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Chemotactic Role of Neurotropin 3 in the Embryonic Testis That Facilitates Male Sex Determination

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Chemotactic Role of Neurotropin 3 in the Embryonic Testis That Facilitates Male Sex Determination[1]

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ABSTRACT

The first morphological event after initiation of male sex determination is seminiferous cord formation in the embryonic testis. Cord formation requires migration of pre-peritubular myoid cells from the adjacent mesonephros. The embryonic Sertoli cells are the first testicular cells to differentiate and have been shown to express neurotropin-3 (NT3), which can act on high-affinity trkC receptors expressed on migrating mesonephros cells. NT3 expression is elevated in the embryonic testis during the time of seminiferous cord formation. A trkC receptor tyrophostin inhibitor, AG879, was found to inhibit seminiferous cord formation and mesonephros cell migration. Beads containing NT3 were found to directly promote mesonephros cell migration into the gonad. Beads containing other growth factors such as epidermal growth factor (EGF) did not influence cell migration. At male sex determination the SRY gene promotes testis development and the expression of downstream sex differentiation genes such as SOX-9. Inhibition of NT3 actions caused a reduction in the expression of SOX-9. Combined observations suggest that when male sex determination is initiated, the developing Sertoli cells express NT3 as a chemotactic agent for migrating mesonephros cells, which are essential to promote embryonic testis cord formation and influence downstream male sex differentiation.

developmental biology, early development, embryo, Sertoli cells, testis

INTRODUCTION

Embryonic testis differentiation is initiated with the morphological event of seminiferous cord formation [1, 2]. The expression of SRY within the indifferent gonad triggers a cascade of events resulting in testis differentiation [1]. Both SF-1 [3] and SOX-9 [4] are speculated to act in concert with SRY to influence male sex differentiation [5]. Seminiferous cords are formed after migration of pre-peritubular cells from the adjacent mesonephros to enclose primordial germ cell and pre-Sertoli cell aggregates within the indifferent gonad [6, 7]. After seminiferous cord formation Dax-1 expression is down-regulated [8], and SOX-9 expression is up-regulated in Sertoli cells [4, 9].

Migrating mesonephros cells are essential for seminiferous cord formation [10–12]. In the absence of a mesonephros, the indifferent testes do not have the capacity to form seminiferous cords [10]. Migration of mesonephros cells through ovaries toward a testis explant increased the expression of SOX-9 within the ovary and promoted the formation of primitive cord structures [13]. Therefore, the mesonephros cells appear to produce factors that alter both gene expression and the morphology of the differentiating gonad. In particular, the migrating mesonephros cells appear to influence Sertoli cell expression of SOX-9. The male sex determining gene, SRY expression, is necessary for mesonephros cell migration [14]. A hypothesis tested in the current study is that a paracrine growth factor potentially downstream of SRY acts as a chemotactic agent to regulate mesonephros cell migration. Several growth factors have been postulated to be downstream of SRY and important in seminiferous cord formation [15–18]. An example is the observation that fibroblast growth factor-9 (FGF-9) knockout mice were found to have impaired testis differentiation and aberrant mesonephros cell migration [16].

Recent experiments have demonstrated that neurotropins may be a likely candidate for critical events involved in seminiferous cord formation [18–19]. Neurotropins have recently been shown to be present in the embryonic testis at the time of seminiferous cord formation [17–19]. Neurotropin-3 (NT3) is expressed by Sertoli cells at this critical stage of development, whereas the NT3 high-affinity receptor, trkC, is expressed in the migrating mesonephros cells [18, 19]. Although NT3 and trkC are expressed, the brain-derived neurotropin factor (BDNF) and trkB have negligible expression, and nerve growth factor (NGF) and trkA are primarily expressed at later stages of embryonic testis development [18, 19]. TrkC mutant knockout mice demonstrate reductions in the number of seminiferous cords during embryonic testis development [20]. The current study examines the potential functions of NT3 and its receptor during the critical process of mesonephros cell migration and seminiferous cord formation.

MATERIALS AND METHODS

Collection of Embryonic Gonads and Gene Expression Analysis

Gonads were collected from timed pregnant Sprague-Dawley rats obtained from Charles River Laboratories (Wilmington, MA). The plug date was considered to be embryonic Day 0 (E0). The number of tail somites in the embryos were counted to confirm the age of the embryo. Only embryos that had 15–17 tail somites were used for studies in rats at age E13. Gonads were extracted with Tri-reagent (Sigma, St. Louis, MO) to obtain RNA. Cyclophilin (1B15) is a constitutively expressed gene used

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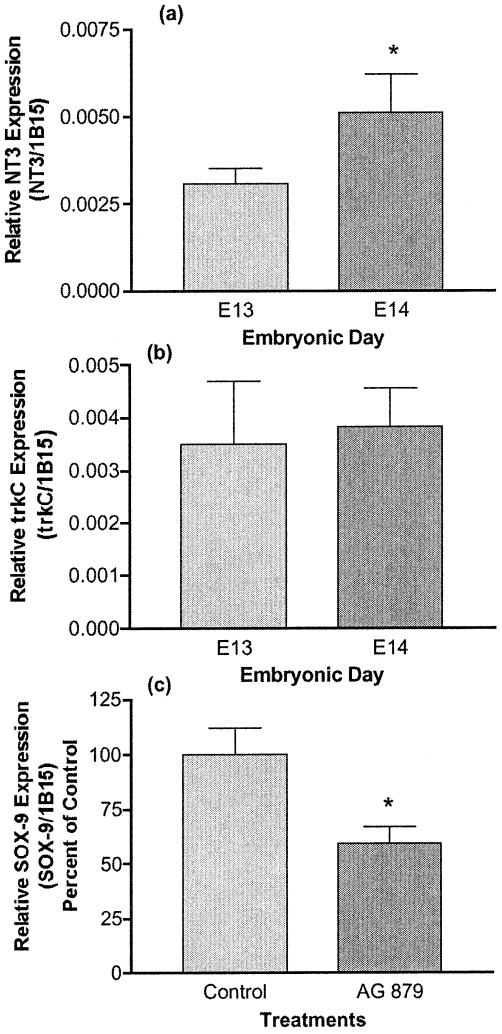

FIG. 1. Quantitative reverse transcriptase-PCR analysis for mRNA levels of (**a**) NT3 and (**b**) trkC in age E13 and E14 testis. Effects of 20 μM tyrophostin inhibitor, AG879, on mRNA levels of (**c**) SOX-9 measured in E13 testis organ cultures. Data are normalized with the constitutively expressed gene cyclophilin (1B15). These data represent approximately 18 testis organ pairs for each observation with the mean ± SEM presented. Statistical difference is indicated by * at $P < 0.05$ with the Student *t*-test.

to normalize the amount of NT3, trkC, and SOX-9 transcripts for each developmental age. A procedure previously reported was used to conduct quantitative polymerase chain reaction (PCR) analysis [21]. The primers used for NT3 were 5′ primer (5′-ATG CAG AGC ATA AGA GTC AC-3′) and 3′ primer (5′-GCC TAC GAG TTT GTT GTT TTC-3′); the primers used for trkC were 5′ primer (5′-CAT GGT TCC AGC TCT CTA ACA CAG-3′) and 3′ primer (5′-ACC AGT CAC CAC TAG CCA AGA ATG-3′); the primers used for SOX-9 were 5′ primer (5′-AGA AAG ACC ACC CCG ATT-3′) and 3′ primer (5′-TGC TGA TGC CGT AAC TGC C-3′).

Embryonic Testis Organ Culture

E13 testes with attached mesonephros were dissected, placed on Millicell CM filters (Millipore, Bedford, MA) under drops of medium floating on the surface of media as previously described [18] and cultured for 72 h. AG879 (20 μM) was used to treat the testis organ cultures. Several doses were used and 20 μM was found to be optimal without having potential pharmacological effects of higher doses. Approximately 100 μM has been used in previous cell culture experiments to inhibit neurotropin signaling in cell lines [22]. Genomic DNA was collected (from embryo tails) and PCR was conducted for SRY [18] to determine the sex of E13 gonads. Organ cultures were digitally imaged and then fixed for histology analysis. Controls for the organ cultures were treated with the same concentration of solvent (i.e., DMSO) used to suspend the compound AG879.

Organ Culture Cell Migration Assays

A cell migration assay was used as previously described [23]. Briefly, the mesonephros were dissected from the gonad, stained with 5(6)-carboxyfluorscein diacetate, succinimidyl ester (CFSE; Molecular Probes, Inc., Eugene, OR), and placed in apposition to the unstained indifferent testis in a drop of media on a filter floating on the surface of 0.4-ml culture medium. The mesonephros and testis aggregate were cultured for 3 days and treated with 20 μM AG879 daily. Cocultured organs were imaged using a confocal microscope following fixation. For the chemotaxis experiment, E13 ovaries were used. The mesonephros was dissected from ovaries and stained as described above. Eight to 10 agarose beads (Cibacron, Sigma; 80–110 μm in diameter) containing NT3 (Amgen Inc., Thousand Oaks, CA) or other proteins were placed on the free side of the ovaries. As controls, BSA (Gibco, Grand Island, NY), or epidermal growth factor (EGF) (Sigma), or FGF-9 containing beads were used. The beads were prepared as previously described [24]. Briefly, beads were washed in 10 volumes of PBS twice and mixed with an equal volume of a 10 μg/ml solution of respective growth factor or BSA. The mixture was incubated for at least 2 h at 4°C prior to use to allow equilibration through diffusion into the beads. Following a 72-h culture, the mesonephros ovary aggregates were fixed and imaged for fluorescently labeled cells in the ovary using a Bio-Rad MRC 1024 laser scanning confocal microscope equipped with krypton/argon laser (Bio-Rad, Hercules CA). The images were obtained by constructing a *z*-series 3-dimensionally using 13–15 optical sections covering 15–20 μm from the most central portion of the organs using LaserSharp software 3.1 (Bio-Rad). In 4 separate experiments, 13 ovary + mesonephros pairs were used.

RESULTS

Previous immunohistochemistry localized NT3 to Sertoli cells during seminiferous cord formation and trkC receptor to the mesonephros and migrating mesonephros cells [18, 19]. Expression of NT3 increased from E13 to E14 (Fig. 1A) in the rat embryonic testis. At this same developmental period, no significant changes in the expression of trkC receptor expression was observed (Fig. 1B). Therefore, NT3 and its receptor are postulated to be important in initiating mesonephros cell migration to form seminiferous cords during early testis morphogenesis.

To determine the effects of NT3 and trkC genes on seminiferous cord formation and testis morphogenesis, a tyrophostin inhibitor, AG879, specific for the trk receptors [22], was used in E13 rat testis organ cultures. AG879 completely inhibited seminiferous cord formation in E13 testis organ cultures (Fig. 2, A and B). Therefore, AG879 likely alters the ability of mesonephros cells to migrate by preventing signaling of NT3 through its trk receptor. Migration assays using fluorescently dyed mesonephros cells were developed to determine whether the NT3 inhibitor altered mesonephric cell migration. Mesonephros from indifferent gonads were dissected and stained with a fluorescent dye and then placed in direct opposition to an indifferent testis. In the controls not treated with AG879, fluorescent cells migrated from the mesonephros into the developing testes (Fig. 2, C and E). In contrast, organ cultures treated with AG879 had few (2–3 cells) to no cells that migrated into the testes (Fig. 2, D and F). Observations suggest that inhibition of NT3 actions prevents the migration of mesonephros cells into the gonad.

Alteration of cell migration into the differentiating testis is speculated to inhibit the expression of genes involved in testis determination. Specifically, the expression of SOX-9 is up-regulated after seminiferous cord formation within the Sertoli cells [9]. It is not known what factors cause this increased expression of SOX-9, but cells migrating from the mesonephros correspond with an enhanced SOX-9 expression. Because AG879 treatment inhibited mesonephros cell migration into the testis, it was speculated that SOX-9 expression may be altered. SOX-9 mRNA expression was measured in organ cultures in the absence or presence of

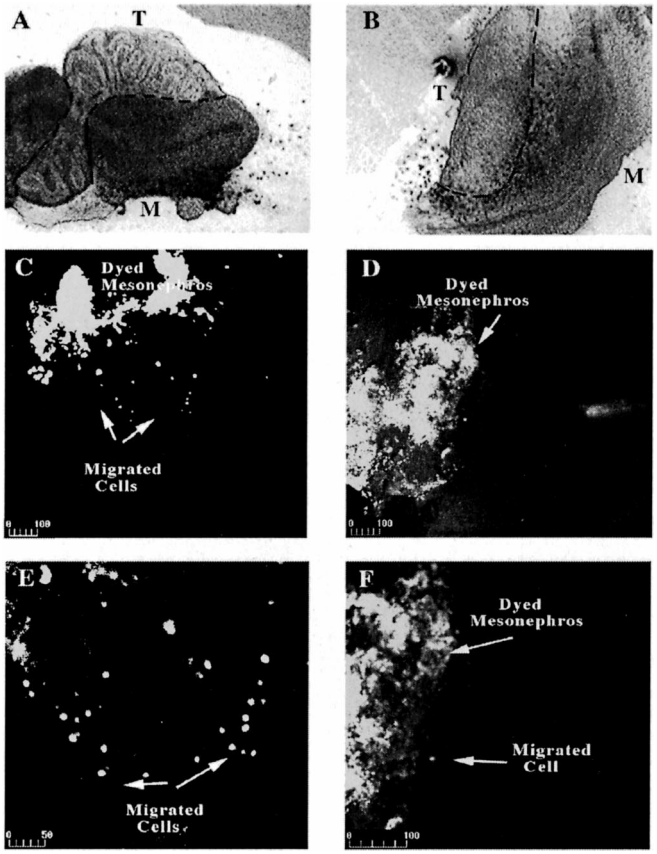

FIG. 2. Morphological analysis of E13 testis organ cultures incubated in the absence (**A**) or presence (**B**) of 20 μM tyrophostin inhibitor, AG879, on seminiferous cord formation. The dashed line indicates the boundary between the testis (T) and mesonephros (M). Effects of (**C**) and (**E**) no treatment or (**D**) and (**F**) 20 μM tyrophostin inhibitor, AG879, on migration of cells from a fluorescent mesonephros into the differentiating testis. These organ cultures represent the results of 36 testis organ pairs for each experiment.

AG879. In E13 organ cultures at 16–17 tail somites treated with AG879, there was a reduction (40%) in the amount of mRNA for SOX-9 compared with that of control testis organ cultures (Fig. 1C). No apparent reduction in Sertoli cell number was observed (data not shown). Inhibition of NT3 actions with AG879 appears to cause a reduction of SOX-9 gene expression in the differentiating testis.

Observations suggest a role for NT3 and its trkC receptor in the regulation of mesonephric cell migration into the differentiating testis during morphological male sex determination. Inhibition of the actions of NT3 resulted in perturbation of seminiferous cord formation, altered expression of SOX-9. A final experiment examined the role of NT3 as a chemotactic factor for the migrating mesonephros cells. Agarose beads containing NT3 or control proteins were placed on the outer surface of an embryonic ovary that had fluorescently dyed adjacent mesonephros. When beads contained a control protein (i.e., BSA) or EGF, no mesonephros cell migration was observed in the ovary (Fig. 3, A and B). When NT3 was placed in the beads, the mesonephros cells migrated into the ovary (Fig. 3C). As previously suggested [16], FGF-9 beads also caused cell migration into the ovary (Fig. 3D). In vivo, no mesonephros cell migration occurs into the embryonic ovary at this developmental period. Histological analysis of ovaries into which NT3 had promoted mesonephros cell migration revealed no cord formation (data not shown). Therefore, NT3 appears to act as a chemotactic factor to promote mesonephros cell migration into the embryonic testis (Fig. 4).

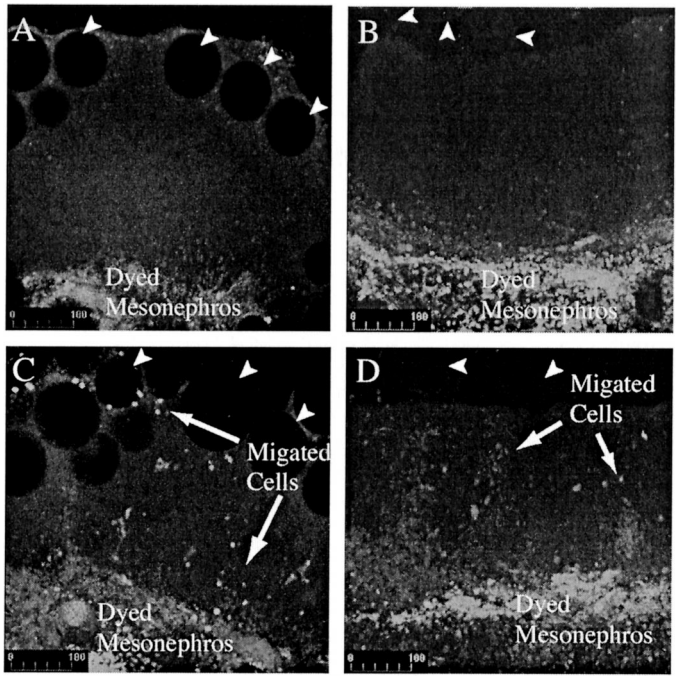

FIG. 3. Effect of agarose beads (arrowheads) containing BSA (**A**), EGF (**B**), NT3 (**C**), or FGF-9 (**D**) on migration of cells from fluorescently stained mesonephros into an E13 ovary. These organs represent 7–13 organ pairs for at least three separate experiments.

DISCUSSION

Previous studies have suggested the testis at the time of male sex determination produces a chemotactic agent to promote mesonephros cell migration into the testis that is required for seminiferous cord formation [1, 2]. This will likely be an early downstream event of SRY and Sertoli

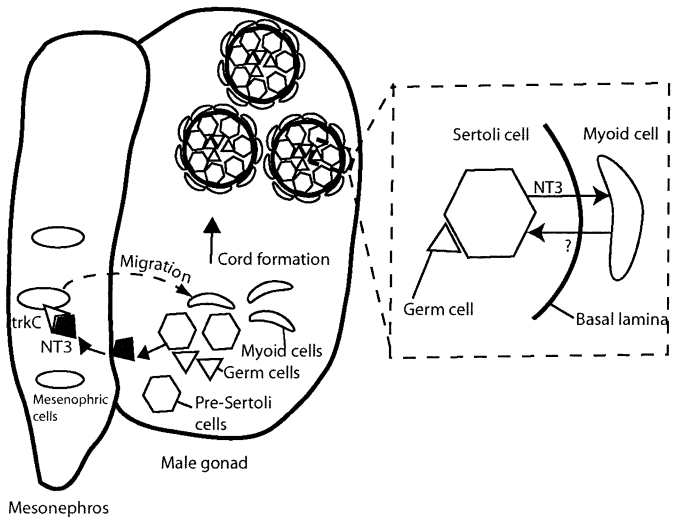

FIG. 4. Proposed model for the role of NT3 in mesonephric cell migration and cord formation. Sertoli cells secrete NT3 as a chemotactic agent that acts on trkC-expressing mesonephric cells to induce migration to the male gonad. Migrating mesonephric cells differentiate into various cell types, including peritubular myoid cells. Sertoli and myoid cells interact through paracrine factors to form seminiferous cords that encapsulate germ cells.

cell differentiation. The current study demonstrates the potential that NT3 may have a role in this early stage of male sex determination and acts as a chemotactic agent (Fig. 4). Observations presented demonstrate that NT3 expression increases during seminiferous cord formation, whereas trk C does not change. NT3 and trkC are both expressed at the appropriate developmental period and in the appropriate cell types to have a role in embryonic testis morphogenesis [18, 19]. Previous examination of neurotropin expression demonstrated that NT3 and trkC are highly expressed during this critical period of embryonic rat testis development, whereas BDNF and trkB have negligible expression, and NGF and trkA are expressed at higher levels later in embryonic testis development [18]. This is in contrast to the adult testis, in which all these genes are expressed [25]. Additional studies using receptor subtype-specific inhibitors have suggested that trkC was critical for cord formation [18, 19]. The trkA receptor is present in the embryonic testis during cord formation, and so may in part, compensate for trkC. The role of the other neurotropins (i.e., NGF and trkA) with NT3 and trkC remains to be fully elucidated.

Mice that lack the trkC receptor gene have testes with fewer seminiferous cords, and initially, fewer interstitial cells [20]. The presence of some cords in the trkC knockout mouse testis may be due to the ability of NT3 to act with reduced affinity on the trkA receptor. Attempts to produce double trkA and trkC knockout mice demonstrated these animals generally die in utero prior to the time of gonadal development [20]. However, the absence of trkC did cause a dramatic reduction in the number of seminiferous cords formed. This supports a potential role for NT3 in this aspect of embryonic testis morphogenesis. The trkC inhibitor, AG879, was found to block cord formation and mesonephros cell migration into the developing testis. This further supports a role for NT3 in this process. Although the AG879 tyrophostin inhibitor has been shown to be highly effective on trk receptors, potential actions on other pathways remain a possibility and needs to be considered in any data interpretation. Although trkC and trkA are primarily localized on mesonephros and interstitial cells, potential direct actions of AG879 on Sertoli cells need to be considered as a potential variable in data interpretation. It is interesting that the inhibition of cord formation by AG879 was also found to suppress SOX-9 gene expression. SOX-9 is critical for Sertoli cell differentiation and testis determination, and is up-regulated following seminiferous cord formation [9]. Because the AG879 did not alter Sertoli cell number, the suppression in SOX-9 gene expression is likely due to a delay in the progression of Sertoli cell differentiation. The current experiments, combined with experiments in FGF-9 null mice [16], suggest that the migrating mesonephros cells may produce factors that increase or up-regulate the expression of SOX-9. Without this critical cell migration, Sertoli cell gene expression (i.e., differentiation) and male sex determination is compromised.

The ability of NT3 to act as a chemotactic factor for the migrating mesonephros cells was investigated with the use of agarose beads containing NT3. The embryonic ovary does not have mesonephros cell migration at this time of development, and so was used for the experiment. NT3 was found to promote the mesonephros cell migration and supports the chemotactic role of NT3. The use of negative controls (i.e., BSA and EGF) in the beads had no cell migration, whereas the positive control (i.e., FGF 9) did promote cell migration. Future studies will examine the potential role of NGF in the beads and use gonads or mesonephros from trk-null mice. Formation of seminiferous cords requires this cell migration and is an early event downstream of SRY induction of Sertoli cell differentiation [13, 14]. It is interesting that the NT3 promoter has SRY binding sites [26] that are conserved [27, 28]. Whether NT3 may be an early downstream gene to SRY is under investigation.

In summary, a critical cell-cell interaction involving the neurotropin NT3 in embryonic testis development and male sex differentiation was identified (Fig. 4). When male sex determination is initiated by genes such as SRY, the Sertoli cells initiate differentiation, and an initial downstream event appears to be the production of NT3 to act as a chemotactic factor to promote mesonephros cell migration into the testis that then promotes seminiferous cord formation and the initial morphogenesis of the testis. Further elucidation of the factors involved in this cell-cell interaction will provide insight into the initial events in male sex differentiation.

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