

Immunodetection of estrogen receptor in epithelial and stromal tissues of neonatal mouse uterus

(development/hormone/reproductive tract/immunohistochemistry/monoclonal antibodies)

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ABSTRACT The tissue distribution and levels of estrogen receptor in neonatal mouse uterine tissue were determined in epithelial and stromal fractions separated by mild enzymatic treatment. Proteins of the isolated fractions were separated by gel electrophoresis and receptor was detected on immunoblots with monoclonal antibody H-222. Estrogen receptor protein was detectable in samples of reproductive tract tissue from 5- and 10-day-old mice. The level of receptor in 5-day-old animals was lower per unit DNA in epithelial cells than in stroma. Receptor levels were increased in both tissue types after treatment with diethylstilbestrol, but not with progesterone. Receptor protein present in these neonatal tissues was able to bind steroid as evidenced by affinity labeling with tamoxifen aziridine. Immunohistochemistry on sections of uteri from 4- and 10-day-old mice confirmed the biochemical results and indicated lower nuclear straining in epithelial cells than in stromal cells of uteri of 4-day-old mice. These results demonstrated that estrogen receptor protein is present in both epithelium and stroma of the neonatal mouse uterus, but at a higher level in stromal cells.

Exposure to estrogenic agents during development of the reproductive tract in fetal or neonatal life results in the expression of aberrant phenotype and possibly cancer (1). One of the best experimental models for studying these effects is the mouse reproductive tract, in which uterine adenocarcinoma similar to that seen in human subjects can be induced (2). The mechanism for the expression of these lesions is not well understood, although it is clear that hormonally active agents are the prime effectors. Initiation of this carcinogenicity is believed to be a result of the hormonal activity of the compound mediated through a receptor protein mechanism, which results in hypertrophy and hyperplasia of the tissue (3). On the other hand, the event may be independent of the hormonal activity and follow a pattern seen for chemical carcinogens involving metabolic activation and irreversible macromolecular binding. Evidence to argue against the receptor and hormonal activity hypothesis was found from steroid autoradiography studies indicating that epithelial cells of the neonatal reproductive tract lacked estrogen receptors (4). However, these cells exhibit DNA synthesis after exposure to estrogens (5), suggesting that the growth responses in the uterine epithelium of neonatal mice may not be mediated by estrogen receptors. Such findings are important since they counter the hypothesis that the carcinogenicity is an estrogen-specific process governed by receptor binding. Alternatively, some of the estrogen receptor responses such as growth stimulation may not be receptor mediated but may occur through other cellular reactions.

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In an attempt to understand the intracellular mechanisms of estrogen action and specificity toward the onset of toxicity/carcinogenicity in the reproductive tract, we have investigated the cell-specific presence of the estrogen receptor by using monoclonal antibody H-222 to the estrogen receptor protein (6). This approach was taken to eliminate problems that could occur involving detection sensitivity by autoradiographic techniques in certain cell types such as the epithelium. We used two approaches: (i) separation and isolation of uterine epithelial cells and biochemical analysis of receptor protein from these tissues by immunochemistry and ligand affinity labeling, and (ii) immunohistochemical detection of the receptor protein within specific cell types of the neonatal reproductive tract.

MATERIALS AND METHODS

Animals and Tissue Preparation. Mice (CD-1:CRL) were obtained from the National Institute of Environmental Health Sciences breeding colony and weaned just prior to sacrifice or treatment. Animals were killed and reproductive tracts were removed and placed in F-12 medium/Dulbecco's modified Eagle's medium phenol red-free medium. Tracts were rinsed with fresh medium and epithelial and stromal tissues were separated as described (7). Separated tissues were homogenized at 0°C in a glass/Teflon pestle homogenizer with an equal volume of buffer [10 mM Tris-HCl, pH 7.6/1.5 mM EDTA/10% (vol/vol) glycerol/0.4 M KCl] containing soybean trypsin inhibitor (50 µg/ml), 0.25 mM antipain, and 0.25 mM leupeptin. The homogenate was then centrifuged at 4°C at 105,000 × g for 50 min in a 41.1 ti rotor (Beckman). The supernatant was decanted and either used directly for gel electrophoresis or underwent reaction with [³H]tamoxifen aziridine (New England Nuclear) for affinity labeling of estrogen receptor.

Sample Preparation and Gel Electrophoresis. Estrogen receptors were precipitated from samples prepared as described above with 4 vol of cold acetone at -70°C for 30 min. Precipitates were solubilized in electrophoresis sample buffer with mini Dounce homogenizers. Samples were analyzed as described (8) on 9-15% exponential gradient polyacrylamide gels. Affinity labeling with [³H]tamoxifen aziridine (21 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) of estrogen receptor protein has been described (8). Protein concentrations were measured by the Bradford method (9) using rabbit immunoglobulin as the standard. DNA was measured as

Abbreviation: DES, diethylstilbestrol.

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described (10) with calf thymus DNA as a standard. Pure mouse α -fetoprotein was a gift of George Mizejenski (New York State Laboratories, Albany, NY). Gel samples were transferred to nitrocellulose sheets by diffusion blotting (8) and probed with a monoclonal antibody (H-222) to the estrogen receptor, which was a gift from Chris Nolan (Abbott) (6).

Epithelial Cell Isolation. Epithelial cells were isolated as reported (7) with the following modifications. The concentration of trypsin used for tissue separations from 5- and 10-day-old animals was reduced from 0.5% to 0.1%, and the trypsin incubation was terminated by addition of medium containing soybean trypsin inhibitor (10 μ g/ml). The epithelial fragments were separated under a dissecting microscope to eliminate nonepithelial fragments from the preparation. To avoid possible loss of epithelial cells from tissues of the younger mice, the Percoll gradient centrifugation step was eliminated. Fibroblastic cell outgrowth was rare in the epithelial preparations, indicating that they were relatively pure (unpublished observation).

Immunohistochemistry. Uteri were removed from 4-day-old mice and fixed with ice-cold 3.7% formaldehyde (1 vol of 37% formaldehyde solution and 9 vol of 0.1 M phosphate buffer, pH 7.2) for 1 hr. The tissue was washed overnight at 4°C in phosphate-buffered saline (PBS) containing 7.5% sucrose, embedded in O.C.T. compound (Ames, Elkhart, IN) and frozen in acetone on dry ice. Cryostat sections ($\approx 4 \mu$ m thick) were incubated with 1% bovine serum albumin in PBS for 30 min to reduce background staining. Sections were treated with primary antibody of Abbott ER-ICA monoclonal antibody kit (Abbott), which was diluted 1:2 with 2% bovine serum albumin in PBS. After washing in PBS, sections were incubated for 45 min with peroxidase-labeled F(ab')₂ [HRP-F(ab')₂] prepared from sheep IgG antibody to rat immunoglobulin (Amersham). To minimize nonspecific binding, HRP-F(ab')₂ was diluted 50 times with 1% bovine serum albumin in PBS containing (each at 20 μ g/ml) antipain, chymostatin, and leupeptin, and then acetone powder of mouse liver was added to the solution for a final concentration of 50 mg/ml. The mixture was shaken gently overnight at 4°C and centrifuged for 5 min at 10,000 $\times g$. The supernatant was filtered through a 0.22- μ m Millex filter (Millipore) before use.

Tissue sections were next incubated with imidazol-3,3'-diaminobenzidine tetrahydrochloride (DAB) solution according to the method of Straus (11). The DAB reaction was carried out for 10–30 min; then the sections were washed with distilled water, dehydrated with ethanol, cleared with xylene, and mounted in balsam.

RESULTS

Uterine tissues were separated into epithelial cell contents and stromal fractions and analyzed by NaDodSO₄/PAGE and immunoblots were probed with monoclonal antibody H-222 or normal rat serum. Fig. 1 shows autoradiographs of samples of epithelium and stroma from mouse uteri postnatal day 5 (section 1), day 10 (section 2), and day 21 (section 3). Samples of day-5 epithelium and stroma (section 1) each contained estrogen receptor protein recognized by monoclonal antibody H-222 in both tissue compartments. The stromal tissue had a stronger signal even though equal amounts of protein were added, suggesting a lower concentration of receptor in epithelial tissue. A similar pattern was present at day 10 (lane 2) with epithelium apparently containing less receptor than stromal tissue. One day-10, epithelial sample had a specific receptor band at 26 kDa. The accompanying stroma did not show this band. Epithelial cells isolated on day 21 (lane 3) have 65-kDa receptor protein and a small amount of 54-kDa protein recognized by monoclonal antibody H-222.

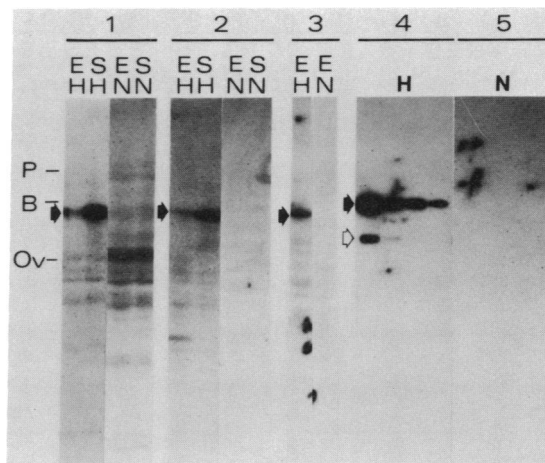


FIG. 1. Gel electrophoretic separation and immunoblot analysis of estrogen receptor from isolated epithelial and stromal cell fractions. Tissue samples are denoted as epithelial (E) and stroma (S) from reproductive tracts from animals of the following ages: day 5 (section 1) containing 300 μ g of protein per gel lane, day 10 (section 2) containing 145 μ g of protein, and day 21 (section 3) containing 250 μ g of protein. Immunoblot analysis of these gels utilized either H-222 estrogen receptor monoclonal antibody (H) or normal rat serum control (N). Section 4 is 24, 12, 6, and 3 ng per lane of partially purified mouse uterine estrogen receptor probed with monoclonal antibody H-222 (H); section 5 is a control blot of the same partially purified receptor preparation probed with normal rat serum (N). Position of the marker protein standards is given on the left: ovalbumin, 45 kDa (Ov); bovine serum albumin, 66 kDa (B); phosphorylase b, 97.4 kDa (P). Solid arrows denote gel position of 65 kDa and open arrow indicates 54-kDa estrogen receptor forms. Results are representative of three separate experiments.

This 54-kDa proteolytic fragment was not seen in either tissue type of the day-5 and day-10 samples. Partially purified mouse uterine estrogen receptor (3–24 ng) was analyzed with monoclonal antibody H-222 (section 4) or normal rat serum (section 5). The pattern seen with the partially purified receptor shows the native form (65 kDa) and a proteolytic fragment at 54 kDa. Serum from an immunized rat used as a control (section 5) illustrates the specificity of the reaction. Sensitivity of ≈ 1 ng of receptor protein can be achieved with this technique (8). It is possible that α -fetoprotein may be present in these neonatal mice. Because α -fetoprotein has a molecular mass similar to that of the estrogen receptor and is also capable of binding steroids (see Fig. 3), we investigated the possible reactivity of the H-222 antibody with α -fetoprotein. Dot blot analysis (data not presented) showed no reactivity of monoclonal antibody H-222 with pure mouse α -fetoprotein, even at a concentration of 10 μ g.

We next determined whether estrogen treatment led to an increase in the level of estrogen receptor in neonatal mice, as reported for adult animals (12). Day-5 and day-10 mice were injected with a 10- μ g/kg dose of diethylstilbestrol (DES) in saline and tissues were removed 18 hr later (Fig. 2). Separated epithelial and stromal tissue from uteri of uninjected (lane 1) and estrogen-treated (lane 2) day-5 mice were analyzed with H-222 antibody or with control serum (lanes 3 and 4). Estrogen treatment led to an increase in the apparent amount of estrogen receptor in the epithelium (lanes 1 and 3). However, the receptor level remained lower in the epithelium than in the stroma. Similar treatment of day-10 mice (lanes 5 and 6) gave a similar pattern. Progesterone treatment (1 mg/kg) had no apparent effect on estrogen receptor levels in either tissue type (lanes 9 and 10).

An affinity-labeling procedure was used to determine whether the protein identified on immunoblots (Figs. 1 and 2) was capable of binding steroid. Tissue samples were affinity-

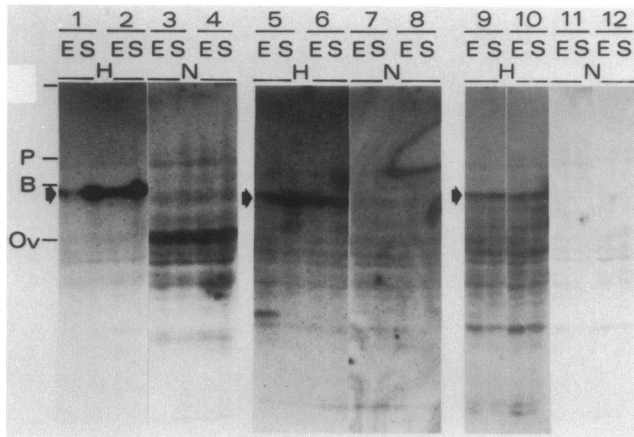


FIG. 2. Estrogen receptor contents in neonatal epithelial and stroma cells after pretreatment with DES or progesterone. Lane 1, contents from 5-day reproductive tract epithelial (E) and stromal (S) cells treated with saline. Lane 2, contents of 5-day epithelial or stromal cells 18 hr after a single dose (10 $\mu\text{g}/\text{kg}$) of DES; protein concentration, 300 μg per sample lane probed with monoclonal antibody H-222 (H). Lanes 3 and 4, control blots of samples in lanes 1 and 2. Lane 5, contents of 10-day reproductive tract epithelial (E) and stromal (S) cells injected with saline; lane 6, same cell fractions from animals 18 hr after dose (10 $\mu\text{g}/\text{kg}$) of DES, protein concentration 145 μg of protein per lane. Lanes 7 and 8, control blots (N) of identical samples in lanes 5 and 6. Lanes 9 and 10, gels of epithelial and stromal reproductive tract cells from 10-day-old mice injected with saline (lane 9) or progesterone (1 mg/kg) (lane 10) and killed 18 hr later; each sample lane contained 180 μg of protein. Lanes 11 and 12, same as lanes 9 and 10 except probed with control normal rat serum (N). Positions of marker proteins shown on left are same as in Fig. 1. Arrows denote position of 65-kDa estrogen receptor protein. Results are representative of two separate experiments.

labeled with [^3H]tamoxifen aziridine proteins separated by NaDodSO₄/PAGE and gels were analyzed by fluorography (Fig. 3). Partially purified receptor was affinity-labeled and analyzed concomitantly at concentrations of 1–16 ng (lanes 9–13). All banding in the partially purified preparations marked by arrows was competitive and displaceable with unlabeled DES. A nondisplaceable band is marked by the asterisk at 42 kDa. The day-5 samples (lanes 1–4) indicate a labeled receptor form (heavy arrow) at 65 kDa in samples from both epithelium and stroma. This receptor form is more abundant in the stroma than in epithelium. These results are similar to those shown by immunoblotting in Fig. 1. The day-5 epithelial samples (lanes 1 and 2) have a large amount of a nonspecifically labeled component at 50–52 kDa. A minor amount of this component is seen in the stromal sample (lanes 3 and 4). Day-10 uterine epithelium and stroma, which were affinity-labeled, are shown in lanes 5–8. Specific nondisplaceable labeled receptor forms are seen in both samples. Interestingly, there are three additional smaller forms seen in both samples. Two forms correspond to proteolytic fragments seen in partially purified estrogen receptor preparations of 54 and 37 kDa (8). The third is similar to the 26-kDa band shown by immunochemical analysis in Fig. 1. It is apparent, particularly in data from day-5 mice shown here and day-4 mice (not presented), that the epithelial samples have a ligand-binding component that could produce a very high nondisplaceable background. Only by electrophoretic separation of the sample proteins can the displaceable specific binding component in the sample be detected.

Immunohistochemistry was used to confirm the biochemical observations by using a modification procedure with the Abbott ER-ICA monoclonal antibody kit. The pattern of immunostaining in uteri from day-4 mice is shown in Fig. 4 (Upper). Strong staining was seen in stromal cells, as ex-

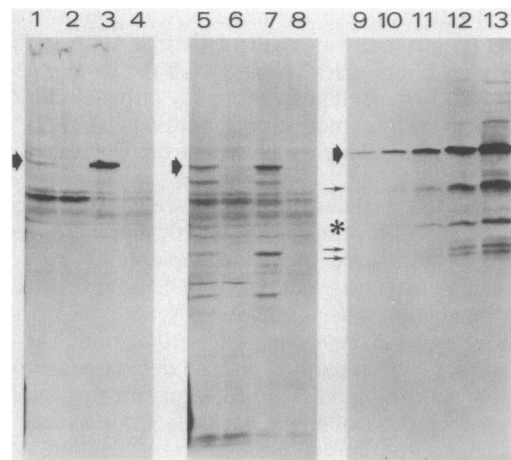


FIG. 3. Tamoxifen aziridine affinity labeling of estrogen receptor of mouse uterine epithelial and stromal cells. Aliquots of 300 μg of protein extracted from 5-day epithelial cells labeled for 3 hr at 4°C as described (8) with 30 nM [^3H]tamoxifen aziridine (lane 1) or with labeled tamoxifen aziridine and 10 μM unlabeled DES (lane 2). Stromal samples from the 5-day-old preparations after labeling with tamoxifen aziridine (lane 3); stromal sample labeled the same as in lane 3 except with addition of 10 μM unlabeled DES. Identical analysis of 10-day-old epithelial (lanes 5 and 6) and stroma (lanes 7 and 8) cell proteins affinity-labeled with tamoxifen aziridine (lanes 5 and 7) and unlabeled DES (lanes 6 and 8). Lanes 9–13, affinity labeling of partially purified estrogen receptor at 0.5, 1, 2, 4, and 8 ng of receptor protein. Asterisk denotes a protein nonspecifically affinity-labeled by tamoxifen aziridine. Heavy arrow marks position of 65-kDa estrogen receptor form and light arrows mark positions of proteolytic fragments as described (8). After electrophoresis, the gels were fixed, treated with EN³HANCE, and dried. Results are representative of two separate experiments.

pected from the biochemical analyses. The staining was uniform throughout the stroma with all cells showing similar localization in nuclei. Compared to the stromal cells, the epithelial cells did not show a uniform immunostaining pattern. The staining in epithelial cell nuclei was random and showed a range of staining intensity that comprised 27% \pm 3% of the epithelial cell population. In all cases, the nuclear epithelial cell staining was less intense than in stromal cells. These data support the biochemical analyses indicating that the epithelial cells have estrogen receptor, but the levels are lower than in stromal cells. In addition, the results demonstrate that the lower levels detected in epithelial cells are not due to a uniform lower estrogen receptor expression in all epithelial cells, but an individual lower or absent expression, thereby resulting in a decreased specific estrogen receptor expression in this cellular fraction.

DISCUSSION

One effect of estrogen treatment results in growth of the reproductive tract. For years this growth response has been considered to be mediated by estrogen binding to its receptor protein (12). A number of tissues possess estrogen receptors but show no growth response, although specific protein stimulation occurs. However, investigation of immature and adult animals indicated that every tissue that responds to estrogen with a growth response possesses estrogen receptors. The exception to these findings was in neonatal reproductive tract tissues, where a growth response was reported in epithelial cells with no detectable presence of estrogen receptor. These studies utilized the steroid autoradiography technique (4).

Our studies utilizing a monoclonal antibody indicate the receptor protein is present in both epithelium and stroma. A

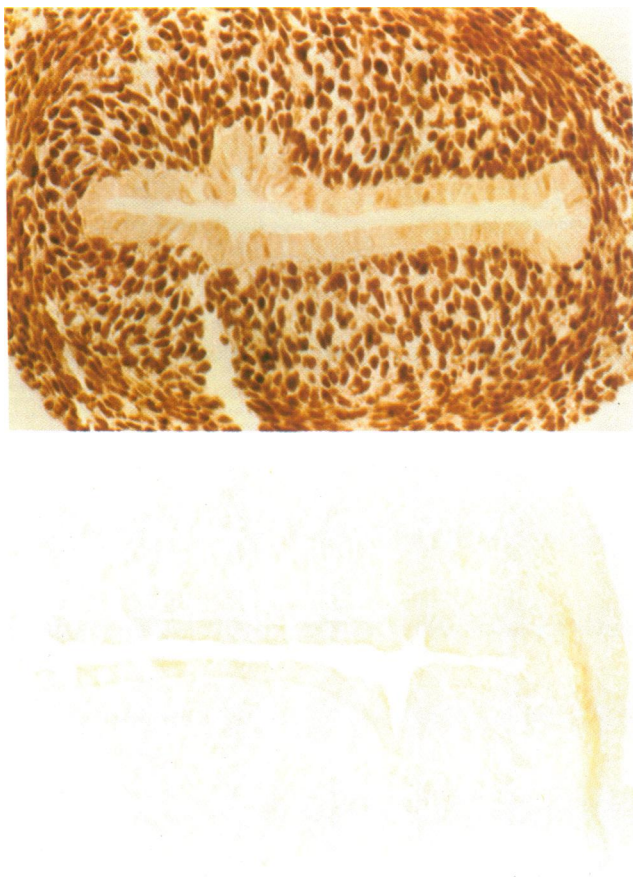


FIG. 4. Tissue localization of estrogen receptor by immunocytochemistry in 4-day mouse reproductive tract tissue. (Upper) Section of reproductive tract after reaction with monoclonal antibody (Abbott ER-ICA monoclonal antibody kit). (Lower) Section of reproductive tract after reaction with control serum. Details and modifications of immunocytochemistry procedure are described in *Materials and Methods*. Results are representative of five independent analyses.

comparison of the detection of receptor proteins between the two techniques of steroid autoradiography and immunocytochemistry is difficult since each detects a different property of the protein. One involves the peptide structure itself; the other involves the ability of the protein to specifically bind the ligand. The detection of the receptor peptide structure by use of a monoclonal antibody is most specific. Results reported in these biochemical studies indicated both properties are present since immunoreactivity was demonstrated as well as ligand binding by the use of affinity labeling. In the report suggesting that epithelium lacks receptors, appreciable uptake of labeled estradiol was seen but not specific displaceable binding (fig. 7 of ref. 4). An explanation for the reported absence of detectable specific ligand binding may be the background labeling seen in the epithelial cells, which could cause problems regarding detection of specific binding. As we point out, only after the affinity-labeled receptor is separated by gel electrophoresis can the competitive binding be detected. Therefore, it is not surprising that such small amounts of displaceable labeling would be missed.

Although the receptor protein may be present in day 4–5 neonate reproductive tract, it may not be functional as far as a hormonal response. Data to support a functional response arise from studies indicating stimulation of epithelial DNA

synthesis (4) at early ages as well as uterine growth, enzyme, and protein stimulation (13). Secondly, results in the current report indicate that treatment of neonates with DES results in an increase in estrogen receptor levels similar to that seen in immature or adult tissues (14). Most interesting of our results were the distribution and levels of estrogen receptor in the epithelium of 4- to 5-day neonates. In the past, biochemical studies have used tissue homogenates and cell mixtures to determine receptor levels under a variety of experimental paradigms. Quantification of estrogen receptor levels and normalization per unit DNA have then been used to estimate the number of sites per cell (3). Such an approach may be misleading in neonates since per unit DNA the levels of estrogen receptor in epithelial cells are lower than stroma. The developmental pattern indicates the amount of estrogen receptor progressively increases, and by 15 days of age, levels in the epithelium and stroma are similar (S.Y., R.R.N., J.A.M., and K.S.K., unpublished data). Moreover, epithelial estrogen receptor expression is advanced by treatment with estrogen and thus is present prematurely in epithelial tissues (R.R.N., S.Y., K.S.K., and J.A.M., unpublished data). Thereby, the continued exposure of fetal or neonatal animals to estrogen, which results in toxicity of the reproductive tract, would result in estrogen receptor expression in affected tissues.

This study illustrates, with development of techniques and reagents such as the estrogen receptor antibody, that past questions regarding receptor protein localization and quantity may require reevaluation to determine whether a small group of cells are present that possess the receptor components. Accordingly, a small group of cells possessing the receptor protein may be the site of origin and govern the target site specificity for the toxicity. This is not unlike earlier studies indicating that the cell of origin for mammary carcinoma is the intermediate cell (15). Only with the advent of a marker for early cellular carcinogenicity and its correlation to the cellular presence of estrogen receptor protein can a direct relationship be developed.

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