Effects of Restrained Fetal Movement on the Development of the Rat Hip Joint

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ABSTRACT The effect of fetal movement on the development of the hip joint was examined by restraining the leg movement using exo utero operation in rat fetuses. At embryonic day (E) 16.5, when the hip joint cavity starts to form, one side of the hind limb was sutured onto the embryonic membrane. After exo utero development to E18.5, the hip joint of the operated side was compared morphologically with those of the unoperated side, sham-operated and unoperated in utero controls. The largest diameter of the femoral head (FH) and the gross morphology of the joint cavity of the operated side was not different from those of controls. By light microscopy (LM), the surface of the control E18.5 FH and acetabulum was smooth and covered by thin scale-shaped cells which were partially pyknotic, and oval-shaped cells underneath were rather regularly arranged in a thickness of a few cells. By transmission electron microscopy (TEM), scale-shaped cells covered the FH surface incompletely, and the spaces in-between were filled with the intercellular matrix. Oval-shaped cells underneath had well developed rough endoplasmic reticulums. By scanning electron microscopy (SEM), mounds and grooves on the FH surface were evident at E17.0 but became unclear at E18.5. Subsurface collagen fibers formed a coarse meshwork at E17.0 but formed bundles at E18.5. On the operated side, by LM, the surface of the FH and acetabulum was irregular and lined by spindle-shaped cells, whereas the underlying mesenchymal cells also showed an irregular cell shape and arrangement. By TEM, collagen fibrils in intercellular spaces were dense but did not generally form bundles. By SEM, the FH surface was rugged with banks and subsurface collagen fibers did not form bundles but remained as a dense meshwork. These results suggest that the fetal hind limb movement influences development of the surface structure of the FH and acetabulum and that this system may be useful to study prenatal etiology of the congenital dislocation of the

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Abbreviations: CDH, congenital dislocation of the hip; FH, femoral head; LDFH, largest diameter of the femoral head.
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Congenital dislocation of the hip (CDH) is one of the most common congenital skeletal deformities. The prevalent type which makes up 98% of the cases of CDH is represented at birth by a dysplasia of the hip consisting of a flat acetabular roof and an underdeveloped proximal end of the femur, i.e., by rather slight anomalies that predispose to dislocation after birth (Warkany, 1971). The mechanism(s) of the delayed prenatal development of the hip joint in patients of CDH still remain unclear. In addition to systemic growth disturbance, intra-uterine mechanical influences have been considered to be possible factors (Silence, 1992). In our previous study on the development of the hip joint in human embryos and fetuses, we observed morphological maturation of the surface of the femoral head (FH) in the fetuses from crown-rump length (CRL) 58 mm to 168 mm (Kihara et al., 1998). Although this result may indicate that the development of the hip joint depends on the genetic program, most of the movements of human fetuses described during the course of gestation have already emerged before 16 weeks (Pillai and James, 1990), which is well before the gestational period for CRL 168 mm (Nishimura, 1983). Furthermore, movements of the lower extremity are relatively active (Kozuma, 1991). Therefore, it is possible that fetal movements influence the development of the hip joint and thus the disturbance of the articular movements of the hip joint by various causes may be involved in the etiology of CDH.

Making a model animal of CDH is helpful for clarification of the etiology and development of the prevention and treatment. Sijbrandij (1965) reported on the effect of immobilizing the hip joint in post-natal rats for the dislocation of the hip joint. They described that progressive acetabular dysplasia and anatomical abnormalities of the head and neck of the femur occurred during the post-natal period. However, there has been no systematic experimental study to our knowledge that examined the specific effect of the disturbance of fetal movement of the lower limb on the development of the hip joint.

In the present study, we studied the effects of fetal movement of the hind limb on the formation of the hip joint in rats, and in particular, the development of the joint cavity and FH during the prenatal period. We restricted movements of the hind limb of rat fetuses and observed the effects using a technique of *exo utero* development (Muneoka et al., 1986; Hatta et al., 1994a, 1994b; Sekimoto et al., 1997; Zhang et al., 1998). We tied the one-sided hind limb onto the embryonic membrane to restrict the range of motion at the hip joint and compared the development of the operated hip joint with that of the unoperated or sham-operated controls. This study revealed that leg movement influences the development of the surface of the FH and acetabulum and suggested that the operated rat fetuses may be useful in investigating the involvement of mechanical stress as a prenatal etiology of CDH.

**MATERIALS AND METHODS**

**Morphological analysis of the normal development of the hip joint in rats**

Animals used in this study were Jcl: Wistar white rats (CLEA Japan Inc., Tokyo, 9–15 weeks of age) and were maintained at the Institute of Experimental Animals of Shimane Medical University in accord with the institutional guidelines. An estrous female was placed in the same cage with a potent male at 9:00 p.m., and we
checked for a vaginal plug (VP) every hour from 10:00 p.m. until 2:00 a.m. and defined embryonic day 0 (E0.0) when we found a VP. The females were sacrificed by overdose of ether anesthesia and rat fetuses were obtained between E16.0 and 18.5 every 12 hours. Subsequently, body weight (BW) and CRL of fetuses were measured and the hind limbs of the one side were fixed for observation by light microscopy (LM) with 10% formalin neutral buffer solution. The hind limbs of the other side were fixed for electron microscopic observation with a mixture of 2.5% glutaraldehyde (GA) and 2% paraformaldehyde (PA) in 0.1 M phosphate buffer (PB) (pH 7.4). The formalin fixed specimens were embedded in paraffin and serial sagittal sections were made. Sections were stained with hematoxylin and eosin and observed under a light microscope (LM). The largest diameter of the FH (LDFH) was measured on the largest section for each specimen.

Parts of the specimens fixed with 2.5% GA and 2% PA were decalcified and embedded in epoxy resin for transmission electron microscopy (TEM). Ultra-thin (60 nm) sections were made and observed with a JEOL JEM 1200 EX TEM. The other parts of the FH for scanning electron microscopy (SEM) were cut into halves. The one halves of the FH were prepared for conventional SEM observation (Otani et al., 1993; Hirotani and Ito, 1975). The specimens were postfixed with buffered 1% osmium tetroxide (OsO₄) at 4°C and stained with 1% tannic acid solution followed by buffered 1% OsO₄. They were dehydrated in a graded series of ethanol, dried with a Hitachi HCP-2, coated with Pt-Pd using an Eiko VX-10A and observed with a Hitachi S-800 SEM at 15 kV. The other halves of the FH were digested with 2 N sodium hydroxide (NaOH) at 20°C for 12–48 hours to reveal the subsurface meshwork structure of collagen fibers (Ohtani et al., 1991) of the FH. These specimens were further prepared for SEM observation as described above.

**Fetal operation and exo utero development**

Animals used were the same as above, and a female was housed overnight with a male. When a VP was found the next morning, the noon of that day was designated E0.5. In our pilot histological study of the rat hip joint, the mesenchymal cells became condensed at the region of the future acetabulum and the femur at E16.5 (Fig. 1A). Lacunae came into the interstitial region that consisted of mesenchymal cells and the articular cavity of the hip joint was largely formed at E17.0 (data not shown). We therefore decided to perform the operation at

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**Fig. 1**

*A*: Light micrograph of a sagittal section of the normal hip joint at E16.5. The mesenchymal cells are condensed at the region of the future acetabulum and the femur but the joint cavity has not yet formed. Scale bar is 100 μm.

*B*: Operated fetus (left) and its schematic representation (right) for restriction of the fetal movement of the hip joint.

The arrow on the left panel indicates the forelimb.
E16.5. At E16.5, pregnant rats were anaesthetized with 50 mg/kg body weight pentobarbital for exo utero fetal operation. The uterine wall was cut longitudinally at the side opposite to the entrance of the uterine artery and the site of the placenta to expose the fetuses covered by the embryonic membrane. The hind limb of the one side was banded at the knee joint or more distally to the embryonic membrane with a 9-0 thread for the ophthalmic surgery, whereas the other side was left unoperated. The hind limbs were tied in situ and were not forced into any specific abnormal positions. In some cases, sham-operation was performed. The holes which were made on the embryonic membrane during the operation were closed with hemostatic ox collagen fleece (Alcon, Humacau, Puerto Rico) in order to prevent leakage of amnion. The left panel of Figure 1B shows the tying at the hind limb of an E16.5 rat fetus onto the fetal membrane supported by styptic as schematically drawn in the right panel. Approximately 15 ml of Hanks' solution (37°C) was injected into the peritoneal cavity before the closure of the abdominal wall to prevent the adhesion (Hatta et al., 1994b). At E18.5, the dams were sacrificed with an overdose of diethyl ether and fetuses were obtained. The operated hip joints of E18.5 fetuses were observed with LM, TEM and SEM as described above and compared with those of in utero normal, exo utero unoperated, or exo utero sham-operated fetuses. When unspecified, all of these controls exhibited essentially the same findings in the measurements and morphological analysis.

Statistical analysis
The numbers of samples examined are shown in Tables 1 and 2. All the measured values are described as the mean ± standard deviation and statistically analyzed by Student's t-test.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>CRL, BW and LDFH of normal rat fetuses</th>
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<tbody>
<tr>
<td></td>
<td>Embryonic days</td>
</tr>
<tr>
<td></td>
<td>E16.0</td>
</tr>
<tr>
<td>No. of fetuses</td>
<td>23</td>
</tr>
<tr>
<td>CRL (mm)</td>
<td>13.0±0.80</td>
</tr>
<tr>
<td>BW (g)</td>
<td>0.28±0.02</td>
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<tr>
<td>No. of fetuses</td>
<td>6</td>
</tr>
<tr>
<td>LDFH (mm)</td>
<td>0.26±0.01</td>
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</table>

Values are all mean ± standard deviation.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>CRL, BW and LDFH of the operated rat fetuses after exo utero development</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>No. of fetuses</td>
</tr>
<tr>
<td></td>
<td>Operated at E16.5</td>
</tr>
<tr>
<td>Operated dams</td>
<td>13</td>
</tr>
<tr>
<td>Operated side</td>
<td>13</td>
</tr>
<tr>
<td>Non-operated side</td>
<td>(3)**</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>3</td>
</tr>
</tbody>
</table>

*: LDFH was measured on 11 live fetuses from 3 dams.
Values are all mean ± standard deviation, and there is no significant difference between the groups in each measurement.

( )**: Non-operated side was analyzed on a part of the operated fetuses and therefore these numbers are included in those of the operated side.
**RESULTS**

**Systemic and gross hind limb development in control and operated fetuses**

The results of the measurement of BW, CRL and LDFH during the normal late-prenatal development of rats are summarized in Table 1. BW and CRL increased gradually from E16.0 to E18.5, whereas LDFH increased until E17.5 but it lagged between E17.5 and 18.5. In the experiment of the fetal operation, a total of 36 live fetuses were obtained at E18.5 (Table 2). Although many of the operated hind limbs had defects of the ankle, congestion or a bulla, these abnormalities were all distal to the knee joint and there were not significant differences in the measurement of the FH as shown below. BW and CRL of the operated fetuses (1.25 ± 0.13 g, 20.4 ± 1.10 mm, respectively) were not significantly different from those of normal E18.5 (1.25 ± 0.10 g, 22.0 ± 1.13 mm) and sham-operated fetuses (1.16 ± 0.14 g, 20.0 ± 1.76 mm). The LDFH of the operated hind limbs (0.49 ± 0.05 mm) was the same as those of normal (0.45 ± 0.03 mm), sham-operated (0.44 ± 0.03 mm) or unoperated *exo utero* developed (0.46 ± 0.01 mm) fetuses. These results suggest that the operation does not disturb developmental increase of BW, CRL and LDFH.

**Morphology of the cavity of the hip joint and FH of control and operated fetuses**

In the LM study, the cavity of the hip joint was largely formed at E17.5 (Fig. 2A), and further developed to enclose the neck of the FH at E18.5 (Fig. 2B). In both the control and operated fetuses, the joint cavity developed essentially equally, gross morphology of the FH was not significantly different, and there was no case with apparent dislocation or subluxation in either the control or operated fetuses (Fig. 2 and data not shown). The morphology of the limbs, round ligament, and acetabular shape and angle were not significantly different either between the control and operated fetuses (data not shown). However, we observed significant differences in the surface region of the FH and acetabulum between the control and operated fetuses. In the surface region of the control FH at E17.5 (Fig. 2A), squamous or spindle-shaped cells almost completely covered the joint surface of the FH and two or three layers of spindle or oval-shaped cells were underneath the surface. At E18.5, the surface of the FH and acetabulum became smooth and was incompletely covered by the very flat squamous cells in one or two layers (Fig. 2B). A part of these superficial cells were pyknotic. Underneath the surface of the FH, oval-shaped cells were rather regularly arranged in a thickness of a few cells. In the E18.5 FH of the operated fetuses, the surface layer was irregular and composed of spindle-shaped cells, whereas the underlying mesenchymal cells also showed a relatively irregular cell shape and arrangement (Fig. 2C). Correspondingly, the surface of the acetabulum in the E18.5 operated fetuses was also irregular (Fig. 2C). The sham-operated fetuses showed the surface structure of the FH and acetabulum essentially the same with that of normally developed fetuses (Fig. 2D).

In the TEM observation, spindle-shaped cells covered the surface and occupied the subsurface region of the FH in E17.0 control fetuses (Fig. 3A). These cells were closely located to each other and the intercellular space contained sparse and separate collagen fibers which ran randomly (Fig. 3A). In the control E18.5 fetuses, scale-shaped or squamous cells incompletely covered the surface of the FH. The cartilage matrix filled the intercellular space, overlayed the surface cells and directly contacted with the joint cavity. Beneath these one or two surface squamous cell layers, large oval-shaped cells were located. These cells had well developed rough endoplasmic reticulum suggesting active protein synthesis and were more separated from each other than at E17.0 by the matrix which contained an increasing amount of collagen fibers (Fig. 3B). These surface and subsurface cells had characteristic nuclei with condensed chromatin along the nuclear membrane, which
Fig. 2 Light micrographs of the hip joints in normal unoperated E17.5 (A), E18.5 (B), operated E18.5 (C) and sham-operated E18.5 (D) fetuses. Top panels show gross morphology of the joint and bottom panels show the cell shape and arrangement at the joint surface region in each case. Asterisks indicate the regions which are magnified in lower panels. Although there are not significant differences in the gross morphology in the E18.5 hip joints, the surface region in operated fetuses (C) apparently differs from those of E18.5 controls (B, D) and looks somewhat similar to that of E17.5 (A). See text for the detail. Scale bars are 100 μm in top panels and 50 μm in lower panels.
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Fig. 3 Electron micrographs of the surface region of the FH in normal unoperated E17.0 (A), E18.5 (B) and operated E18.5 (C) fetuses. The right panels show higher magnified views of the parts marked by asterisks in the left panels. Note that the cell shape and arrangement, nuclear morphology and distribution pattern of collagen fibers in the matrix in operated fetuses are different from those of both normal E17.0 and E18.5 fetuses (see text for details). Scale bars are 2 μm.
Fig. 4  Scanning electron micrographs of the FH surface (A, C, E) and subsurface structure of the collagen fibers after digestion of the matrix (B, D, F) in normal unoperated E17.0 (A, B), E18.5 (C, D) and operated E18.5 (E, F) fetuses. The FH surface of the operated fetuses (E) is irregular with unfilled banks and the subsurface collagens (F) remain as a meshwork but does not form bundle- or sheet-like structures which are observed in normal E18.5 fetuses (D). Scale bars are 5 µm (A, C, E) and 2 µm (B, D, F).
together with pyknotic appearance by LM suggest the apoptotic changes in these cells during the normal course of cartilage formation in the FH surface. At E18.5, the collagen fibers tended to distribute unevenly and to form bundles which ran generally parallel to the joint surface (Fig. 3B, data not shown). In the operated E18.5 fetuses, spindle- but sometimes irregular-shaped cells incompletely covered the FH, and two or three oval- or box-shaped cell layers were located under the surface cell layer (Fig. 3C). These cells were more separated from each other than at E17.5, but generally less separated than in the E18.5 controls (Fig. 3C vs. 3A and 3B). The collagen fibers in the matrix were denser than in the E18.5 controls, but generally homogeneously distributed (Fig. 3).

In SEM observation, the FH surface of E17.0 fetuses was rough and grooves ran along the border of the surface cells. Flat mounds on the surface appeared to correspond to the nuclei of surface mesenchymal cells (Fig. 4A). At E18.5, the mounds on the surface became unnoticeable and grooves were unclear. Collagen fibers formed bundles which filled the intercellular space and overlayed the cells (Fig. 4C). The surface of the FH of the operated fetuses was rugged. Various sizes of intercellular banks which were generally wider than in E17.0 but narrower than in E18.5 controls remained unfilled with the collagen fibers (Fig. 4E).

After digestion of the matrix to reveal subsurface meshwork structure of collagen fibers in the FH, there were differences among the three groups: control E17.0, E18.5, and the operated E18.5 fetuses. The collagen fibers ran randomly and separately to form a coarse meshwork in the control E17.0 FH (Fig. 4B). In the control E18.5 FH, fibers attached with each other and formed bundle- or sheet-like structures which were generally parallel to the joint surface (Fig. 4D). In contrast, collagen fibers in the operated E18.5 FH did not form bundle- or sheet-like structures but remained as a random meshwork, although the density of the fibers was higher and consequently the meshwork was finer than in the E17.0 controls (Fig. 4F).

**DISCUSSION**

CDH in children is an important disease to recognize because if it is left untreated it results in hip dysplasia (Walker, 1983). The etiology of CDH has remained unclear, but it may be related to prenatal factors including intrauterine malposition (breech) (Sival et al., 1993; Luterkort and Marsal, 1985), multiple pregnancy, oligoamnios (Sival et al., 1990), ligamentous laxity attributable to either physiologic effects of hormones or a genetic trait (Wilkinson, 1992), and collagen disorder (Silence, 1992), as well as postnatal factors such as abnormal anatomic alignment of the hip, positioning the hip extended and adducted (Wilkinson, 1963). It has been suggested that some prenatal factors of CDH are related to the disturbance of intrauterine movements, especially those of the lower limbs (Silence, 1992). Several studies reported that the experimental dysplasia of the hip joint using postnatal young rabbits and rats was caused by fixation of the hind limbs and the wedge-shaped resection of the acetabulum (Wilkinson, 1963; Sijbrandji 1965; Eronen et al., 1978). However, these experiments were only able to examine the disturbance of movements of the hind limbs as an etiology of dislocation of the hip during the post-weaning period, and the causes of dislocation of the hip during prenatal and weaning periods have remained unknown. Furthermore, the normal prenatal development of the joints has been argued to be driven either by genetic program alone or also modified by intrauterine movements (Mital and Millington, 1971; Persson, 1983). In our previous study (Kihara et al., 1998), we observed morphological maturation of the surface of the FH in human fetuses during mid gestation. However, since fetal movements in human fetuses emerge fairly early in gestation, we could not determine whether this morphological maturation
was caused purely by genetic program or influenced by fetal movements. Therefore, in the present study, we aimed to experimentally examine the involvement of the fetal movements in the normal prenatal development of the hip joint and, furthermore, as one of the prenatal etiologies of CDH.

We first observed the normal development of the hip joint of the rat fetuses between E16.0 and E18.5, during which the hip joint including the joint cavity and the FH are largely formed. Next, we compared the development of the hip joint in fetuses that were operated to restrict the motion of the one side of hind limbs with that in controls. Since it was reported that the movement of the hind limb of rat fetuses was first observed at E16 (Narayanan et al., 1971) and we observed the beginning of the joint cavity formation on that day, we operated on the fetuses at E16.5 and let the fetuses further develop exo utero to just before term to observe the effects (Muneoka et al., 1986; Hatta et al., 1994a, 1994b; Sekimoto et al., 1997; Zhang et al., 1998).

There were no significant differences in CRL, BW, LDFH and gross morphology of the FH and the hip joint cavity between the fetuses operated at E16.5 to develop exo utero until E18.5 and the E18.5 fetuses developed normally in utero as well as the sham-operated exo utero-developed fetuses. These results indicate that the restriction of movements of the hind limb and the following exo utero development up to E18.5 does not affect the systemic development as well as the gross development of the FH and hip joint. However, the operated side of the E18.5 FH showed surface structures significantly different from those of the control E18.5 fetuses.

The surface of the FH of the operated fetuses was more irregular than that of the control fetuses. The surface cells of the FH of the operated fetuses did not change the cell shape to the flat, squamous one with characteristic nuclei which suggest apoptotic changes and is typical of those in the E18.5 controls. Furthermore, the surface and subsurface cells were not separated from each other by the matrix as in the control. However, the rough ER developed equally well in these cells between the operated and control fetuses, suggesting that protein synthesis per se occurs similarly in the operated and control FH. Consequently, the intercellular matrix had denser collagen fibers in the operated than in the control fetuses. Nevertheless, interestingly, the surface of the FH of the operated fetuses was not covered by the collagen fibers and relatively deep intercellular grooves were left unfilled. Furthermore, the subsurface collagen fibers in the operated E18.5 FH remained as a random meshwork albeit finer than in the E17, 5 controls, but did not form bundle- or sheet-like structures which were parallel to the joint surface as in the E18.5 controls. Although a similar orientation of bundles parallel to the surface has also been reported in humans (Robinson and Cameron, 1956; Cameron and Robinson, 1958; Little et al., 1958), it has been controversial as to whether the orientation in the network structure of collagen fibers in the FH is guided by the compression and tension forces to which they are subject, or purely by genetic program (Zarek and Edwards, 1963; Sledge, 1968; Mital and Millington, 1971). The present study showed that the subsurface collagen fibers in the FH of the operated side did not form bundle- or sheet-like structures seen in controls and remained as a random meshwork which are normally observed in younger fetuses. This suggests that fetal movement is indeed involved in the formation of mature organization of collagen fibers in the surface region of the FH.

The present findings thus suggest that the restriction of fetal movement of the hind limb influences prenatal development of the joint surface of the FH and acetabulum as well as the three-dimensional structure of collagen fibers in the matrix. Although disturbed fetal movements appeared to delay the maturity of the chondrocytes and the matrix in the surface of the FH and acetabulum, cell shape and arrangement as well as collagen fiber structure were not simply delayed but, more correctly, abnormal. This, together with the fact that gross development of the hip joint was not affected by the disturbed fetal movements, suggest that the proper development and maturation of the hip joint occurs by cooperative effects between the development driven by
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genetic programs and appropriate modification at least partially by mechanical stress due to fetal movements.

We did not observe any apparent dislocation or subluxation of the hip joint in the present operated fetuses. This may mean that the disturbance of movement of the hind limb alone does not cause overt malformation. In addition to the environmental factors, genetic factors are mixed in the etiology of many cases of CDH, as deduced from twin studies (Idelberger, 1951; Carter and Wilkinson, 1964; Wilkinson, 1992). Genetic predisposition has been considered to be the major factor in the etiology of acetabular dysplasia, being present in 40% of cases of unilateral CDH (Tonnis, 1939). The present operated fetuses of ICR mice have no significant genetic predisposition for CDH. Therefore, the present experimental system may serve as a model which has prenatal environmental but not genetic predisposition. As mentioned above, the FH and acetabulum of the present operated fetuses were not simply delayed in development but exhibited abnormal morphology. Since exo utero-developed fetuses can be born by Caesarean section and fostered (Hatta et al., 1994b), it is thus intriguing to further analyze whether this prenatal abnormality indeed serves as a predisposition for dislocation of the hip by giving birth to the operated fetuses and adding postnatal factors such as malpositioning of the hip joint.

In conclusion, the present fetal operation using exo utero development system caused significant prenatal changes in the surface morphology of the FH and acetabulum and may be beneficial in the examination of the prenatal etiology and pathogenesis of the CDH.

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