ENGRAFTING FETAL LIVER CELLS INTO MULTIPLE TISSUES OF HEALTHY ADULT MICE WITHOUT THE USE OF IMMUNOSUPPRESSANTS

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Abstract: We have shown the fetal liver cell engraftments into multiple tissues of adult healthy mice, achieved without suppressing the animals' immune systems. Fetal cells from the livers of male *C57Bl/6J Black* lineage mice at day 13 to 15 of gestation were injected intravenously into female adult *CC57W/MY White* mice. The grafting was evaluated by Y-chromosome-specific PCR, cytometric analysis of fluorescently stained donor cells, and histological analysis. All the methods consistently showed the presence of multiple engraftments randomly distributed through the various organs of the recipients. After 60 days, the grafts still constituted 0.1 to 2.75% of the tissues. The grafted cells did not change their appearance in any of the organs except the brain, where they became enlarged. Inflammatory reactions were not detected in any of the histological preparations. The frequency of engraftments was higher in the liver, indicating that similarity between the donor and recipient cells facilitates engraftment. The high inherent plasticity of fetal liver cells underlies their ability

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to integrate into healthy recipient organs, which can be governed by environmental conditions and connections with neighboring cells rather than by the initial cellular developmental programs. The fact that fetal liver cells can be grafted into multiple tissues of healthy animals indicates that they can be used to replace the natural loss of cells in adult organisms.

Key words: Fetal cells, Transplantation, Engraftment, Immune suppression

INTRODUCTION

The regeneration and replacement of damaged tissues is a major guest of current research, as these procedures could be used in the treatment of numerous human pathologies. The replacement of dead cells is required in the treatment of ailments affecting various organs and tissues, including neurodegenerative disorders, characterized by a loss of neuronal cells; type II diabetes mellitus, associated with degeneration of pancreatic islet cells; rheumatoid arthritis, in which cartilage is destroyed; degenerative disorders of the liver and kidneys as well as many others [1-4]. Fetal cells have attracted considerable attention in the field of regenerative therapeutics due to their high plasticity, self-renewal and multilineage differentiation capacities. Indeed, it has been shown that they are able to proliferate and differentiate quickly in response to environmental cues, survive excision and dissection, graft more efficiently due to a lack of long extensions and strong intercellular connections, and deliver protection by the secretion of specific growth hormones and protective factors [1, 5]. Fetal cells have been successfully engrafted in a number of specifically targeted organs and tissues such as the skin [6, 7], the immune system [8], the bone marrow [9], and the lymphopoietic system [10], as well as in the brain when damaged by neurodegeneration [2] and in the heart in cardiac transplant cases [11]. In all these cases, the recipient organs were either severely damaged or destroyed prior to transplantation. In order to achieve successful transplantation, the immune system was often suppressed [12, 13] or the transplantation was carried out *in utero* [14, 15].

In our research, we selected fetal liver cells as a model system characterized by a high cellular plasticity [5, 16]. The transplantation was carried out into healthy adult animals without the suppression of their immune system and without any induced damage of their organs preceding transplantation. Fetal liver cells from *C57Bl/6J Black* lineage mice were transplanted into a very distant lineage of *CC57W/MY White* mice, which is an example of allogenic transplantation. Consequently we monitored the fetal cell engraftments in the numerous organs and tissues through the whole body of the selected group of recipients. The fetal liver cells were collected from embryos on day 13 to 15 of gestation, when the fetal liver is enriched with a wide range of hematopoietic stem cells, including T- and B-cell progenitors [17-19]. At this stage, the cells actively proliferate and exhibit significant plasticity. In later development, they become distributed in

the bone marrow and the other immune organs of the embryos [20]. The pattern of their engraftments in the body and the reaction of the healthy host organisms towards them have a general implication for developmental cell biology, providing an insight into how the primary cells can overcome the immune barrier, graft to multiple organs and survive in the host tissues possessing different properties than their own. Our results, showing the multiple engraftments of fetal hematopoietic cells in adult organisms, have a therapeutic potential for treating cases with multiple organ and tissue damage, such as radiation injuries affecting the whole body [21]. The engraftment of fetal cells in multiple tissues of healthy animals also has potential to restore the natural loss of cells occuring during the normal life span.

MATERIALS AND METHODS

Laboratory animals
Male *C57Bl/6J Black* lineage mice were used as the donors, and female *CC57W/MY White* lineage mice were used as the recipients in our transplantation experiments. All the mice were 6 weeks old and had a weight of 20-23 g, and were provided by the Animal Center, Institute of Immunology, Vilnius, Lithuania. All the animals were maintained at 22 ± 2°C. Food and water were provided *ad libitum*. All the procedures were carried out in accordance with the guidelines of the European Union, and with the approval of the Ethics Committee on Animal Experimentation, Institute of Immunology, Vilnius, Lithuania. The mouse blood was analyzed on a Hemavet 850 analyzer (CDC/Drew scientific, USA). Only those animals meeting the blood health standards were used in further experiments. In the PCR tests, 18 mice received fetal transplants intravenously, and at the same time the control group of 12 mice received an adult male liver cell suspension. A further 2 mice were injected with fetal cells stained with PKH26 fluorescent dye (Sigma, USA).

Preparation and transplantation of the fetal liver cells
Fetal cell suspensions were prepared using *C57Bl/6J Black* lineage mouse livers from embryos at day 13 to 15 of gestation [20]. As shown previously, they have the phenotype of c-kit (CD117) ±; Thy-1.1 (CD90) Lo; Sca-1 +; Mac-1 (CD11b) Lo; 6B2 (anti-B220) (CD45)-; CD3 -; CD4 -; CD5 -; CD8 -; Ter119 -; 8C5 (anti-GR1) -; MHC-II -; MHC-I Lo. [20, 22-24]. 1 ml of RPMI-1640 serum-free medium was added into a 10-ml tube containing one fetal liver. The liver was gently triturated using a glass rod, and then the media was added up to the 3-ml mark. The sample with the re-suspended liver was centrifuged at 280 g for 10 s. Only cells residing in the supernatant were collected, and their concentration and viability was assessed using a Neubauer counting chamber and the trypan blue exclusion assay [20]. Cells with a viability of not less than 80% were used for transplantation. The cells were centrifuged at 400 g for 7 min and suspended into a saline solution at a concentration of 10×10^6/ml. In the experiments with the Y-chromosome positive and fluorescently stained cells, 200 µl of cell

suspensions were injected into *CC57W/MY White* lineage mice into the tail vein using a 1-ml micro-fine plus insulin syringe with a 0.33 x 12.7 mm needle (Beckton Dickinson, Ireland).

Staining fetal cells with PKH26 fluorescent dye
For the staining with PKH26 dye, the fetal liver cells were selected at their proliferating stage. According to the manufacturer, PKH26 dye remains stable in the cells for up to 100 days *in vivo*, and is therefore widely used in cell-tracing experiments [25, 26]. We followed the PKH26 kit manufacturer's recommendations for the optimization of the staining procedure. We carried out optimization experiments by assessing the cell viability after staining and found that 2×10^{-6} M dye per 1×10^7 cells in a 2-ml total volume provided the optimal cell recovery. The cell viability was evaluated by the ethidium bromide exclusion assay. Cell staining uniformity was optimized to the maximum values if the highest viability after staining was achieved. This was supported by fluorescence activated cell sorting analysis (FACS) data.

Preparation of parenchymal hepatocytes from male *C57Bl/6J Black* lineage adult mice
Parenchymal hepatocytes were isolated from *C57Bl/6J Black* lineage adult mouse livers (8-10 weeks old) using a standard two-step collagenase perfusion method [27]. After perfusion, the cells were centrifuged at 50 g for 1 min and filtered through a nylon mesh to remove aggregated cells and residual tissues. The cell viability was between 80 and 90%, as determined by trypan blue dye exclusion. Freshly isolated cells were diluted and re-suspended into solution to a concentration of 10×10^6/ml. The control mice received an infusion of hepatocytes in a 0.2-ml saline solution through the tail vein [28].

Recipient mouse organ preparation
The spleens, kidneys, lymph nodes, thymuses, bone marrow, spinal cords, brains, lungs and livers of the recipient mice were weighed and re-suspended within 3 hours or preferably shoter periods after experimental animal vivisections. We strictly followed the previously described protocols [20]. Another part of each organ was immediately frozen on dry ice and kept at -70ºC for further histochemical analysis.

Preparation of the recipient organ cell suspensions
In order to produce cell suspensions of the recipient organs, a part of each organ from the recipient mice was placed into a 10-ml glass tube containing a 1-ml saline solution. The tissue was gently triturated using a glass rod. Then, 1 ml of trypsin-EDTA solution A (0.25% trypsin and 1:5000 EDTA; Biological Industries, Israel) was added and the cell suspensions were incubated at 37ºC for 15-30 min. An additional 4 ml of RPMI-1640 medium containing 10% fetal calf serum (FCS) was added. The cell suspensions were rigorously triturated using a pipette, and centrifuged at 280 g for 15 s. The supernatants were collected and

centrifuged again at 400 g for 10 min. The final pellets were suspended in saline solution and subjected to FACS analysis. Cell suspensions from healthy untreated organs were used as negative controls.

FACS analysis
FACS analysis was performed on a FACS Calibur cytometer (Becton Dickinson, USA) using a 450-nm FL-2 filter. The procedures were carried out in accordance with the standard flow cytometry protocols (Becton Dickinson, USA). The results were analyzed using the "Cell Quest" program. Stained samples were compared with unstained controls; false positive unstained cells were discounted from the sample analysis. The percentage of engrafted cells stained with PKH26 in the selected organs was calculated as a ratio of the number of stained cells to the total number of cells in the organs. The latter was determined by multiplying the quantity of cells in the fragment subjected to FACS analysis to the weight ratio of the fragment and the whole organ. The experimental error in the FACS analysis experiments constituted ca. 10%.

Polymerase chain reaction (PCR) analysis of the engraftments
The fetal cells were evaluated by PCR analysis for the presence of the Y chromosome, and only Y-chromosome positive cells were used for transplantation. Subsequently, the engraftment was also evaluated by using PCR analysis. The whole genomic DNA was purified from the animal spleens, kidneys, lymph nodes, thymuses, spinal cords, brains, lungs, bone marrow, blood and livers using a genomic DNA extraction kit (MBI Fermentas, Lithuania). PCR primers were selected as described previously [29]. This included the testis-specific Y-chromosome pseudogene (TSPY) forward: 5'-tcc ttg ggc tct tca tta ttc tta ac-3' and reverse: 5'-gag aac cac gtt ggt ttg aga tg-3' primers. PCR reactions were carried out in 25-μl volumes with 2 μl of extracted DNA (0.01 μg/μl). 35 cycles of PCR were conducted, each as follows: 94ºC for 45 s; 55ºC for 30 s; and 72ºC for 1 min. PCR products of 100 bp were evaluated using 1.5% agarose gel electrophoresis (MBI Fermentas, Lithuania) and visualized with a UV system (HeroLab, Germany). Engraftment values (EV) were defined as the number of recipient cells engrafted with one Y-positive transplanted cell. These were estimated comparing the gel electrophoresis bands of our test samples with calibration gel electrophoresis performed by mixing known concentrations of PCRs of XY and XX karyotype cell suspensions in a series of dilutions. The intensities of the bands were analyzed using the Gel Quantifier Image Analysis Software (AMPL software; Australia). The detectable limit of EV was 10^6, and the accuracy of the measurements was within an order of magnitude. In all experiments, female mouse DNA was used as a negative control.
The statistical analysis was carried using the non-parametric Mann-Whitney test with "Analyse-it" software (UK). The EVs in the liver were compared with the values for the other organs.

Histological analysis

The sections from the cryopreserved organs were cut out using a RM2125 microtome (Leica, Germany). Both unstained and hematoxilin-eosin (Becton Dickinson, USA) stained sections were prepared. The slices were analyzed under a Leica fluorescent microscope (Leica, Germany) at a 300 x magnification; the numerical aperture of the objective lenses was 30 x. All the measurements were performed on standard objective glasses without immersion at room temperature. The fluorescent samples were imaged using 450-nm filters.

RESULTS

PCR analysis of the engraftments

The engraftments of the male allogenic murine transplant cells into the organs of *CC57W/MY White lineage* female mice were evaluated by PCR analysis, monitoring TSPY on the Y chromosome. The mice injected with the transplant cells remained healthy and exhibited normal behavior. On days 15, 30 and 60 following the cell injections, their organs were subjected to DNA extraction procedures. This DNA was processed as described in the Materials and Methods, and used for PCR analysis. The positive control group of female mice received mature male liver cell suspensions, while female mice that did not get any injections were used as the negative control group. The TSPY-specific PCR products visualized on 1.5% agarose gel electrophoresis are shown in Fig. 1.
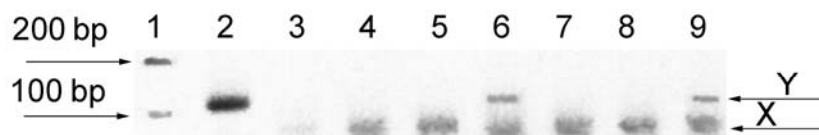

Fig. 1. Visualisation of the TSPY-specific PCR product on 1.5% agarose gel electrophoresis. Lane 1 corresponds to the ladder (GeneRules Ladder Mix™); lane 2 is the TSPY-specific PCR product of the male DNA; lane 3 is that of the female DNA; lanes 4 to 9 are the PCR products of the livers of female *CC57W/MY White* lineage mice injected with fetal transplants from male *C57Bl/6J Black* lineage mice. Only lanes 6 and 9 display positive TSPY-specific PCR products, respectively corresponding to 15 and 30 days after transplantation.

Only the female mice with the Y-chromosome transplant engraftments were characterized by the TSPY-specific band, while the animals without the donor grafts and the negative controls did not display this band. The EVs for a number of the organs, as determined from the PCR product gel analysis, are summarized in Tab. 1. The results demonstrate that within the recipient groups of mice, the Y-chromosome TSPY marker was detected in all the analyzed organs, but not

Tab. 1. The engraftment values for the fetal liver cells from male *C57Bl/6J Black* lineage mice in the recipient organs of female *CC57W/MY White* lineage mice.

Recipient mice	Spleen	Kidney	Liver	Lymph nodes	Lungs	Brain	Spinal brain	Thymus	Bone marrow	Blood
Day 15										
1	0	0	10000	0	0	0	0	0	0	0
2	1000	0	10000	0	0	0	0	0	0	0
3	0	100	0	1000	0	0	0	0	0	0
4	0	0	0	10000	10000	10000	0	0	0	0
5	1000	0	10000	0	0	0	0	0	0	0
6	0	0	10000	0	0	0	0	0	0	0
Positive controls	0	0	10000	0	0	0	0	0	0	0
Negative controls	0	0	0	0	0	0	0	0	0	0
Day30										
1	0	0	0	0	7000	0	0	0	0	0
2	0	0	7000	0	0	0	10000	10000	0	0
3	0	0	0	0	0	10000	0	0	0	0
4	10000	0	0	0	1000	0	0	0	0	0
5	0	0	7000	0	0	0	0	0	0	0
6	0	0	1000	0	0	0	10000	0	0	0
Positive controls	0	0	10000	0	0	0	0	0	0	0
Negative controls	0	0	0	0	0	0	0	0	0	0
Day 60										
1	0	0	8000	7000	5000	7000	10000	7000	0	0
2	0	0	0	0	10000	0	0	0	0	0
3	0	0	0	0	0	9000	0	0	0	0
4	0	0	8000	0	0	0	0	0	0	0
5	0	1000	0	0	0	0	0	0	0	0
6	0	0	1000	0	0	0	0	0	0	0
Positive controls	0	0	10000	0	0	0	0	0	0	0
Negative controls	0	0	0	0	0	0	0	0	0	0

in the blood and bone marrow. The EVs for different organs varied from 100 to 10000. The appearance of the marker in the organs was rather random within the studied group of mice. The most frequent site of engraftment was the liver, as seen in 10 of the 18 recipients, although the number of grafted cells (EVs $\geq$ 7000) was not the highest for this organ. The engraftments in other organs (listed in Tab. 1) were detected in 2 to 5 of the recipients. It is important to note that the engraftments were found in a number of organs in the same animal; in one female mouse tested on day 60, we found 6 concurrent engraftments. Over the 60 days of our experiment, the total number of engraftments remained similar at each tested time point, which indicates that the donor cells were implanted within the first 15 days and not rejected up to the 60th day. At each time point, the distribution of grafts remained random. The statistical analysis of the engraftments in the liver compared to other organs gave values of p = 0.07 for the liver vs. the brain, 0.088 for the liver vs. the lungs, 0.01 for the liver vs. the spleen, 0.002 for the liver vs. the kidney, 0.015 for the liver vs. the lymph nodes, 0.046 for the liver vs. the spinal cord, and 0.007 for the liver vs. the thymus. This clearly demonstrated that the most frequent engraftments of fetal cells were in the liver. The positive control group of 12 female mice (4 mice for each time point), which received the adult male liver cell suspensions, always showed the presence of the Y-chromosome TSPY band only in the liver. Their EVs were consistently equal to 10000.

Imaging the grafted cells in the histological preparations of the recipient organs
The donor cell engraftments in the recipient organs were visualized using fluorescence and optical microscopy. The representative images of the histological preparations from the kidneys, liver and brain are shown in Fig. 2. Similar fluorescence and optical images of the engraftments in all the other studied organs except the blood and bone marrow were also acquired (data not shown). In all the images of all the tested organs, there were no noticeable inflammatory reactions to the transplants after 60 days. As shown in Fig. 2, even after such an extended period of time, the morphological appearance of the donor cells in the recipient kidney and liver closely resembles that of the initial transplants. The deviation in the shape of the donor cells was only observed in the brain where the cells were substantially enlarged, undergoing transformation.

Evaluating the engraftments of the fluorescently stained donor cells
The results of the PCR analysis were confirmed in independent FACS experiments by quantifying the grafts of fetal cells stained with the fluorescent dye PKH26 in the same organs and tissues as in the PCR experiments. The distribution of the engraftments was assessed at the latest time point of 60 days. The evaluation was performed by cytometric analysis. The results are presented in Fig. 3 as the percentage of PKH26-stained cells engrafted in selected organs, determined as described in the Materials and Methods. The same random

distribution of grafts in the multiple tissues was revealed as in the PCR experiments. The donor grafts were found in the lymph nodes, thymus, kidneys, liver, brain, spinal cord, lungs and heart, showing no specific organ preference. The percentage of engrafted cells varied between 0.1 and 2.75% of the total number of cells from each organ subjected to FACS analysis. Consistently with the PCR results, the engraftments were not detected in the blood and bone marrow of the recipient mice.
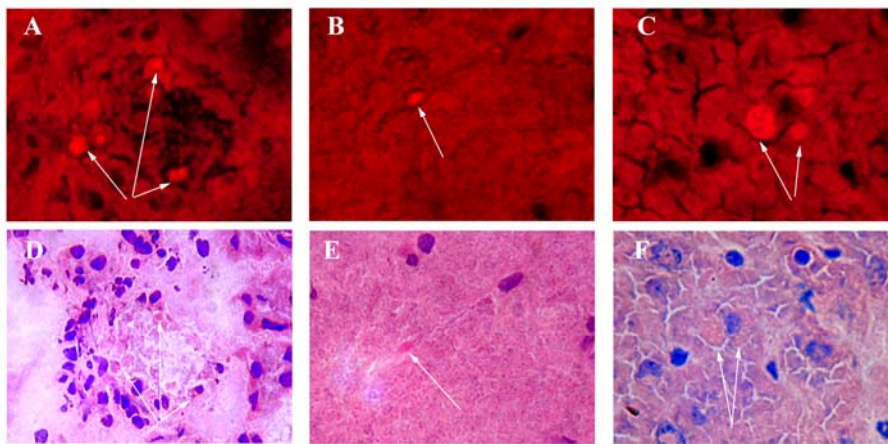

Fig. 2. Histological preparations of the female *CC57W/MY White* mouse organs engrafted with fluorescently stained fetal liver cells from male *C57Bl/6J Black* lineage mice. Histological preparations of the kidneys (A, D) brain (B, E) and liver (C, F) of female *CC57W/MY White* mice are shown. The samples were taken on day 60 after transplantation with fluorescently stained fetal liver cells from male *C57Bl/6J Black* lineage mice. The fluorescence and optical images at a 300 x magnification are presented in the upper and lower panels, respectively. The arrows indicate the fluorescently stained donor cells. Scale bars are 16 μm.
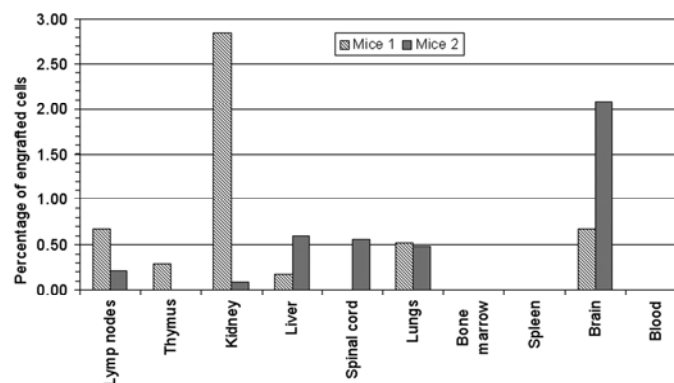

Fig. 3. Engraftment of fluorescently stained fetal liver cells from male *C57Bl/6J Black* lineage mice in the recipient organs of *CC57W/MY White* lineage mice, according to FACS data. The percentage of the engrafted cells in each organ on day 60 after transplantation is shown along the *y* axis. The errors of experimental measurements constitute ca. 10%.

DISCUSSION

We found that mouse fetal liver cells are able to graft into multiple tissues of adult allogenic mice without the need for suppression of the immune system of the recipients prior to transplantation. Consistent results were shown by three independent methods (the PCR assay to monitor the localization of Y-chromosome-containing donor cells in the female hosts; the cytometric analysis to measure the distribution of fluorescently labeled cells in the recipient tissues; and the fluorescent and optical microscopic imaging of the recipient tissues). The latter demonstrates that in all the analyzed tissues except in the brain, the grafted cells did not change their primary appearance even after 60 days of engraftment. The donor cells grafted in the brain tissues were noticeably enlarged, compared to predecessors, which might indicate that they had entered the transformation stage. The fact that grafts were found in the brain also suggests that fetal cells are able to cross the blood-brain barrier and be mobilized in brain tissues due to their low immunogenicity. However, the donor fetal liver cells were found neither in the blood nor in the bone marrow, demonstrating that due to their inherent plasticity they can adopt a new program of development, dependent on the environmental conditions of the host tissues rather than on their initial properties. We observed the largest population of fetal cells in the recipient liver, indicating that the similarity between the donor and host cells facilitates the engraftment of the latter. In two mice the engraftment of fetal cells in the thymus correlated with their wider distributions in other organs, reaching a total of 6 organs in one animal. As the thymus is an important immune organ in which T cells are matured, it may facilitate the survival of the transplants in the host tissues. The rate of engraftments in all the organs was in 0.1 to 2.75% range, which is comparable with the values of 0.125 to 0.257% previously reported for the allogenic transplantation of liver cells to the recipient human and mouse livers measured by real-time PCR for Y-chromosome sequences [29]. During our monitoring period (days 15-60 after transplanation), we did not observe an acute inflammatory reaction of the hosts towards the donor cells in any of the histological preparations of the analyzed organs. A lack of rejection by the hosts results in a high survival rate of the donor cells. The mechanisms underlying the mobilization of the primary cells in the recipient tissues remain poorly understood. It has been proposed that a number of chemokines, cytokines, and adhesion molecules such as selectins and integrins can be involved in the inter-cellular connections [30]. In the adult organism, the tissue cells produce organ-specific recognition and adhesion molecules on their surfaces. The mobilization of the primary cells in different donor tissues may indicate that they are able to produce a range of recognition molecules required by their environment. As in the fetal liver there are different types of hematopoietic cells, among them cells with a very high plasticity can be populated, which can be adapted for tissue regeneration and repair. The fact that fetal liver cells can be grafted into multiple tissues of healthy animals indicates

that they can restore the natural loss of cells in adult organisms during their life span.

In conclusion, the complex studies of fetal cell transplantation into adult mice were performed monitoring the distribution of the donor cells through the whole body. We have convincingly demonstrated that fetal liver cells are able to form engraftments in multiple organs of sex-mismatched allogenic adult mice. The engraftments are very stable, surviving up to 60 days without immune rejection from the hosts. The fetal cells were mobilized in different donor tissues due to their high inherent plasticity. This indicates that during the early stages of the fetal liver formation, the stem cells can adopt a variety of developmental programs governed by current environmental conditions and connections with neighboring cells rather than by their primary source of origin.

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