Characterization of stage-specific embryonic antigen-1 (SSEA-1) expression during early development of the turkey embryo

SUSAN D’COSTA and JAMES N. PETITTE*#
Department of Poultry Science, North Carolina State University, Raleigh, North Carolina, USA

ABSTRACT SSEA-1 is a carbohydrate epitope associated with cell adhesion, migration and differentiation. In the present study, SSEA-1 expression was characterized during turkey embryogenesis with an emphasis on its role in primordial germ cell development. During hypoblast formation, SSEA-1 positive cells were identified in the blastocoel and hypoblast and later in the germinal crescent. Based on location and morphology, these cells were identified, as PGCs. Germ cells circulating through embryonic blood vessels were also SSEA-1 positive. During the active phase of migration, PGCs in the dorsal mesentery and gonad could no longer be identified using the SSEA-1 antibody. The presence of PGCs at corresponding stages was verified using periodic acid Schiff stain. Pretreatment of PGCs with trypsin, α-galactosidase and neuraminidase did not restore immunoreactivity to SSEA-1. In general, expression was not limited to the germ cell lineage. SSEA-1 was also detected on the ectoderm, yolk sac endoderm, gut and mesonephric tubules. During neural tube closure, SSEA-1 was expressed by the neural epithelium of the fusing neural folds. Later SSEA-1 was detected in regions of the developing spinal cord. Enzyme pretreatment unmasked the epitope on some neural crest cells and cells in the sympathetic ganglion. The temporal and spatial distribution of SSEA-1 in the turkey embryo suggests a role in early germ cell and neural cell development. The absence of SSEA-1 on turkey gonadal germ cells was different from that observed for the chick. Therefore, while features of avian germ cell development appear to be conserved, expression of SSEA-1 can vary with the species.

KEY WORDS: PGCs, SSEA-1, PAS, turkey, nervous system, embryo, neural crest

Introduction

Germ cells have been a subject of study for biologists for almost a century. As potentially immortal cells, they give rise to gametes and provide the only continuity from one generation to the next. In the vertebrate embryo, the development of the gametes occurs through a complex, often stereotyped, process beginning with the emergence of primordial germ cells (PGCs). Avian PGCs like other vertebrate germ cells are extragonadal in origin and undergo a circuitous journey to reach the presumptive gonad. In birds, the germ cells originate in the epiblast (Eyal-Giladi et al., 1981; Karageç et al., 1996), and unlike mammalian germ cells are not clustered in a particular region, but are scattered around the area pellucida of the prestreak blastoderm with a majority in the central region (Ginsburg and Eyal-Giladi, 1987; Kagami et al., 1997). As development continues, these cells translocate by an unknown mechanism to a lower layer, the hypoblast (Ginsburg and Eyal-Giladi, 1986). During gastrulation the hypoblast is displaced anteriorly and carries the PGCs to an extraembryonic region known as the germinal crescent (Swift, 1914; Ginsburg and Eyal-Giladi, 1986). Later, these cells are found circulating temporarily in the vasculature (Swift, 1914; Fujimoto et al., 1976); and subsequently, they exit the circulation near the vitelline blood vessels and actively migrate through the dorsal mesentery to reach the gonadal ridge (Ando and Fujimoto, 1983).

The current picture of avian germ cell development in birds has been based predominantly on studies using chick embryos. This

Abbreviations used in this paper: PGCs, primordial germ cells; SSEA-1, Stage-Specific Embryonic Antigen-1; H&H, Hamburger and Hamilton (1951) stages of development; G&B, Gupta and Bakst method of staging embryos (1993); PAS, periodic acid-Schiff; ES, embryonic stem; EG, embryonic germ; PBS, phosphate buffer saline; ABC-AP, avidin biotin conjugated alkaline phosphatase; AP, alkaline phosphatase; NBT, nitro blue tetrazolium chloride; BCIP, 5-bromo-4-chloro-3-indoly phosphate.
was facilitated by the observation that chick germ cells in the
germinal crescent, blood and early gonad can be identified using
periodic acid-Schiff (PAS) staining (Meyer 1960, 1964). However,
in the pre-primitive streak embryo, PAS staining is not specific for
germ cells and consequently could not be used. Recently, antibod-
ies such as EMA-1, FC10.2 and SSEA-1 that recognize carbohy-
drate epitopes have been applied as immunological markers to
trace the temporal and spatial development of PGCs in the early
development. The objective of this study was to characterize the
expression of SSEA-1 in the developing turkey embryo with an
emphasis on its expression in the germline.

Results

Identification and localization of primordial germ cells

Stages VII - X (G&B): 0-11 h of incubation

At oviposition (stage VII G&B) the turkey blastoderm consists of
three regions. The central region or the area alba, is made up of
large opaque cells. Surrounding it is the relatively transparent area
pellucida which is in turn surrounded by the area opaca. SSEA-1
cells were detected in the area alba and area pellucida of
the unincubated turkey embryo (stage VII/VIII G&B). Based on
immunohistochemical staining of whole blastoderms from stages
VII-IX (G&B), SSEA-1 labeled an average of 26 cells in the
dorsal mesentery and genital ridge (Takagi et al., 1992) also express the SSEA-1 epitope (Martin and
Lock, 1983; Resnick et al., 1992; Pain et al., 1996). Upon differentiation, these cells are no
longer positive. Recent analysis of the morphogenetic de-
velopment of the prestreak turkey embryo revealed significant differences from that ob-
served for the chick and quail (Gupta and
Bakst, 1993). Although the turkey is a com-
mercially important species in many parts of
the world, there is a lack of studies on primor-
dial germ cell development. Turkey PGCs
have been recognized in the germinal cres-
tent and gonad using the standard PAS stain-
ing (Reynaud 1967, 1969, 1971), but no work
has been published detailing early germ cell
development. The objective of this study was to characterize the
expression of SSEA-1 in the developing turkey embryo with an
emphasis on its expression in the germline.

Identification and localization of primordial germ cells

Stages VII - X (G&B): 0-11 h of incubation

At oviposition (stage VII G&B) the turkey blastoderm consists of
three regions. The central region or the area alba, is made up of
large opaque cells. Surrounding it is the relatively transparent area
pellucida which is in turn surrounded by the area opaca. SSEA-1
cells were detected in the area alba and area pellucida of
the unincubated turkey embryo (stage VII/VIII G&B). Based on
immunohistochemical staining of whole blastoderms from stages
VII-IX (G&B), SSEA-1 labeled an average of 26 cells in the
dorsal mesentery and genital ridge (Takagi et al., 1992) also express the SSEA-1 epitope (Martin and
Lock, 1983; Resnick et al., 1992; Pain et al., 1996). Upon differentiation, these cells are no
longer positive.

Recent analysis of the morphogenetic de-
velopment of the prestreak turkey embryo revealed significant differences from that ob-
served for the chick and quail (Gupta and
Bakst, 1993). Although the turkey is a com-
mercially important species in many parts of
the world, there is a lack of studies on primor-
dial germ cell development. Turkey PGCs
have been recognized in the germinal cres-
tent and gonad using the standard PAS stain-
ing (Reynaud 1967, 1969, 1971), but no work
has been published detailing early germ cell
development. The objective of this study was to characterize the
expression of SSEA-1 in the developing turkey embryo with an
emphasis on its expression in the germline.

Results

Identification and localization of primordial germ cells

Stages VII - X (G&B): 0-11 h of incubation

At oviposition (stage VII G&B) the turkey blastoderm consists of
three regions. The central region or the area alba, is made up of
large opaque cells. Surrounding it is the relatively transparent area
pellucida which is in turn surrounded by the area opaca. SSEA-1
cells were detected in the area alba and area pellucida of
the unincubated turkey embryo (stage VII/VIII G&B). Based on
immunohistochemical staining of whole blastoderms from stages
VII-IX (G&B), SSEA-1 labeled an average of 26 cells in the
dorsal mesentery and genital ridge (Takagi et al., 1992) also express the SSEA-1 epitope (Martin and
Lock, 1983; Resnick et al., 1992; Pain et al., 1996). Upon differentiation, these cells are no
longer positive.

Recent analysis of the morphogenetic de-
velopment of the prestreak turkey embryo revealed significant differences from that ob-
served for the chick and quail (Gupta and
Bakst, 1993). Although the turkey is a com-
mercially important species in many parts of
the world, there is a lack of studies on primor-
dial germ cell development. Turkey PGCs
have been recognized in the germinal cres-
tent and gonad using the standard PAS stain-
ing (Reynaud 1967, 1969, 1971), but no work
has been published detailing early germ cell
development. The objective of this study was to characterize the
expression of SSEA-1 in the developing turkey embryo with an
emphasis on its expression in the germline.

Results
was observed. The numbers had grown to approximately 300-350. Some of the cells were in the epiblast, and others were present in the hypoblast or between the two layers. Once again the positive cells varied in their staining intensities.

**Stages XI (G&B) - 4 (H&H): 12-36 h of incubation**

After incubation of the embryo for approximately 12 h, a complete hypoblast is present along the ventral surface of the epiblast. Between the epiblast and hypoblast is the blastocoel. Immunohistochemical staining of sectioned stage XI (G&B) embryos identified 40-50 strongly labeled cells in the blastocoel and hypoblast (Fig. 1B). The positive cells in the blastocoel were loosely attached to the hypoblast below it.

Embryos at stage XI (G&B) and stage 2 (H&H) that were separated into epiblast and hypoblast prior to staining showed light staining of cells in the epiblast. Some cells apparently ingressing from the epiblast were more darkly stained than the other cells. These stained cells were either alone, in groups of two, or were associated with other cells. The remaining SSEA-1 positive cells were detected on the hypoblast (Fig. 1D). By stage 4 (H&H), SSEA-1 positive germ cells could be identified in the anterior region of the embryo (Fig. 1C), corresponding to the developing germinal crescent. PAS positive germ cells were also identified in the germinal crescent of whole-mount embryos. At earlier stages, viz. in pre-primitive streak embryos, it was not possible to identify germ cells using the PAS staining. The PAS reaction was non-specific and all blastodermal cells were darkly stained.

**Stages 11-15 (H&H): 3-3.5 days of incubation**

Turkey PGCs circulating through the vasculature expressed the SSEA-1 epitope (Fig. 2B). As in other birds, they were larger in size than the accompanying blood cells. The presence of PGCs in blood smears was also verified using PAS. The PAS positive turkey germ cells appeared similar in morphology to the SSEA-1 positive germ cells. They were larger than blood cells, had an eccentric nucleus, and contained large amounts of glycogen (Fig. 2A).

**Stages 20 -30 (H&H): 4.5-9 days of incubation**

Immunohistochemical staining of cryosections of stage 20 (H&H) (day 4.5) turkey embryos with anti-SSEA-1 labeled cells of the yolk sac endoderm situated in the vicinity of the vitelline blood vessels (Fig. 4A). However, the turkey PGCs migrating through the dorsal mesentery could no longer be traced with the antibody (Fig. 2D). Sections stained with PAS verified the presence of turkey germ cells in alternate sections; a majority of PAS positive germ cells were identified in the dorsal mesentery migrating towards the germinal ridge (Fig. 2C). A few PGCs were identified exiting outside of the dorsal aorta. Clusters of PAS positive germ cells were also identified in the smaller vitelline blood vessels. At corresponding stages of development, the positive control chick embryos contained germ cells migrating through the dorsal mesentery. These chick germ cells were clearly SSEA-1 negative (Fig. 2G) and PAS positive.

At stage 24, (H&H) (day 6) the turkey germ cells are situated in the germinal ridge proper. Double staining of embryonic sections with SSEA-1 antibody and PAS staining confirmed the presence of PGCs in these sections. These germ cells are SSEA-1 negative and, because they still contain glycogen granules, are PAS positive (Fig. 4C).
At stages 27-30 (H&H) (day 8 to 9), turkey primordial germ cells in the gonad could not be identified using the SSEA-1 antibody (Fig. 2F). Germ cells in alternate sections were PAS positive but contained reduced amounts of glycogen (Fig. 2E). As previously reported (Halter et al., 1996; Karagenç et al., 1996), and in the present study, chicken gonadal germ cells at day 5.5 (stage 27/28 H&H) and 7.5 (stage 31 H&H) were SSEA-1 positive (Fig. 2H) thereby confirming that the immunohistochemical staining procedure used in this study was successful in detecting the SSEA-1 epitope. PGCs obtained after dissociation of the gonad with EDTA showed similar results, i.e. chick PGCs expressed the epitope whereas the turkey gonadal PGCs were SSEA-1 negative. Therefore, the absence of staining for turkey PGCs was not due to poor antibody penetration.

**Pretreatment of germ cells**

Immunohistochemical studies by Takagi et al. (1997) showed that pretreatment of paraffin sections with 0.1% trypsin increased the number of SSEA-1 positive germ cells in porcine genital ridges. Cryosections of day 8.5 (stage 27/28 H&H) turkey embryos were pretreated with 0.01% and 0.005% trypsin for 5 and 10 min respectively. Higher concentrations of trypsin resulted in over digestion of the section. This treatment did not improve the detection of SSEA-1 on germ cells. However, SSEA-1 labeled migrating neural crest cells and cells in the sympathetic ganglion (Fig. 3E).

Cho et al. (1996) have shown that a decrease in SSEA-1 expression upon differentiation of F9 teratocarcinoma cells is due to an increase in α-galactosylation of membrane glycoproteins and the subsequent masking of the SSEA-1 epitope by galactose. Hence, to test if the turkey PGCs in the gonad were masked by galactose residues, gonadal cells from day 8.5 (stage 27/28 H&H) turkey embryos were also treated with α-galactosidase. This did not improve the detection of SSEA-1 on germ cells. The double staining with PAS after immunostaining verified the presence of PAS positive germ cells in the samples. In addition, the pretreatment of sections containing the urogenital region of the embryo with neuraminidase did not alter the reactivity of the germ cells to the SSEA-1 antibody.

**SSEA-1 expression in neural tissues of turkey embryos**

At stage 8+ (H&H) SSEA-1 was expressed in the neural groove and neural epithelium in the regions where the neural tube had folded and fused (Fig. 3A). In the same embryo where neural tube closure is not complete, SSEA-1 is expressed in the groove of the neural folds (nf). (C) Expression in the dorsal region of neural tube (nt) at stage 20 (H&H). (D) SSEA-1 immunoreactivity is observed predominantly in the ventricular zone (vz), mantle zone (mz) and marginal zone of the stage 27/28 (H&H) embryo. The epitope was not detected in the developing ventral horn (vh). (E) Pretreatment with trypsin enhanced the expression on neural crest cells (arrowhead) in the dorsal region of the embryo and cells in the sympathetic ganglion (sg) (stage 27/28 H&H). (F) Neuraminidase treatment of stage 24 (H&H) embryonic sections unmasked the epitope on neural crest cells (arrowhead) in the body wall, some of the SSEA-1 positive neural crest cells (arrow) were in the ectoderm. Sections in Figure 3D,E, and F have been counterstained with PAS stain. Bar, 50 μm.

Fig. 3. SSEA-1 expression in the developing turkey spinal cord. (A) During neural tube closure at stage 8+ (H&H) SSEA-1 is expressed by neural epithelium (ne) and the non-neural ectoderm (ec). The endoderm (en) is SSEA-1 negative. (B) In the same embryo where neural tube closure is not complete, SSEA-1 is expressed in the groove of the neural folds (nf). (C) Expression in the dorsal region of neural tube (nt) at stage 20 (H&H). (D) SSEA-1 immunoreactivity is observed predominantly in the ventricular zone (vz), mantle zone (mz) and marginal zone of the stage 27/28 (H&H) embryo. The epitope was not detected in the developing ventral horn (vh). (E) Pretreatment with trypsin enhanced the expression on neural crest cells (arrowhead) in the dorsal region of the embryo and cells in the sympathetic ganglion (sg) (stage 27/28 H&H). (F) Neuraminidase treatment of stage 24 (H&H) embryonic sections unmasked the epitope on neural crest cells (arrowhead) in the body wall, some of the SSEA-1 positive neural crest cells (arrow) were in the ectoderm. Sections in Figure 3D,E, and F have been counterstained with PAS stain. Bar, 50 μm.
was also expressed in the roof plate. The pretreatment of stage 24 (H&H) embryonic sections with neuraminidase unmasked the epitope on some neural crest cells. Very few of the SSEA-1 positive neural crest cells were in the dorsal region of the embryo; a majority of the stained neural crest cells were observed in the body wall. Some of the cells were beneath the ectoderm of the body wall, while others had migrated into the ectoderm (Fig. 3F). The appearance of the epitope following pretreatment indicated that the epitope is sialylated on some migratory neural crest cells.

As development proceeded at stage 27-28 (H&H), SSEA-1 was detected on the ventricular zone, the dorsal mantle zone, and the marginal zone of the spinal cord (Fig. 3D). SSEA-1 was sparsely expressed on the roof plate. The lateral motor column (ventral horn) and the floor plate lacked SSEA-1 expression.

Treatment of day 8.5 (stage 27/28 H&H) turkey cryosections with trypsin significantly improved the detection on neural crest cells. Neural crest cells in the dorsal region of the embryo and cells in the sympathetic ganglion were clearly identified (Fig 3E).

SSEA-1 expression in the other somatic tissues of the avian embryo

At stage 20 (H&H), SSEA-1 was sparsely expressed on the mesonephric tubules of the turkey, the staining was much stronger from stages 24 (H&H) onwards (Fig. 4C). SSEA-1 staining was also seen in the luminal surface of the gut (Fig. 4B) and developing intestine at stage 24 (H&H).

SSEA-1 was also expressed in the developing nervous system of the positive control chick embryos. It was detected in the alar plate of the stage 21 (H&H) (day 4) embryo. At stage 27/28 (H&H) (day 5.5) SSEA-1 was detected in the dorsal mantle layer. In addition, SSEA-1 was also expressed on the chicken mesonephric tubules at stage 27-31 (H&H) and the cells lining the lumen of the gizzard, and intestine of chick embryos (stage 27) (Fig. 4D).

No staining was observed in the negative control slides that lacked primary or secondary antibody.

Discussion

In the present study, the temporal and spatial pattern of SSEA-1 expression during the development of turkey from oviposition until stage 30 has been traced. In addition, the development of germ cells from oviposition until colonization of the gonad has been followed using a combination of immunohistochemistry and the PAS technique.

Early origin of germ cells in the turkey embryo

At oviposition (stage VII G&B), the turkey embryo is 2-5 cells thick. In addition to the area pellucida and area opaca, the turkey embryo has a centrally placed area alba which consists of clusters of large opaque cells (Gupta and Bakst, 1993). In the unincubated stage VII-VIII (G&B) embryo, SSEA-1 labeled cells were found scattered around the central region of the blastoderm. These cells were isolated or in groups of two. The presence of these cells was not restricted to the area alba. As development proceeds, the number of positive cells increases. At stage X (G&B) during hypoblast formation, some of the SSEA-1 positive cells were faintly stained whereas other cells were darkly stained. Concomitant with complete hypoblast formation, SSEA-1 positive cells were observed in the turkey blastocoel and the hypoblast. Some of the cells in the turkey epiblast were stained on their dorsal and ventral surfaces similar to that previously observed for the chick embryo (Karagenç et al., 1996). Based on the temporal and spatial pattern of SSEA-1 expression and the morphology of the SSEA-1 positive cells, it appears that SSEA-1 most likely also labels prestreak germ cells in the turkey embryo. The development of germ cells in the turkey embryo is similar to that found in the chick embryo. In the unincubated stage X (EG&K) chick embryo approximately 20 EMA-1/SSEA-1 positive cells are scattered around the area pellucida. During hypoblast formation (stages XI-XIII EG&K) the number of these cells increases (Karagenç et al., 1996). The location of SSEA-1 positive cells in the turkey embryo during early development is similar to EMA-1 and SSEA-1 staining in the chick embryo (Urven et al., 1988; Karagenç et al., 1996). Analogous to chick embryos, PAS staining was not specific to germ cells in the preprimitive streak turkey embryo (Urven et al., 1988; Karagenç et
involved in the movement of avian germ cells across the endothelium. It thus seems probable that SSEA-1 might also be involved in the requirement for migration through the dorsal mesentery.

Passive migration of turkey PGCs

During their journey to the gonad, avian PGCs temporarily circulate through the embryonic blood vessels. Turkey PGCs circulating through the vasculature were identified using both PAS and SSEA-1. SSEA-1 (also known as the Lewis X antigen and CD15) has also been implicated in the movement of blood cells across the endothelium. SSEA-1 is expressed on human granulocytes, while a sialylated form is expressed on monocytes (Thorpe and Feizi, 1984). Before their extravasation at the time of inflammation, leukocytes move to the edge of the capillaries and begin to roll along the endothelium. A family of cell adhesion proteins, E-, L-, and P-selectin, aid in this rolling process. These adhesion proteins are expressed on both blood cells and endothelial cells. One of the ligands for the selectins is sialyl-Le$^a$. The asialo SSEA-1 sequence also binds to the selectin P and E although less strongly. The binding of selectins to its ligand sialyl-Le$^a$ is involved in adhesion of blood cells to the endothelium; the first step in the process of extravasation. Gomperts (1994) suggested that if chick PGCs expressed the SSEA-1 epitope then it could be possible that the PGC-endothelial cell interaction could be similar to the neutrophil-endothelial cell interaction. PGCs have been observed in the small vitelline vessels around the embryo. According to Ukeshima et al. (1991), these PGCs extend filopodia that come in contact with the inner side of the endothelial cell and adhere to the blood vessels. After adhesion, they move out through the gaps of the endothelial cells. The movement of germ cells across the endothelium bears some resemblance to the extravasation of blood cells. In the present study, SSEA-1 was detected on turkey germ cells during their passive migration through the vasculature. Earlier studies by Karagenç et al. (1996) have also detected SSEA-1 on circulating chicken PGCs. It thus seems probable that SSEA-1 might also be involved in the movement of avian germ cells across the endothelium.

Turkey gonadal germ cells

After the turkey germ cells exit out of the vasculature and begin migrating to the gonad, they no longer express the epitope. This was a surprising observation, and it was suspected that the SSEA-1 epitope was being masked. However, pretreatment with α-galactosidase, trypsin or neuraminidase did not improve the detection of gonadal PGCs. This loss of antigenicity occurs earlier in the turkey than in the chicken or other mammalian embryos. In the present study, chick PGCs continued expressing the epitope long after they colonized the gonad, viz. stage 31. Murine PGCs, however, stop expressing the epitope once they colonize the gonad (14.5 dpc) (Donovan et al., 1986). Treatment of 15.5 dpc mouse sections with neuraminidase restored the reactivity of germ cells, thereby indicating that the epitope was sialylated following the colonization of the gonads (Donovan et al., 1987). However, this does not appear to be the case in the turkey, since neuraminidase treatment of turkey germ cells had no effect. The disappearance of SSEA-1 from murine PGCs is coincident with the time at which these cells stop dividing and enter into meiotic prophase and the time at which germ cells lose their ability to give rise to teratocarcinomas (Stevens 1964; Fox et al., 1981). The reason for the premature downregulation of the epitope on turkey PGCs is unknown but the timing suggests that SSEA-1 is not a conserved requirement for migration through the dorsal mesentery.

This species variation in carbohydrate moieties on gonadal PGCs in the chick and turkey embryo is not unique. Yoshinaga et al. (1992) have also observed a variation in binding capacity of lectins to sugar residues on chick and quail germ cells. Some lectins bound to both quail and chick PGCs. Conversely, others like Griffonia simplicifolia II bound only to chick PGCs, and the lectin from Wisteria floribunda bound only to quail PGCs. This also suggests species differences in the carbohydrate residues on germ cells.

SSEA-1 expression on the developing nervous system

Some of the fundamental processes involved in the establishment of the nervous system include proliferation, cell-cell adhesion and migration. The pattern of SSEA-1 expression in the turkey provides evidence suggesting an involvement of SSEA-1 in the above processes. In the turkey, SSEA-1 was expressed by the multipotent and proliferating neural epithelial cells and migratory neural crest cells. Later its expression was restricted to the dorsal component of the neural tube suggesting that the antigen might be involved in the development of the sensory component of the nervous system. At later stages of development, pretreatment with enzymes unmasked the epitope on migratory neural crest cells. SSEA-1 has also been implicated in the development of the nervous system of other species (Dodd and Jessel, 1986; Oudega et al., 1992). These observations together support the role of SSEA-1 in the development of the vertebrate nervous system.

In general, immunohistochemical localization of SSEA-1 confirms the presence of the epitope on different migratory cell populations in the turkey embryo. A combination of immunohistochemical and PAS staining indicated that turkey PGCs express SSEA-1 during prestreak stages of development, in the germinal crescent, and during passive migration in the embryonic circulation. However, upon active migration from the blood to the gonad, SSEA-1 expression ceased. Therefore, until a universal gene product that is specific to avian germ cells is identified, the usefulness of SSEA-1 and other immunological markers of avian germ cell development must be evaluated for each species.

Materials and Methods

Turkey (Meleagris gallopavo) embryos obtained from a commercial source (Goldsboro Milling, Goldsboro, North Carolina) were used for this study. Embryos from stages VII to XI (0-12 h of incubation) were staged according to Hamburger and Hamilton (1951).

Embryos at specific stages corresponding to important landmarks during the migratory route of germ cell development were used for this study. The number of embryos examined at different stages were as follows: stage VII-X: 11 embryos, stage XI-4: 36 embryos, stage 8-15: 13 embryos and stage 20-30: 28 embryos. Studies were performed on whole blastoderms, separated epiblast/hypoblast, blood smears (during the circulatory period of germ cell development), dispersed gonadal cells, and on cryosections of embryos from day 0 - day 9 of incubation. Prior to any staining, specimens were fixed overnight in 4% paraformaldehyde in PBS (pH 7.2) at 4°C.

Cryosections

Frozen sections of gelatin-embedded materials were obtained using the procedure described by Stern (1993). Briefly, after fixation, specimens were washed well in PBS and transferred to 5% sucrose/PBS at 4°C overnight. They were transferred to 20% sucrose/PBS at 4°C until the

al., 1996) and, hence, could not be used to study early germ cell development.
specimen sank. They were then infiltrated in 7.5% gelatin in 15% sucrose/PBS at 38°C for 5 h and embedded in the same gelatin sucrose solution. The gelatin block containing the embryo or tissue was frozen in OCT compound and sectioned at 16 μm. Sections were collected on Probe on Plus® microscope slides (Fisher Scientific, Pittsburgh, Pennsylvania). After removing the gelatin from the sections using PBS at 37°C, the sections were washed in PBS and used for immunohistochemistry. Some alternate sections were stained with PAS as described below.

**Collection of gonadal PGCs for immunohistochemistry**

Gonads (n=10) were collected from day 8.5 (stage 27/28 H&H) turkey embryos and dissociated in 0.02% EDTA in PBS at room temperature for 20 min. The contents were then gently vortex-mixed, the clumps were allowed to settle and the cell suspension was collected. To the remaining clumps EDTA solution was added once again, and the above procedure was repeated. The cells were washed in PBS and placed on coverslips (Fisher Scientific) and dried and fixed in 4% paraformaldehyde. The coverslips were then used for immunohistochemical staining with the SSEA-1 antibody. PGCs collected from day 8.5 (stage 30 H&H) chick embryo gonads (n=11) were used as positive controls for the experiment.

**Periodic acid-Schiff staining**

Embryos (stage 4 H&H) and cryosections were oxidized in periodic acid solution (aqueous solution 1g/dl) (Sigma, St. Louis, Missouri) for 5 min. They were then rinsed in several changes of deionised water for 10 min and stained in Schiff reagent (Sigma) (Parasazinide HCI 1%, sodium bisulfitie 4% in hydrochloric acid 0.25 mol/L) for 15 min. Samples were once again washed several times in tap water and mounted in aqueous mounting medium made from 10 g of gelatin dissolved in 60 ml of water at about 75°C to which 70 ml of glycerin and 1 ml of phenol were added.

Air dried blood smears (stages 11-15 H&H) were fixed in fresh formalin-ethanol fixative solution for a minute, rinsed in running tap water for a minute, immersed in periodic acid for 5 min at room temperature and rinsed several times in distilled water. The slides were immersed in Schiff reagent for 15 min and then rinsed in running tap water for 5 min. Some smears were counterstained with hematoxylin and rinsed in running tap water. Smears were air dried and mounted in Permunt.

**Immunohistochemistry with SSEA-1 antibody**

Immunohistochemical studies were carried out using the Vectastain ABC-AP kit (Vector Laboratories, Burlingame, California). Blastodermus, epiblast, hypoblast, blood smears or cryosections were rinsed thrice in PBS and then blocked in 1.5% normal goat serum in PBS for 20-30 min to eliminate non-specific staining. Subsequently, cryosections were incubated for half an hour in primary monoclonal antibody against SSEA-1 (clone MC 480 obtained from the Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City, Iowa 1: 1000 diluted ascites). Other samples were incubated for an hour in antibody solution. After a rinse in PBS, they were incubated in biotinylated secondary antibody (30 min) then rinsed in PBS and incubated in Vectastain ABC-AP reagent (30-45 min). After a final wash in PBS they were stained in the alkaline phosphatase substrate NBT/BCIP solution (Amresco, Solon, Ohio). In order to prevent endogenous AP staining, 3.75 mM levamisole (Vector Laboratories) was added to substrate solution. Samples were mounted in an aqueous mounting medium. The number of cells expressing SSEA-1 in the embryos (stages VII - XI G&B) were counted.

Embryos from day 0 - day 9 of incubation were sectioned and then stained except stage 4 (H&H) embryos. Stage 4 (H&H) turkey embryos were stained prior to being sectioned. The incubation periods and washes were prolonged and 1% Triton X-100 was added to the PBS to help the penetration of the antibody. Incubation in normal goat serum, primary and secondary biotinylated antibodies and ABC-AP solution was extended to 48 h at 4°C. Washes were carried out overnight at 4°C. Embryos were stained in NBT/BCIP solution, embedded in gelatin and cryosectioned.

Cryosections of the trunk region of day 4 (stage 21 H&H) and day 5.5 (stage 27/28 H&H) chick embryos and urogenital region of day 7.5 (stage 31 H&H) chick embryos were used as positive controls for the immunohistochemical staining procedure. Turkey embryonic sections, blastodermus, epiblasts and hypoblasts incubated in solutions lacking either the primary or secondary antibody were used as negative controls to check for non-specific staining.

**Antigen retrieval**

**Pretreatment with trypsin**

Cryosections containing the urogenital region of day 8.5 (stage 27/28 H&H) turkey embryos were placed in PBS at 37°C for 25 min, rinsed in PBS and treated with either 0.01% trypsin for 5 min or 0.005% trypsin for 10 min at room temperature. After treatment with trypsin, the slides were rinsed in PBS and used for immunohistochemistry. The sections were then counterstained with PAS.

**α-Galactosidase treatment of gonadal PGCs**

Gonads from ten turkey embryos (day 8.5) were isolated in DMEM and 10% FBS on ice. They were washed twice in PBS and germ cells were collected using 0.02% EDTA. The cell suspension was then treated with green coffee bean α-galactosidase (Sigma, St Louis, Missouri) in HEPES buffer (10 mM HEPES, pH 6.5, 0.15 M NaCl, 5 mM CaCl₂) according to the procedure described by Cho et al. (1996). Approximately 5×10⁵ gonadal cells were treated with 100 milliunits of enzyme for one hour at 37°C. Enzyme that was inactivated by boiling for 20 min was used as control. The cells were then washed twice in PBS and placed on coverslips. After drying, they were fixed in 4% paraformaldehyde in PBS and used for immunohistochemistry and then counterstained with PAS.

**Neuraminidase treatment of paraffin sections**

Stage 24 (H&H) turkey embryos were decapitated and fixed overnight in 4% paraformaldehyde at 4°C. The embryos were rinsed in PBS then dehydrated, embedded in paraffin and sectioned. The sections (5 microns) were dewaxed, rehydrated and rinsed in PBS. Alternate sections containing the urogenital region of the embryo were treated with Neuraminidase from Vibrio cholerae (1 unit/ml; Boehringer Mannheim, Germany) for one hour at 37°C. The treated and untreated sections were washed in PBS and used for immunohistochemistry according to the procedure described above.

Following immunohistochemical staining the sections were rinsed in tap water and placed in periodic acid for 6 min. The sections were then rinsed in water for 10 min and stained in Schiff reagent for 15 min. After rinsing in tap water, the sections were mounted in the above aqueous mounting medium.

**Acknowledgments**

The authors thank Dr. S.L. Pardue, Dr. J. Grimes and Goldsboro Milling for providing the turkey eggs. This work was supported, in part, by funds provided under Project Number NC 01868 of North Carolina State University.

**References**


