Seminiferous Cord Formation and Germ-Cell Programming

Epigenetic Transgenerational Actions of Endocrine Disruptors

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ABSTRACT: The molecular and cellular control of embryonic testis development was investigated through an analysis of the embryonic testis transcriptome to identify potential regulatory factors for male sex determination and testis morphogenesis. One critical factor identified is neurotropin 3 (NT3). At the onset of male sex determination, Sertoli cells initiate differentiation and express NT3 to act as a chemotactic factor for mesonephros cells to migrate and associate with Sertoli-germ cell aggregates to promote cord formation. Promoter analysis suggests that NT3 may be an initial downstream gene to SRY and helps promote testis morphogenesis. Endocrine disruptors were used to potentially interfere with embryonic testis development and further investigate this biological process. The estrogenic pesticide methoxychlor and antiandrogenic fungicide vinclozolin were used. Previous studies have shown that methoxychlor and vinclozolin both interfere with embryonic testis cord formation and cause increased spermatogenic cell apoptosis in the adult testis. Interestingly, transient *in vivo* exposure to endocrine disruptors at the time of male sex determination caused a transgenerational phenotype (F1-F4) of spermatogenic cell apoptosis and subfertility. This apparent epigenetic mechanism involves altered DNA methylation and permanent re-programming of the male germline. A series of genes with altered DNA methylation and imprinting are being identified. Observations reviewed demonstrate that a transient embryonic *in utero* exposure to an endocrine disruptor influences the embryonic testis transcriptome and through epigenetic effects (e.g., DNA methylation) results in abnormal germ-cell differentiation that subsequently influences adult spermatogenic capacity and male fertility, and that this phenotype is transgenerational through the germ-line. The novel observations of transgenerational epigenetic endocrine disruptor actions on male reproduction critically impact the potential hazards of these compounds as environmental toxins. The literature reviewed provides insight into the molecular and cellular control of embryonic testis development, male sex determination, and the programming of the male germ-line.

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INTRODUCTION

Transgenerational Epigenetic Phenomena

Transgenerational effects of irradiation, chemical treatments (e.g., chemotherapy), and environmental toxins such as endocrine disruptors have been observed over the last decade. Most transgenerational observations are simply the effects of the agent on the gestating mother and subsequent actions on the offspring associated with the F1 generation.[1–3] Transgenerational effects for multiple generations have not been as thoroughly studied and will require transmission through the germ-line. In the context of the current review, transgenerational denotes germ-line transmission to multiple generations, minimally the F2 generation. The ability of an external agent to induce a transgenerational effect is through an epigenetic phenomenon involving DNA methylation or stable chromosomal alterations.[4–6] Transgenerational effects of irradiation were the first to be identified, and some have been shown to be transmitted through the germ-line to multiple generations.[4,5,7] These are often associated with mutagenesis and tumor formations in subsequent generations. The treatment of cancer with chemotherapeutics also has been shown to cause transgenerational effects,[8–10] but the impact on multiple generations has not been thoroughly investigated. Recently, nutritional effects on the F1 generation have been observed.[11] Environmental toxins such as endocrine disruptors have also been shown to influence the F1 generation after parental exposure,[9,12–14] but studies have not demonstrated transgenerational effects on multiple generations. However, the potential impact of such transgenerational effects of endocrine disruptors has been discussed.[15]

Epigenetic alterations that lead to transgenerational transmission of specific genetic traits or molecular events (e.g., imprinting) were recently identified.[16–19] These observations have led to the conclusion that re-programming through an altered methylation state of the germ-line is responsible. The impact this has on human health and evolutionary importance is significant.[16,17] Recent investigations of the DNA methylation state of primordial germ cells (PGCs) have indicated that as PGCs migrate down the genital ridge, de-methylation (i.e., erasure of methylation) begins and upon colonization in the early gonad complete de-methylation is achieved.[20–22] This has primarily been observed through analysis of specific imprinted genes.[23] During the period of sex determination in the gonad, germ cells undergo re-methylation involving sex-specific determination of the germ cells. Although de-methylation may not require the gonad somatic cells,[21] re-methylation of the germ-line appears to be dependent on association with the somatic cells in the gonads.[20,22] Due to this unique property of germ cells to undergo de-methylation and re-methylation during the period of sex determination in the developing gonad, the ability of an environmental agent such as an endocrine disruptor to influence through an epigenetic process the germ-line is postulated. This epigenetic effect on the germ-line could re-program the germ cell through an event such as altered DNA

imprinting. This epigenetic effect could cause a transgenerational effect on subsequent generations through the germ-line. Because re-methylation of the germ-line appears to depend upon gonadal somatic cells, an alteration in somatic cell function by an agent such as an endocrine disruptor could indirectly influence germ-cell re-methylation.

Recent observations identify for the first time a transgenerational effect of endocrine disruptors on testis development and adult spermatogenic cells.[24] The endocrine disruptors vinclozolin and methoxychlor have been shown to alter testis morphogenesis at the same time that germ-cell re-methylation occurs. Transient embryonic exposure of the endocrine disruptors at the time of gonadal sex determination promoted an adult testis phenotype of increased spermatogenic cell apoptosis and decreased sperm concentration, and this phenotype was transmitted out to the fourth generation (F1-F4).[24] This transgenerational phenotype was apparently due to altered DNA methylation of a number of genes identified.[24]

Reproductive Toxicology and Endocrine Disruptors

Many reports have suggested that environmental endocrine disruptors, which act to mimic estrogens or act as antiestrogens or antiandrogens, are detrimental to reproduction and may promote abnormalities such as a decrease in sperm count, an increase in testicular cancer,[25,26] and an increase in abnormalities in sex determination in many species.[27] Examples of the environmental endocrine disruptors that have been targeted for adverse effects on the reproductive systems in humans and animals are pesticides (e.g., methoxychlor), fungicides (e.g., vinclozolin), a range of xenoestrogens, and certain phthalates. Most of these chemicals are ubiquitous in the environment, and both humans and animals are exposed to them daily. Many of these compounds and endocrine disruptors can be metabolized into both estrogenic and antiandrogenic activities.[28] Recently, methoxychlor and vinclozolin were used[29–31] as model endocrine disruptors[32] that have both estrogenic and antiandrogenic metabolites.[28]

Many environmental endocrine disruptors are weakly estrogenic and elicit their actions through estrogen receptors. The two mammalian receptors for estrogen (ER-α and ER-β) are widely distributed throughout the reproductive tract and during fetal gonad development.[33,34] ER-β is present in higher concentrations within the fetal testis and ovary, whereas ER-α is present mainly within the uterus.[35,36] During fetal testis development ER-β is first expressed in Sertoli and myoid cells after seminiferous cord formation.[37] In rats, ER-β has also been localized to pre-spermatogonia, which may explain the proliferative actions of estrogen on early postnatal gonocyte cultures.[38] The importance of ER-α was further delineated when knockout mice[39] and human males[40] lacking expression of this gene were found to be sterile. Fetal development of the testis in these experiments was not altered. Early embryonic testis morphology in a double knockout remains to be examined.[41] Neonatal exposure to estrogen alters ER-α and ER-β expression during postnatal testis and hypothalamic/pituitary development.[42,43] Interestingly, neonatal exposure to the estrogenic compound diethylstilbestrol promotes abnormal testis and male reproductive tract development. Therefore, actions of estrogenic endocrine disruptors on estrogen receptors may impair normal fetal gonadal development or stimulate inappropriate differentiation of cells, leading to infertility. Although the estrogen recep-

tors are thought to have a role in testis development,[44–46] the specific functions remain to be elucidated. Treatment of males with estrogens during early fetal life may alter responsiveness to androgens by changing androgen receptor (AR) expression patterns.[47,48]

Antiandrogenic endocrine disruptors can also influence fetal gonad development. AR expression is very similar to ER-β expression in the developing testis.[36,49] However, AR is stage dependent, whereas ER-β expression appears to be more constitutively expressed.[36] AR is detected in Sertoli, myoid, and pre-spermatogonial cells just after cord formation.[50] AR also can be detected in interstitial cells late in development. It is proposed that AR is present in cells that migrate from the mesonephros and enables cord formation to occur.[50] Therefore, inappropriate expression or actions of AR through treatment by endocrine disruptors may affect the process of morphological sex differentiation (cord formation). Antiandrogens such as flutamide[51] or cyproterone acetate (CPA)[52] administered to pregnant rats at different ages of gestation impair fertility in the male offspring. Both flutamide and CPA block the ability of androgens and epidermal growth factor (EGF) to stabilize the wolffian duct.[53] Testosterone has been demonstrated to increase the expression of EGF receptor in the developing testis.[53] Therefore, perturbation of AR may also cause inappropriate expression and action of growth factors in the testis. A commonly used antiandrogenic endocrine disruptor is vinclozolin, which is used as a fungicide in the wine industry.[54,55] Vinclozolin has been shown to act as an environmental antiandrogen and influence gonad development and fertility.

Vinclozolin is an antiandrogenic compound that is metabolized into butenoic acid and enanilide derivatives termed M1 and M2, respectively.[56] The affinity of the metabolites for the androgen receptor is 10–15 times (i.e., Ki 10–100 μM) greater than that of the parent compound.[57] Exposure of neonates to antiandrogenic compounds causes abnormalities in sexual differentiation and gonad formation.[57,58] Peripubertal exposure to antiandrogens delays puberty, inhibits development of androgen-dependent tissues, and alters androgen receptor function in the male rat.[59–61] Embryonic and early postnatal exposure can influence subsequent male sexual differentiation and fertility.[62–65] Embryonic exposure periods at the time of testis formation appear to be the most sensitive exposure period to the antiandrogens.[66] Evidence with a variety of toxic compounds indicates that metabolites of estrogenic substances such as p,p'DDE (metabolite of DDT) act as an antiandrogen and inhibit the transcription of androgen-regulated genes.[67] Interestingly, a recent report suggests the existence of antagonistic and synergistic interactions between vinclozolin and androgens.[68] Therefore, the impact of toxic compounds has become more complicated, and their estrogenic and antiandrogenic effects on reproduction and gonadal development need to be investigated.

An example of an endocrine disruptor with mixed estrogenic and antiandrogenic activity is methoxychlor, which has been used to determine the effects of endocrine disruptors on mammalian reproduction. Methoxychlor is a chlorinated hydrocarbon pesticide currently used in the United States as a replacement for DDT.[69] Methoxychlor can be metabolized by the liver into two demethylated compounds (i.e., mono-OH-M and bis-OH-M). The most active estrogenic metabolite is 2,2-bis-(*p*-hydroxyphenyl)-1, 1, 1-trichloroethane (HPTE).[32,70,71] Other methoxychlor metabolites appear to have antiandrogenic activity.[28] HPTE is weakly estrogenic[72–74] and stimulates the expression of estrogen receptors.[75] Recently it was found that the estro-

genic metabolite of methoxychlor, HPTE, has differential effects on ER-α and ER-β, being an ER-α agonist and ER-β antagonist.[76,77] Other methoxychlor metabolites also have differential effects on ER-α and ER-β.[77] Therefore, in examining the actions of methoxychlor or HPTE, thought must be given to ER agonist and antagonist activity as well as antiandrogenic activities. Consideration of these differential activities is critical in elucidating the mechanisms of action of endocrine disruptors such as methoxychlor. Previously, methoxychlor metabolites were shown to act differentially on the ER from different species.[78] The effects of methoxychlor during embryonic or early postnatal periods can influence reproductive functions at later adult periods.[79–81] Neonatal exposure to methoxychlor can influence pregnancy,[82,83] ovarian and hypothalamic function,[84,85] reproductive behavior,[86] prostate development,[87] thymus development,[88] and testis development.[89] Therefore, transient embryonic exposure to an endocrine disruptor can reprogram and/or imprint effects that become manifest in the adult on reproductive physiology. One study has shown that the effects on a gestating mother may influence subsequent pregnancies as well.[90] In recent studies methoxychlor was utilized as a model endocrine disruptor to investigate the reproductive toxicology of this class of compound,[29,30] and spermatogenic effects were found in adults after transient embryonic exposure.

Testis Cell Biology and Development

The adult testis is a complex organ that is composed of seminiferous tubules, which are enclosed by a surrounding interstitium. The seminiferous tubules are the site of spermatogenesis, where germ cells develop into spermatozoa in close interaction with Sertoli cells. The Sertoli cells[91] form the seminiferous tubules and provide the cytoarchitectural arrangements for the developing germinal cells.[92] Tight junctional complexes between the Sertoli cells contribute to the maintenance of a blood-testis barrier[93] and create a unique environment within the tubule.[94,95] The majority of Sertoli secretory products[96–101] are hormonally regulated and provide useful markers of Sertoli cell differentiation. Surrounding the Sertoli cells are a layer of peritubular myoid cells that function in contraction of the tubule. The peritubular cell surrounds and forms the exterior wall of the seminiferous tubule. Peritubular cells are mesenchymally derived cells that secrete fibronectin[102] and several extracellular matrix components.[103] Both the peritubular and the Sertoli cells form the basement membrane surrounding the seminiferous tubule, and their interactions are important in germ-cell development. The interstitial space around the seminiferous tubules contains another somatic cell type, the Leydig cell, which is responsible for testosterone production. Leydig cells have a major influence on spermatogenesis through the actions of testosterone on both the seminiferous tubule and the pituitary. Although the Leydig cell has numerous secretory products,[104] the ability of the cell to produce androgen to act on the seminiferous tubules is the most significant secretory product of the cell. Leydig cell androgen production can be directly influenced by the actions of the endocrine disruptor methoxychlor and its metabolite HPTE.[105] Therefore, interaction of all three somatic cells, Sertoli, peritubular, and Leydig, is important for regulation of normal spermatogenic function in the testis.[104]

The process of testis morphogenesis occurs late in embryonic development (embryonic day 13 [E13] in the rat). Prior to morphogenesis of the testis the migration of primordial germ cells occurs, first from the yolk sac to the hindgut and then from

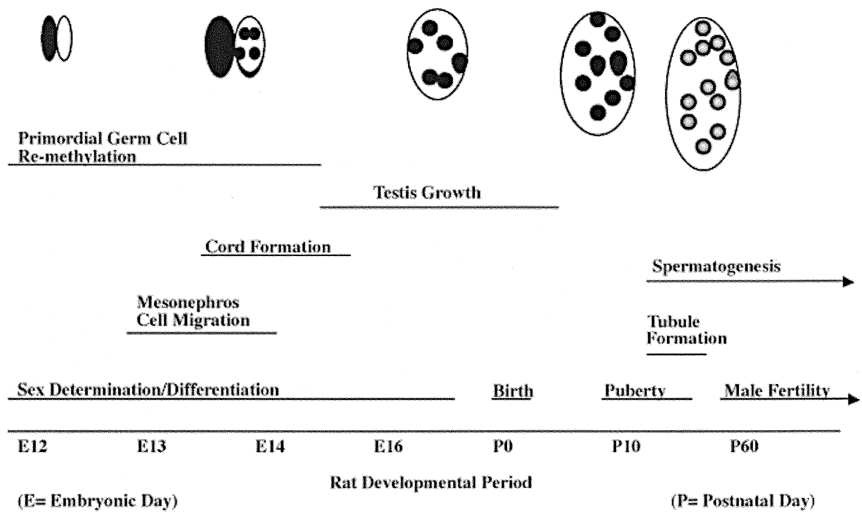

FIGURE 1. Testis development.

the hindgut to the genital ridge (FIG. 1). The gonad is bipotential after germ cell migration and morphologically can be distinguished from the adjoining mesonephros (E12 in rat), but it cannot be identified as an ovary or a testis. Two events occur early on E13 to alter the bipotential gonad. First, Sertoli cells, which are proposed to be the first cells in the testis to differentiate, aggregate around primordial germ cells.[106,107] Secondly, migration of mesenchymal cells occurs from the adjoining mesonephros into the developing gonad to surround the Sertoli cell–germinal cell aggregates. The migrating population of cells are speculated to be pre-peritubular cells.[108,109] The mechanism for this migration is a chemotactic signal from the testis to promote cell migration.[110] Therefore, during early testis development, Sertoli-peritubular cell interactions promote cord formation. The cords neonatally develop into seminiferous cords and at the onset of puberty develop into seminiferous tubules (FIG. 1). Seminiferous cords form as the Sertoli cell–primordial germ-cell aggregates become more organized and are fully surrounded by the migrated mesonephros mesenchymal cells (i.e., pre-peritubular cells). The formation of the seminiferous cords (E14 in the rat) is a critical event in the morphogenesis of the testis because it is the first indication of male sex differentiation. During the process of cord formation, the Sertoli cells undergo morphological changes including: a change in expression of mesenchymal to epithelial cell markers (cytokeratin),[111] a change in expression of cytokeratin 19 to cytokeratin 18 (cytokeratin 19 expressed in the ovary),[112] and expression of MIS, which inhibits the development of the müllerian duct, the precursor of the female uterus, cervix, fallopian tubes, and upper vagina.[113,114] Outside of the seminiferous cords the peritubular layer of cells become identifiable from the interstitium or Leydig cells at E15,[115] and 3βHSD production is detected after E15.[116] This is important because the production of testosterone and androgens by the Leydig cells has been demonstrated to stabilize the wolffian duct

derivatives for normal male duct development.[117] Therefore, appropriate differentiation of somatic cell types in the testis around the time of cord formation is crucial not only for normal development of the testis, but also for the continued presence of the wolffian duct.

Primordial germ cells form aggregates with Sertoli cells prior to cord formation[106,107] and then are localized within the seminiferous cords as testis morphogenesis is initiated (FIG. 1). The germ cells undergo rapid mitosis until the late stages of embryonic development at which time they become quiescent. After birth in the rodent, germ-cell mitosis resumes, and during the onset of puberty and formation of the seminiferous tubules spermatogenesis is initiated (FIG. 1). Germ cells throughout development are in close association with somatic cells (i.e., Sertoli cells). Alteration of somatic cell differentiation could indirectly affect germ-cell development as well as directly affecting the germ cells. Recent studies examined the effects of endocrine disruptors on testis development with a focus on delayed effects on adult spermatogenic cell viability and development (i.e., spermatogenesis).[29–31]

The testis transcriptome (i.e., global gene expression profile) changes during testis development due to differentiation and growth of a variety of different cell types.[118] These changes in the testis transcriptome reflect critical regulatory genes and gene families required to promote normal testis function and development. A variety of functionally related genes such as transcription factors, signal transduction genes, cell cycle genes, cell survival genes, and growth factors will be involved in testis development and part of the transcriptome. Recent observations that endocrine disruptors (i.e., methoxychlor and vinclozolin) affect the testis during development are in part mediated through alterations in the testis transcriptome. The ability of the endocrine disruptor to alter the testis transcriptome and specific genes and gene families is in part one of the mechanisms used to alter fetal and adult testis function and development. Examples of gene families shown to influence embryonic testis development include the epidermal growth factor (EGF) family,[119–121] transforming growth factor-β (TGF-β) family, [122] and neurotropin growth factor family.[123,124]

A previous study demonstrated that a null mutation in a specific gene expressed in the embryonic testis can give the same phenotype as that seen with endocrine disruptor treatment. A mouse knockout model with a null mutation in the trk C receptor for neurotropin 3 (NT3) was used.[124] NT3 is essential for cell migration from the mesonephros and testis morphogenesis. The trk C knockout mouse was analyzed for similar phenotypes as those found with methoxychlor-treated gestating mothers. For trk C knockout animals that survived birth and puberty, testis histology showed a dramatic increase in spermatogenic cell apoptosis[124] and a similar testis phenotype.[30] Therefore, the absence of NT3 actions during embryonic testis development caused a similar phenotype as that of the endocrine disruptor-treated gestating mothers' male offspring.

These previous studies support the concept that altered expression of critical genes in the embryonic testis can cause abnormal testis development and a phenotype similar to that seen with endocrine disruptor treatment of the embryonic testis. This suggests that the affects of endocrine disruptors on the testis transcriptome will likely in part promote the epigenetic effects of methoxychlor on the germ-line and be the causal factor for the transgenerational effects observed.[29]

EPIGENETIC TRANSGENERATIONAL ACTION OF ENDOCRINE DISRUPTORS ON MALE FERTILITY

A recent study using methoxychlor and vinclozolin treatment of gestating mothers showed no major effect on sex determination or gross testis histology throughout development.[29] Animals were exposed *in utero (*E8–E15) and then spermatogenic defects were observed in the pubertal and adult F1 generation (FIG. 1). Treated animals have an increase in spermatogenic cell apoptosis. Similar results are observed at postnatal day 20 or day 60 for both endocrine disruptors. In addition to this decreased spermatogenic cell survival, sperm motility and morphology were also found to be impaired.[29–31] Interestingly, animals exposed *in utero* at E15–E20 (i.e., around birth [E20]) to the same dose of endocrine disruptor had no spermatogenic cell defects.[30,31] Therefore, only exposure during the E8–E15 period of development affected later adult spermatogenesis, and exposure after E15 had no effect on testis development.[29] This correlates with critical processes such as germ cell remethylation, cord formation, and sex determination during the E10–E15 period compared to the growth phase after E15 (FIG. 1). In previous studies of endocrine disruptor actions on the testis using treatment regimens after E15, effects were not seen because the important E10–E15 period was missed. Due to the observation that neither methoxychlor nor vinclozolin transient exposure between E15 and E20 had any effect on postnatal/adult spermatogenesis, the effects observed from E8–E15 are not due to artifacts such as postnatal exposure from milk transfer from exposed mothers during weaning or bedding contamination from the exposed mother because either E8–E15 or E15–E20 would have the same potential transfer. In addition, no effect on total body weight was observed from either endocrine disruptor treatment so that the effects observed are not due to the effects on growth rates. Therefore, the endocrine disruptor effects observed on the F1 generation are due to local effects on testis development and germ-cell maturation during the E8–E15 period and are not a toxic effect in later postnatal development. None of the transgenerational effects described were due to endocrine disruptor toxicity because none of the subsequent gestating mothers or pups were ever exposed; only the F0 gestating mother was exposed. Therefore, the spermatogenic defect observed is due to the effects of the endocrine disruptor on transient embryonic testis development at the time of sex determination and not to exposure artifacts or nonspecific toxicity.[29–31]

Interestingly, the same increased apoptosis rate in spermatogenesis was identified in the F2 generation as the F1 generation.[29] A three- to fivefold increase in apoptosis was observed in P20, P60, and P120 male rats. Epididymal sperm were analyzed from P60 and P120 rats, and an approximately 20% decrease in sperm concentration and forward movement was also observed.[24] Similar observations were observed with the F2 generation of vinclozolin-treated animals at both P20 and P60 ages of development.

Analysis of the F3 and F4 generations demonstrated similar effects on spermatogenesis and sperm with a smaller effect in sperm motility with methoxychlor-treated animals.[24] Therefore, exposure of a gestating mother at the critical time of sex determination and testis morphogenesis (i.e., cord formation) appears to cause a germ-line effect of decreased spermatogenic capacity and decreased sperm viability that is transgenerational in the male. This experiment has now been repeated with numerous different gestating mothers with similar results.[29] An outcross of an

affected treated male (F2 generation) with a wild-type untreated female (VOC) also had reduced sperm motility[29] and increased spermatogenic cell apoptosis, whereas a reverse outcross (RVOC) of a treated female (F2 generation) with a wild-type male had no effect.[29] It is currently speculated that transient exposure at the critical time of testis development is crucial and that a phenomenon such as an imprinted methylation state of the germ-line may be involved. Although genetic mutagenesis (i.e., DNA sequence change) could be a factor, the phenotype observed was present in most males from all generations, suggesting a frequency not possible for random mutagenesis, but instead more indicative of an epigenetic factor such as methylation. This intriguing observation of a transgenerational effect of an endocrine disruptor through the male germ-line will be examined mechanistically in the future.

SUMMARY

The observations that an environmental toxin (e.g., endocrine disruptor) can have an epigenetic effect on the germ-line and cause a transgenerational effect on male reproduction have a significant impact on our understanding of the potential hazards of these compounds in human health as well as that of all other mammalian species. These studies establish a novel mechanism of action not previously appreciated on how environmental toxins may act on a gestating mother to influence her grandchildren and subsequent generations. Elucidation of this phenomenon will allow us to better understand the true hazards of environmental toxins, identify the specific causal agents, and develop appropriate preventive and therapeutic approaches. Independent of the specific compound or agent of interest, the establishment of this potential mechanism of action is critical to our insight into the effects of environmental factors that influence embryonic development and adult reproduction. Although the dose of endocrine disruptors used in a recent study[29] is higher than anticipated environmental exposures, the observation that the germ-line can be reprogrammed and induce transgenerational effects is a critical phenomenon to establish and elucidate. The specific toxicology of individual environmental toxins will need to be established.

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