Dioxins have been reported to exert various adverse effects, including cell-cycle dysregulation in vitro and impairment of spatial learning and memory after in utero exposure in rodents. Furthermore, children born to mothers who are exposed to dioxin analogs polychlorinated dibenzofurans or polychlorinated biphenyls have developmental impairments in cognitive functions. Here, we show that in utero exposure to dioxins in mice alters differentiation patterns of neural progenitors and leads to decreased numbers of non-GABAergic neurons and thinner neocortical layers. This reduction in number of non-GABAergic neurons is assumed to be caused by accumulation of cyclin-dependent kinase inhibitor p27^Kip1 in nuclei of neural progenitors. Lending support to this presumption, mice lacking p27^Kip1 are not susceptible to in utero dioxin exposure. These results show that environmental pollutants may affect neocortical histogenesis through alterations of functions of specific gene(s)/protein(s) (in our case, dioxins), exerting adverse effects by altering functions of p27^Kip1.

Environmental pollutants | cerebral cortex | development | neuronal progenitor cells | cell cycle
---|---|---|---|---
Dioxins are ubiquitous environmental pollutants that have been known to disturb hormonal homeostasis in mammals (1–3). In utero exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), one of the most potent dioxins, has been shown to cause impaired spatial learning and memory in rats (4). Furthermore, in humans, it is reported that children born to mothers who are exposed to dioxin analogs polychlorinated dibenzofurans (PCDFs) or polychlorinated biphenyls (PCBs) have developmental impairments in higher cognitive functions (2, 5). Additionally, a recent report describes the relationship between prenatal exposure level of polycyclic aromatic hydrocarbons and child intelligence at 5 y of age (6). However, the mechanisms by which dioxin exposure in utero affects the higher cortical functions after birth remain undetermined.

Non-GABAergic projection neurons, accounting for 80% of the neocortical neurons, are produced by proliferation/differentiation of neuronal progenitor cells (NPCs) constituting the pseudostatified ventricular epithelium [PVE; roughly coexistent with the ventricular zone (VZ)] along the lateral ventricular surface of the embryonic forebrain. The term PVE has been adopted, because it excludes postmitotic, premigratory neuroblasts of the subventricular zone (7, 8). In mice, the NPCs undergo 11 cell divisions during the period of neocortical histogenesis, with the length of the cell cycle (T_C) increasing by 2-fold from 8 to 18 h, mainly because of prolongation of the G1 phase of the cell cycle (T_G1) (8). During the same period, the proportion of daughter cells that become postmitotic during each cell cycle [quiescent (Q) fraction] increases (9). It is of critical importance that the layer position of the non-GABAergic projection neurons is strongly correlated with the cell cycle of origin (that is, the cell cycle at which a given non-GABAergic neuron becomes mitotically quiescent and starts radial migration to the neocortex) (10). Taken together, the regulated patterns of increase of the T_G1 and Q fractions and strict correlation between the layer position and the cell cycle of origin both strongly suggest a link between cell-cycle regulation of the G1 phase and neuronal cell-class determination (layer destination of projection neurons) (10).

Progression of the cell cycle is precisely controlled by a set of proteins including cyclins, cyclin dependent kinases (CDKs), and CDK inhibitors (11). p27^Kip1, one of the CDK inhibitors, specifically inhibits the activity of cyclin E/CDK2 kinase and inhibits entry of the cells into the S phase (12, 13). Indeed, some of the critical events during the G1 phase of the cell cycle in NPCs are regulated by p27^Kip1: alterations in p27^Kip1 expression in the NPCs result in changes in the Q fraction, thereby altering the number of neurons to be produced and hence, the thickness of the neocortex. Specifically, overexpression of p27^Kip1 in NPCs in vivo increases the Q fraction (that is, promotes differentiation of the NPCs), with a resultant thinner neocortex (14, 15), whereas the lack of p27^Kip1 decreases the Q fraction, resulting in a thicker neocortex (16). It is worthy of note in this context that TCDD has been reported to induce p27^Kip1 and delay the G1 phase of the cell cycle in a hepatoma cell line, fetal thymocytes, and human neuronal cell line (17). Taken together, these observations suggest that in utero exposure to TCDD is likely to alter the proliferative behaviors of the NPCs by inducing p27^Kip1 protein expression, resulting in abnormalities of neocortical histogenesis.

Here, we report that in utero exposure to TCDD indeed modified the p27^Kip1 activities in NPCs to cause neocortical dysgenesis. These observations can be explained by an hypothetical mathematical model where both the increase in Q fraction and the neuronal class switch occur prematurely compared with that under physiological conditions.

Results
TCDD Exposure in Utero Reduced the Size of the Telencephalon and Thickness of the Neocortex as Assessed on Postnatal Day 21. The telencephalon, olfactory bulb, and cerebellum of TCDD-treated mice showed a normal appearance on postnatal day (P) 21 (Fig. 14). However, the forebrains in these TCDD-treated animals were smaller, with the width and length being reduced by 4.78% (9.48 ± 0.063 mm vs. 9.93 ± 0.030 mm in the controls; P < 0.001, n = 10) and 2.29% (8.37 ± 0.048 vs. 8.57 ± 0.035 mm; P = 0.004, n = 10), respectively, compared with the values in the controls. The reductions in the width and length of the telencephalon indicate that TCDD exposure in utero caused roughly 7% reduction of the neocortical surface area. The thickness of the primary somatosensory cortex was reduced by 14.9% (750 ± 29.2 vs. 881.3 ± 13.2 μm; P < 0.001, n = 6) (Fig. 1B); the thickness of the deeper...
cortical layers (layers V–VI) was reduced by 24.1% (366.7 ± 16.67 μm; *P = 0.005, *n = 6), whereas no significant change in the thickness of the superficial layers (layers I–IV) was noted (241.7 ± 9.24 μm vs. 235.6 ± 6.14 μm; *P = 0.38, *n = 6) (Fig. 1C).

TCDD Exposure in Utero Reduced the Number of Non-GABAergic Neurons in the Deeper Cortical Layers as Assessed on Postnatal Day 21. We identified non-GABAergic projection neurons by the lack of positive immunohistochemical staining of the cells with anti-GABA antibody (Fig. 1B and D). The number of non-GABAergic projection neurons in the primary somatosensory neocortex on P21 was significantly reduced in the deeper layers of the TCDD-treated animals by 20.0% compared with that in normal controls (Fig. 1E) (235.6 ± 9.24 vs. 294.3 ± 16.67 μm per 1,000 μm²; *P = 0.037, *n = 3). However, there was no significant difference in the number of non-GABAergic neurons in the superficial layers of the cortex in the TCDD-treated animals compared with that in the controls (Fig. 1E) (149.8 ± 10.9 vs. 166.8 ± 6.14 per 1,000 μm²; *P = 0.246, *n = 3). No alteration in the cell-packing density of the non-GABAergic projection neurons was observed in either the deeper or superficial layers in the TCDD-treated animals. Taken together, we concluded that the reduction in neocortical thickness of the TCDD-treated mice was caused by the reduction in the number of non-GABAergic projection neurons in the deeper cortical layers.

Total Cell Cycle Length of the NPCs Was Not Altered by TCDD Exposure in Utero. Next, we examined the TCDD-treated embryonic forebrains on E12, the time point at which the non-GABAergic neurons of the deeper layers (layers V–VI) are to be produced (10). Histologically, the dorsomedial cerebral wall, the future primary somatosensory neocortex, was normal in the TCDD-treated embryos (Fig. 2A). The S phase zone, where accumulation of the nuclei of the NPCs is observed during the S phase of the cell cycle, in the dorsomedial cerebral wall was located between 60 and 70 μm from the lateral ventricular border on E12 in the TCDD-treated mouse, similar to the finding in the normal control mice (Fig. 2A and B) (*n = 4). At 4 h after exposure to bromodeoxyuridine (BrdU), BrdU-positive nuclei moved to the ventricular surface in both the TCDD-treated and control animals, indicating that the interkinetic nuclear migration in the embryonic forebrain operated normally in the TCDD-treated mice (Fig. 2C) (*n = 3). After 6.5 h exposure to BrdU, virtually all of the nuclei in the VZ were BrdU-positive in both the TCDD-treated and control mice, indicating that the growth fractions in the VZ were nearly equal to 1.0 in both the TCDD-treated and control mice (Fig. 2D) (*n = 3). The results of cumulative BrdU labeling (Fig. 2E) (18) revealed that the total cell-cycle length of the NPCs in the forebrain of the TCDD-treated animals was 10.7 h, not significantly different from that in the controls (Table 1).

TCDD Exposure in Utero Promoted Early Cell Cycle Exit of NPCs. We then identified the NPCs in the Q fraction and P fraction (the fraction of daughter cells that remain proliferative; *P = 1.0 − Q) on E12 by using two S-phase tracers, iododeoxyuridine (IdU) and BrdU (Fig. 2F) (15, 19). In the dorsomedial cerebral wall of the TCDD-treated mice, the IdU-positive nuclei (blue nuclei) were located in the outer margin of the S-phase zone in both the P + Q and Q experiments (Fig. 2F). The distribution patterns of the P + Q and Q cells were not different between the TCDD-treated and control animals (Fig. 2G). An increase in the number of Q cells was observed in the TCDD-treated animals, with an estimated Q fraction of 0.17, which represented a 21.4% increase compared with the value in the controls (Table 2) (*n = 3).

TCDD Exposure in Utero Increased the Nuclear Fraction of the p27kip1 Protein in the NPCs. To elucidate the molecular mechanisms underlying the aforementioned changes, we first investigated the TCDD-induced changes in the mRNA expression levels of cell-cycle regulatory genes in the dorsomedial cerebral wall by dot blot hybridization (Fig. 3A and B) (*n = 5). We observed up-regulation...
Table 1. Lengths of each phase of the cell cycle estimated by cumulative BrdU labeling index

<table>
<thead>
<tr>
<th></th>
<th>Tc - Ts</th>
<th>Tc</th>
<th>Ts</th>
<th>Tg1</th>
<th>Tg2+M</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCDD</td>
<td>7.0</td>
<td>10.7</td>
<td>3.7</td>
<td>5.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Control</td>
<td>6.3</td>
<td>10.5</td>
<td>4.3</td>
<td>4.3</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Tc, length of the total cell cycle; Ts, length of S phase; Tg1, length of G1 phase; Tg2+M, length of G2 + M phase.

Table 2. Q fraction analysis

<table>
<thead>
<tr>
<th></th>
<th>Np+Q</th>
<th>Nq</th>
<th>P fraction</th>
<th>Q fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCDD</td>
<td>14.5</td>
<td>2.6</td>
<td>0.82</td>
<td>0.17</td>
</tr>
<tr>
<td>Control</td>
<td>14.7</td>
<td>2.1</td>
<td>0.86</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Np+Q, number of blue nuclei in the P + Q experiment; Nq, number of blue nuclei in the Q experiment; Q fraction, Nq/Np+q; P fraction, 1 – Q.

of the p27^{kip1} and p15^{ink4b} mRNAs in the E12 forebrain in the TCDD-treated animals compared with the expressions in the controls (Fig. 3B): both p27^{kip1} and p15^{ink4b} are CDK inhibitors that are known to promote exit from the cell cycle (11). We then analyzed the p27^{kip1} and p15^{ink4b} protein levels by immunoblot analysis of lysates of the E12 dorsomedial cerebral walls. The p27^{kip1} and p15^{ink4b} protein levels in the total tissue lysate were not significantly different between the TCDD-treated and control animals. However, when only the nuclear fractions from the dorsomedial cerebral wall were analyzed, the p27^{kip1} protein level was about 2.5-fold higher in the TCDD-treated mice compared with the levels in the controls, the difference being significant (Fig. 3 C and D) (n = 4).

TCDD Exposure in Utero Did Not Reduce the Number of Non-GABAergic Neurons in the Deeper Cortical Layers of the p27^{kip1} Knockout Mice. To further confirm the role of p27^{kip1} in the events associated with TCDD exposure in utero, we repeated the in utero TCDD exposure experiments using p27^{kip1} knockout mice (p27^{−/−}) (20–22). We have previously reported that an increased thickness of the somatosensory neocortex on P21 in p27^{−/−} mice compared with that in the wild-type animals is caused by the overproduction of non-GABAergic projection neurons destined for the superficial neocortical layers (16). Here, neither the layer thickness nor the number in non-GABAergic neurons of the primary somatosensory neocortex on P21 was significantly reduced in the TCDD-treated p27^{−/−} compared with the findings in the controls (i.e., p27^{−/−} mice exposed to corn oil; n = 5) (Fig. 4 B-D).

Discussion

Reduction in the Peak Population Size of NPCs by TCDD Exposure. The neocortical surface area, although influenced by multiple factors such as neuropil expansion and other growth-related parameters, is mostly determined during its ontogeny by the degree of tangential expansion of PVE (9). The size of the PVE, in turn, is virtually exclusively determined by the maximum population size of the NPCs, because dominant constituents of the PVE are the NPCs (9). Thus, the reduction of the neocortical surface area in the TCDD-treated mice (Fig. 1) strongly indicates a decrease in the maximum number of NPCs during neocortical histogenesis. The population size of the NPCs is governed solely by the pattern of ascent of the Q fraction during the early phase of neocortical histogenesis, and the maximum size is reached at the
of note, the progenitor population of the PVE is already committed to their lineage without pluripotency. in fact, our preliminary results indicated that GABAergic neurons and glial cells were also reduced in number, and such reduction was not observed in p27<sup>−/−</sup>. it follows that p27<sup>−/−</sup> is likely to be responsible for determining the size of those neural populations as well. the proliferation/differentiation characteristics of progenitor populations of GABAergic neurons (NPCs of the ganglionic eminence) and glial cells surely deserve further investigation (26).

**Mechanism of Nuclear Accumulation of p27<sup>−/−</sup> After TCDD Exposure.**

The premature increase of the Q fraction, as described in the foregoing paragraphs, is the underlying biological mechanism for the TCDD-induced abnormality of neocortical histogenesis. The increase of the Q fraction seems to be attributable to the nuclear accumulation of p2⁷<sup>−/−</sup>; this hypothesis is lent strong support by the finding that mice lacking the p2⁷<sup>−/−</sup> protein showed almost no alteration of the neocortical thickness after TCDD exposure (Fig. 4). Thus, p2⁷<sup>−/−</sup> may be involved in the cascade of critical events anywhere downstream of the direct effect of TCDD. there has been no report to this date, to the best of our knowledge, on the effect of TCDD on the subcellular localization of the p2⁷<sup>−/−</sup>. the nuclear fraction of p2⁷<sup>−/−</sup> protein is determined by the balance of the protein transportation into and out of the nuclei. However, the expression levels of Jab-1, Akt, and Skp2 proteins, known to be involved in the nuclear transportation and degradation of the p2⁷<sup>−/−</sup> protein, were not found to be altered in the NPCs of the TCDD-treated mice (27–31). Another intriguing observation is the stability of the total cell-cycle length observed, despite TCDD exposure (Table 1). The stabilizing mechanisms of the cell-cycle kinetics shown in *Drosophila melanogaster* (32) may also be involved in the homeostasis of the NPC cell cycle after in utero TCDD exposure.

this report quantitatively evaluates the effects of an environmental pollutant on neocortical histogenesis. in addition, this study is an example of an experimental model where the phenotypic severity of a particular adverse effect of a given environmental substance was found to be dependent on the genotype of the animals, which has profound implications from the viewpoint of toxicogenomics (33). Furthermore, our mathematical model of neocortical histogenesis has been shown to be a powerful tool to examine neocortical dysgenesis after relatively subtle alterations in the decision-making characteristics of the NPCs. we believe that this analytical method would be applicable to various environmental substances that may have adverse effects on human CNS development.

**Materials and Methods**

**TCDD Administration.** TCDD (Cambridge Isotope Laboratory) was dissolved in corn oil at a concentration of 2 μg mL<sup>−1</sup>. a single dose of TCDD solution, or corn oil {0.01 mL [gram body weight (g bw)]<sup>−1</sup>} as control, was administered orally to pregnant c57Bl/6N and p27<sup>−/−</sup> mice on E7 using a disposable feeding needle. the total dose of TCDD administered was 20 μg (kg bw)<sup>−1</sup>. this dose was adopted, because it was expected not to affect the mother in terms of child-rearing behavior. in fact, no adverse effect was observed during pregnancy and the postpartum period.

**Measurement of the Dimensions of the Telencephalon.** Brains from either TCDD- or corn oil-exposed mice on P21 were fixed in 4% phosphate-buffered formaldehyde containing 0.5% glutaraldehyde by transcardiac perfusion. the length and width of 10 telencephalons obtained from either TCDD- or corn oil-exposed P21 mice embryos were measured with micrometer calipers.

**GABA Immunohistochemistry, Cumulative BrdU Labeling Analysis, and Q Fraction Analysis.** GABA immunohistochemistry was performed as described previously using anti-GABA antibody (Chemicon International) (16). Cumulative BrdU labeling analysis (18) and Q fraction analysis (9, 15, 34) were performed as previously described. Detailed methods are described in SI Materials and Methods.
mRNA Expression Analysis and Immunoblot Analysis. Total RNA was isolated from E12 cerebral walls using the RNeasy Protect kit (Qiagen), and the mRNA was purified using the MicroPolyA Pure kit (Ambion) for generating bio-
inated cDNA probes. The biotinylated probes were hybridized to cDNA
expression arrays (GE array Q series Mouse Cell Cycle Gene Array; Superarray).
Immunoblot analyses were conducted using anti-p27Kip1, p15INK4b, cyclin E,
Skp2, β-actin (Santa Cruz Biotechnology), cyclin D1, CDK2, CDK4 (Sigma),
AKT (Cell Signaling Technology), and Jab-1 (GeneTex) antibodies. Detailed
methods are described in SI Materials and Methods.

ACKNOWLEDGMENTS. p27Kip1 knockout mice were provided by Nippon
Roche KK, which were generated by Nakayama K, et al. (20). We acknowl-
edge the assistance of Ms. H. Zaha and the discussions that we held with
Drs. C. Tohyama and T. Goto during the preparation of this manuscript. This
work was supported by Grant-in-Aid for Young Scientists (B) of the Ministry
of Education, Culture, Sports, Science and Technology of Japan (17790723,
20790744, and 22791001 to T.M.) and Grant-in-Aid for Scientific Research (B)
of Japan Society for the Promotion of Science (JSPS) (15390327, 18390302,
and 20390299 to T.T.) and the 21st century Center of Excellence program of
JSPS.
Supporting Information

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SI Materials and Methods

Animal Maintenance. C57BL/6N and p27\(^{\text{Kip1}}\) knockout mice (p27\(^{+/−}\)) were housed in an animal facility maintained under a 12-h light-dark schedule. Conception was ascertained by the presence of a vaginal plug corresponding to embryonic day 0 (E0). p27\(^{−/−}\) mice were routinely genotyped by PCR using WT5 (5′- CCT GGA GCG GAT GGA CGC CAG ACA -3′) and WT3 (5′- CAC CAA ATG CCG GTC CTC AGA GTT -3′) primers for detecting wild-type p27\(^{\text{Kip1}}\) and KOS (5′- GGTT CCT TAG AAA TAG AGA ATG -3′) and KO3 (5′- ATG CCT CAG ACT GGC TTG GGA AAA -3′) for detecting knockout gene. p27\(^{−/−}\) females were crossed by breeding heterozygous (p27\(^{+/−}\)) males.

2.3.7,8-Tetrachlorodibenz-p-dioxin Administration. 2.3.7,8-Tetrachlorodibenz-p-dioxin (TCDD) and the TCDD-treated mice were handled exclusively at an approved facility at the National Institute for Environmental Studies, Tsukuba, Japan, to avoid the confounding effects of environmental contamination on the results.

GABA Immunohistochemistry. Fixed postnatal day (P) 21 brains were embedded in paraffin and sectioned coronally into 4-μm sections with a rotary microtome. GABA immunohistochemistry was performed as described previously using anti-GABA antibody (Chemicon International) (1). The sections were counterstained with cresyl violet. The numbers of GABA-positive and GABA-negative cells were counted in each bin (that is, in sections measuring 250 μm in width and 25 μm in height, lined serially from the pial surface to the white matter in the primary somatosensory area of the neocortex). Gial and non-GABAergic cells were distinguished by their morphological characteristics as described previously (1). Three nonadjacent sections from three brains were analyzed.

Cumulative Labeling Analysis. Pregnant mice were administered bromodeoxyuridine (BrdU) [50 μg body weight (bw)]\(^{−1}\), Sigma] by i.p. injection every 3 h starting from 9:00 AM on E12. The mice were exposed to BrdU for 2, 4, and 6.5 h before killed. Embryonic forebrains were immersion-fixed in 70% ethanol and embedded in paraffin and sectioned coronally at 4 μm; the sections were processed for immunohistochemistry using anti-BrdU antibody (Becton Dickinson) as described previously (2). The sections were counterstained with basic fuchsin. The BrdU labeling index (LI), the ratio of the number of BrdU-labeled nuclei to the total number of nuclei, was used as the analytical index. Analysis was conducted at the dorsomedial cerebral wall in a sector measuring 100 μm in length in the coronal plane. Nuclei of endothelial cells were not counted. The average LI was calculated for each bin across a series of three to four brains obtained from the embryos of two to three litters. The number of nuclei counted for this report was ~80,000.

Q Fraction Analysis. Cells in the Q fraction were identified using two S-phase tracers, iododeoxyuridine (IdU) and BrdU (Sigma), as previously described (3–5). Briefly, pregnant C57BL/6N mice exposed to TCDD or corn oil were administered IdU [50 μg (g dw)]\(^{−1}\] by i.p. injection at 7:00 AM on E12. At 9:00 AM, the animals were divided into two groups: (i) one group in which BrdU [50 μg (g dw)]\(^{−1}\] injection was administered every 3 h and then, the mice were killed 7 h later (Q experiment), and (ii) a second group in which a single BrdU injection was administered followed by killing of the animals 7 h afterward (P + Q experiment). Both sets of forebrains were fixed in 4% phosphate-buffered formaldehyde, embedded in paraffin, sectioned serially into 4-μm sections, and double-immunostained with anti-IdU/BrdU (Becton Dickinson) and anti-BrdU antibodies (AbD Serotec). The number of IdU-positive/BrdU-negative blue nuclei was counted in the dorsomedial ventricular zone (VZ) of six nonadjacent sections from three brains obtained from the embryos of two to three litters.

Expression of Cell Cycle Regulatory Genes. E12 cerebral walls of animals exposed to either TCDD or corn oil were carefully dissected under a dissecting microscope using microscissors and snap-frozen in liquid nitrogen. Total RNA was isolated using the RNeasy Protect kit (Qiagen), and the mRNA was purified using the MicroPolyA Pure kit (Ambion) for generating biotinylated cDNA probes. The biotinylated probes were hybridized to cDNA expression arrays (GE array Q series Mouse Cell Cycle Gene Array; Superarray) in accordance with the manufacturer’s protocol. Signal intensities were quantified by Scanalyze 2 imaging software (Michael B. Eisen, University of California, Berkeley, CA). Signal data for all genes were analyzed and consolidated using the GE Array Analyzer software (Superarray). In each experiment, the signal intensity was subtracted from the background signals and normalized by comparison with the signal intensity of β-actin. The results from experiment conducted in triplicate were analyzed.

Immunoblot Analysis. Snap-frozen E12 cerebral walls were initially washed in ice cold PBS with a proteinase inhibitor mixture (Complete Mini; Roche) and homogenized manually and mechanically by pellet/pestle in ice-cold lysis buffer not containing detergent (10 mM Hepes-KOH, pH 7.8, 10 mM KCl, 0.1 mM EDTA, pH 8.0, 1 mM DTT, Complete Mini as proteinase inhibitor). To optimize the destruction levels of the cellular membrane, samples were stained with H&E and examined under an optical microscope. After centrifugation and removal of the supernatant, lysis buffer with 0.1% Nonidet P-40 (Sigma) was applied to the nuclear fraction. Genomic DNA was fragmented using an ultrasonic sonicator, and SDS/PAGE was performed using 3 μg nuclear protein. Separated proteins were blotted onto supported nitrocellulose membranes using a semidry blotting apparatus (BioRad). Immunoblot analyses were conducted using anti-p27\(^{\text{Kip1}}\), anti-p15\(^{\text{INK4b}}\), anti-cyclin E, anti-Skp2, anti-β-actin (Santa Cruz Biotechnology), anti-cyclin D1, anti-CDK2, anti-CDK4 (Sigma), anti-AKT (Cell Signaling Technology), and anti-Jab-1 (GeneTex) antibodies. The signal intensity of p27\(^{\text{Kip1}}\) was quantified using the Scion Image imaging software (Scion). In each experiment, the signal intensities were standardized by comparison with the intensities of β-actin. The results of experiments conducted in triplicate were analyzed.

Statistical Analysis. All experiments were performed a minimum of three times. Statistical comparison of the datasets was performed by a two-tailed paired Student t test using Microsoft Excel 2003 software.


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