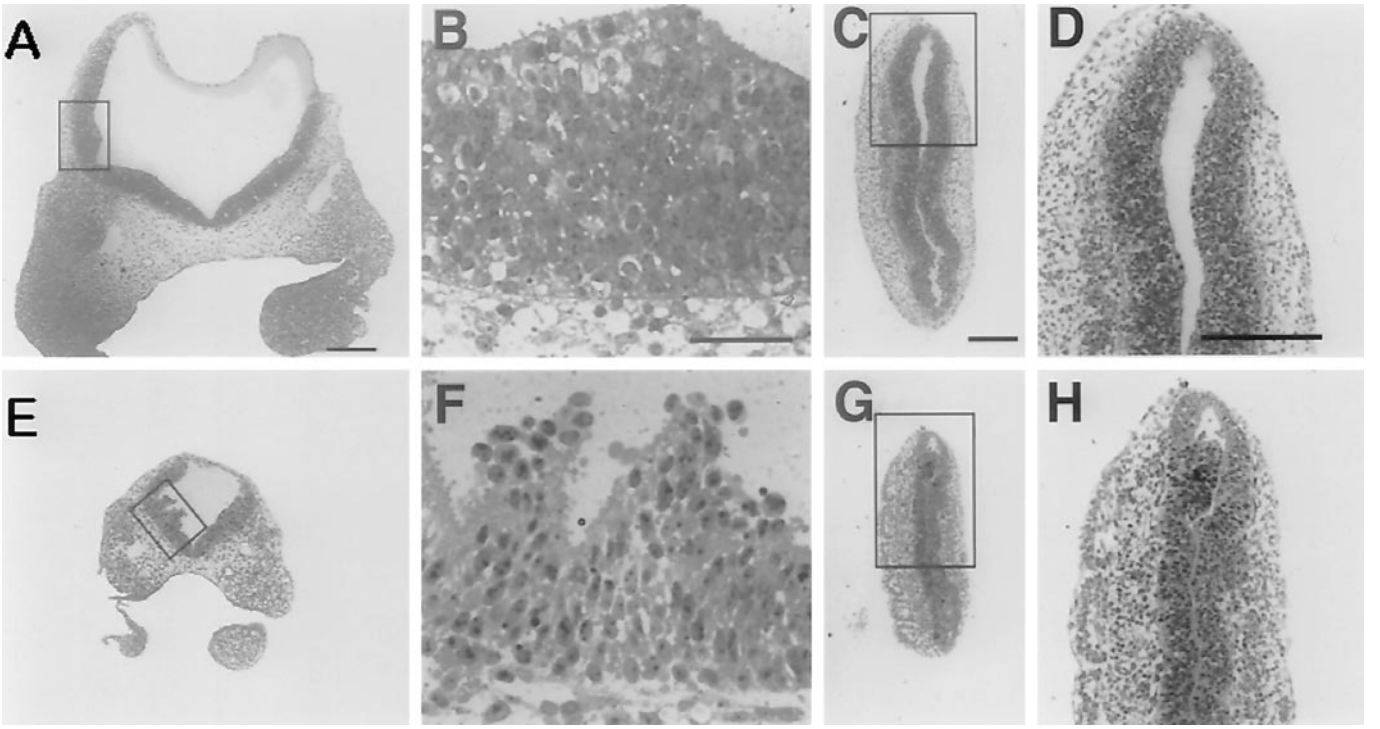

Fig. 4. Transverse sections of cloned fetuses at 10.5 dpc. A fetus cloned with a V zone neural cell was sectioned through the fourth ventricle (*A* and *B*) and the spinal cord (*C* and *D*). Similarly, a fetus cloned with a P zone neural cell was cut through the fourth ventricle (*E* and *F*) and the spinal cord (*G* and *H*). Unlike sections of the V zone fetus (*A–D*), those of the P zone fetus (*E–H*) showed an abnormal (undulated) neural tube with internal protrusions and dispersed cells. (Bars = 200 μm in *A*, *C* and *D*; 40 μm in *B*).

oped from oocytes that received nonneural cell nuclei. This is very unlikely because 96% of P zone cells and 99% of V zone cells that we used were judged to be neural cells.

We found that the fetuses cloned with neural cells had smaller placentas (0.21 g $\pm$ 0.06 g, $n = 10$) than those cloned from other cell types, such as cumulus cells (0.33 $\pm$ 0.08 g) (3), tail-tip cells (0.30 $\pm$ 0.08 g) (25), embryonic stem cells (0.23 $\pm$ 0.02 g) (27), and Sertoli cells (0.40 $\pm$ 0.08 g) (26). Although the placental weights of cloned fetuses were still larger than those of fetuses developed from normally fertilized oocytes (0.14 g), less distinct enlargement of placenta in fetuses cloned with embryonic neural cells may suggest that neural cells are good donor cells to use for cloning.

Cloning mice by using neural cells from adult cerebral cortex was not successful (3). All cloned embryos died either before or shortly after implantation. This may be a result of DNA rearrangements in adult neural cells. Here, we showed that developmental potency of neural cell nuclei is reduced sharply as the cells migrate from immature neural stage (in V zone) to postmitotic differentiated stage (in P zone) of the developing cerebral cortex (Tables 2 and 3). When we used postmitotic differentiated neuron nuclei for cloning, most embryos were retarded in their development and showed abnormalities in their neural tubes, despite the fact that the proportions of activated oocytes, morula/blastocyst embryos, and live fetuses at 10.5 dpc were all comparable to those using V zone cell nuclei (Table 2 and Fig. 3). The marked difference between the nuclei of P zone and those of V zone of the cerebral cortex with respect to their ability to support normal embryonic development suggests that some dramatic changes occur within the nuclei of differentiating neural cells. That five (56%) of nine midterm fetuses cloned with V zone neural cells were abnormal (Table 2) also may be explained by the presence of many early-differentiated neurons in the V zone. Changes in chromatin structure and/or the DNA methylation status of developing neural cells may contribute to the difference in cloning success rates with V and P zone neural cells. However, it has been postulated that DNA rearrangements take place in neural cells even during their early stages of differentiation (17). A sharp drop of the potency to support normal development after their migration from V to P zone (Table 2) may be the result of DNA rearrangement within these cells. It is known that a massive cell death takes place in the P zone as well as in the intermediate zone between V and P zone when DNA-repair enzymes DNA ligase IV and XRCC4 are absent in knockout mice (16). In these mice, DNA rearrangement within their Ig and T cell receptor genes are impaired.

Table 3. Generation of live-born pups by using nuclei of neural cells collected from V and P zones of the fetal cerebral cortex

Donor cell	No. of reconstructed oocytes	No. (%) of activated oocytes	No. (%)* of morulae/blastocysts	No. of embryos transferred	No. (%)* of newborn pups	No. (%)* of pups developed into adults
V zone (premature or early-differentiated)	117	106 (90.6)	42 (35.9)	42	5 (4.3)	5 (4.3)
P zone (differentiated)	182	175 (96.2)	42 (23.1)	42	2 (1.1)	1 (0.5)

*Percentage of pups developed from all reconstructed oocytes.

Somatic DNA rearrangement analogous to the DNA alterations in the immune system may take place in the neural cells when they migrate from V zone to P zone of the cerebral cortex (16, 17). When nuclei of thymus and spleen lymphocytes that have received mostly DNA alterations are used for cloning, normally developed pups cannot be obtained (30). Furthermore, abnormalities of cloned fetuses from P zone cell nuclei were detected particularly in the neural tube and were found as loose cell contact (Fig. 3). These results may suggest that possible DNA rearrangements during neural differentiation arise in genes involved in the cell-to-cell contact during neural development. It is possible that these genes also influence cardiac development.

Interestingly, cadherin-related neuronal receptor (*CNR*) and protocadherin genes with genomic structures similar to Ig and T cell receptor genes (31–34) are expressed as Reelin receptors in maturing neurons in the P zone (35). Furthermore, it is now known that point mutations of *CNR* transcripts are accumulated during the development of the cerebral cortex (36, 37). Thus, loss of the potency of nuclei of developing neural cells to support normal embryo development, as revealed by nuclear transfer technology, seems to reflect somatic DNA rearrangements in these cells.

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