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Original Article

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

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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; Karagenç *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-indoyl-phosphate.

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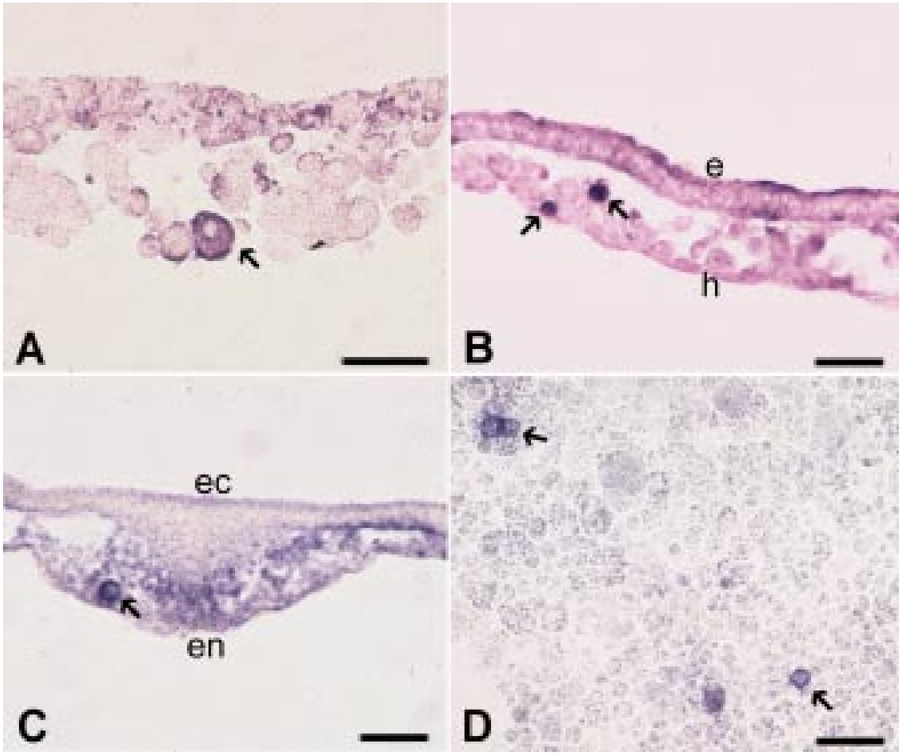

Fig. 1. Immunohistochemical localization of SSEA-1 in the early turkey embryo. (A) *Transverse section of stage VIII (G&B) embryo, an individual PGC (arrow) is located in the ventral surface of blastoderm.* **(B)** *At the time of hypoblast (h), formation germ cells (arrow) can be identified in the blastocoel and the hypoblast of sectioned embryos (stage XI).* **(C)** *SSEA-1 labeling identified germ cells in the germinal crescent region of the stage 4 (H&H) embryo.* **(D)** *SSEA-1 positive germ cells (arrow) on the dorsal side of a dissected hypoblast (st. 2 H&H). Section in Figure 1B is counterstained with aqueous eosin. e, epiblast; h, hypoblast; en, endoderm; ec, ectoderm. Bar, 50 μm.*

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, antibodies such as EMA-1, FC10.2 and SSEA-1 that recognize carbohydrate epitopes have been applied as immunological markers to trace the temporal and spatial development of PGCs in the early blastoderm (Urven *et al.*, 1988; Loveless *et al.*, 1990; Karagenç *et al.*, 1996).

The stage-specific embryonic antigen-1 (SSEA-1) is perhaps one of the best analyzed antigens on primordial germ cells in mammalian and avian embryos. In 1978, a monoclonal antibody to SSEA-1 was developed by the fusion of mouse myeloma cells with spleen cells from a mouse immunized with F9 teratocarcinoma cells (Solter and Knowles, 1978). Characterization of anti-SSEA-1 indicated that it was against a carbohydrate epitope with a galactose (β1-4) N-acetylglucosamine (α1-3) fucose linkage and was similar to the Lewis X antigen (Gooi *et al.*, 1981).

During early embryo development, SSEA-1 is differentially expressed. It is not a lineage-specific marker, as antibody reactivity does not appear to be continuous in any one cell type, rather it is a stage-specific marker for different cell types and varies as development proceeds (Fox *et al.*, 1981). During murine embryogenesis, SSEA-1 is first expressed on the cell surface of the 8-cell embryo (Solter and Knowles, 1978). Later its presence is restricted to the embryonic ectoderm, visceral endoderm, germ cells and the developing nervous system (Fox *et al.*, 1981). In addition to marking murine germ cells from 9.5 dpc until colonization of the gonads (Fox *et al.*, 1981; Donovan *et al.*, 1986), it is also expressed on germ cells of other species. Chick PGCs (Halfter *et al.*, 1996; Karagenç *et al.*, 1996) and porcine germ cells in the dorsal mesentery and genital ridge (Takagi *et al.*, 1997) also express the epitope. Immunohistochemical studies by Oudega *et al.* (1992) and Streit *et al.* (1996) have also localized SSEA-1 expression in the developing rat and chick spinal cord, respectively. Undifferentiated multipotent mouse and chick embryonic stem (ES) cells, murine embryonic germ (EG) cells, and embryonic carcinoma (EC) cells 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 development of the prestreak turkey embryo revealed significant differences from that observed for the chick and quail (Gupta and Bakst, 1993). Although the turkey is a commercially important species in many parts of the world, there is a lack of studies on primordial germ cell development. Turkey PGCs have been recognized in the germinal crescent and gonad using the standard PAS staining (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 labeled 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 blastoderm. These cells were scattered around the central region of the embryo. The intensity of the label varied on individual cells from moderate to very dark. Immunohistochemical staining of transverse sections of stage VIII (G&B) embryos verified the presence of SSEA-1 positive cells in the blastoderm (Fig. 1A).

After incubation of the embryo for 5-11 h the hypoblast, lies beneath the epiblast, covering about one half of the *area pellucida.* At this time (stage X G&B) an increase in the SSEA-1 positive cells 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).

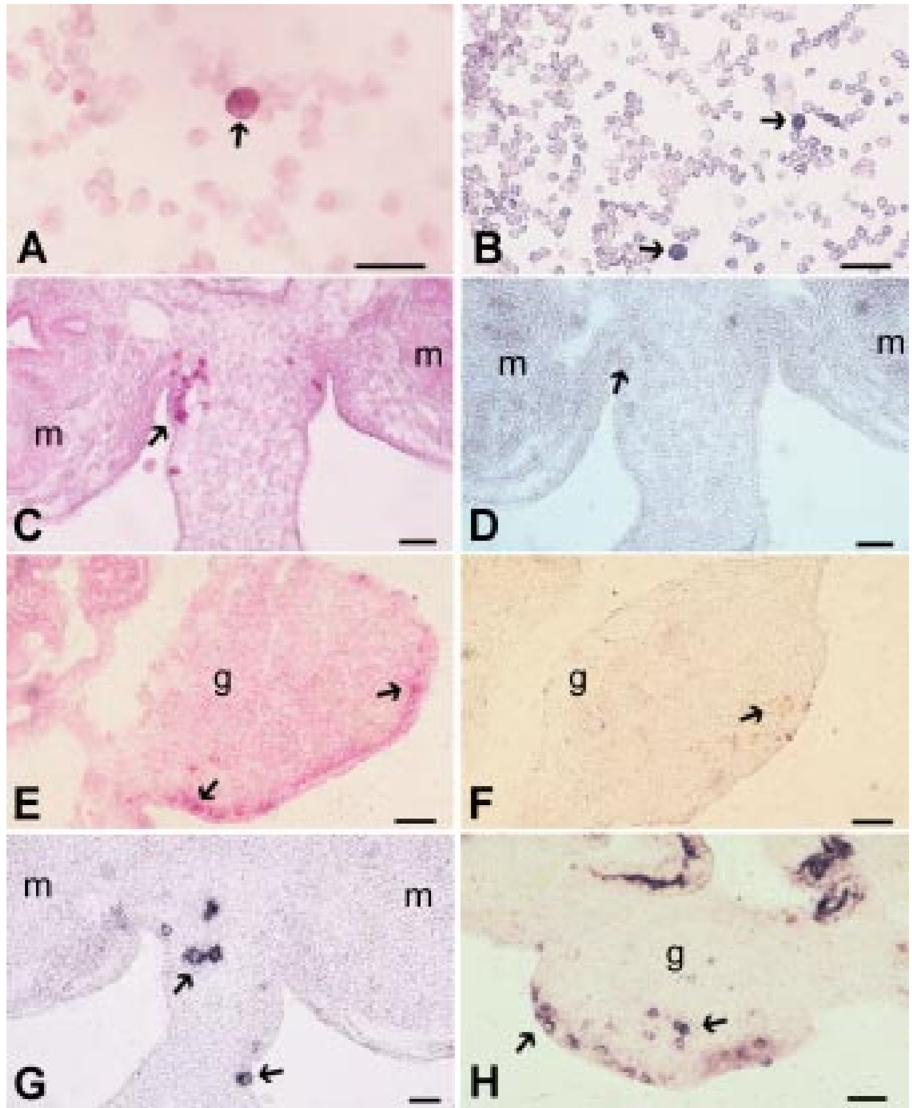

Fig. 2. Identification of avian germ cells (indicated by arrows) using PAS stain and immunohistochemistry. *Circulating turkey PGCs (stage 13 H&H) could be identified among blood cells using both PAS* **(A)** *and SSEA-1* **(B)** *staining. Turkey germ cells migrating towards the genital ridge at stage 20 were PAS positive* **(C)**, *however, they did not express the SSEA-1 epitope* **(D)**. *C and D are alternate sections. Transverse section of stage 30 (H&H) turkey embryo contained PAS positive germ cells* **(E)** *in the gonad (g). Cryosections of the turkey embryo stained with SSEA-1 verified that gonadal germ cells are SSEA-1 negative* **(F)**. *In the positive control chick embryos the germ cells in the dorsal mesentery* **(G)** *at stage 21 (H&H) and gonad* **(H)** *at stage 31 (H&H) continued to express the epitope. m, mesonephros. Bar, 50 μm.*

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 (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).

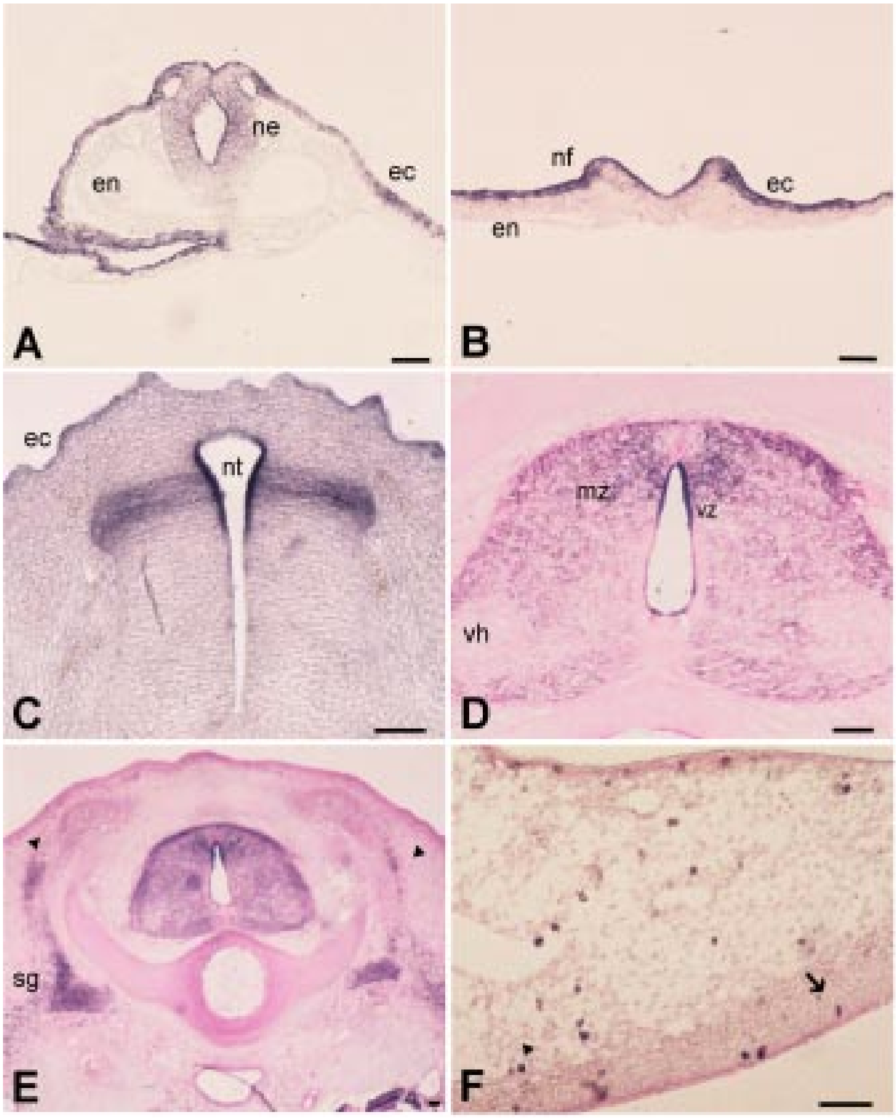

Fig. 3. SSEA-1 expression in the developing turkey spinal cord. (A) *During neural tube closure at stage 8 + (H&H) SSEA-1 is expressed by neural epithelium (ne) and the non-neural ectoderm (ec). The endoderm (en) is SSEA-1 negative.* **(B)** *In the same embryo where neural tube closure is not complete, 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.*

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 (Halfter *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 turkey 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 regions where the folds were not yet in contact, the neural groove stained positive (Fig. 3B). SSEA-1 was also detected along the border of the neural plate and the ectoderm, i.e. the location of the future neural crest cells. The epitope was present on the non-neural ectoderm while the lateral plate mesoderm, somites and endoderm were SSEA-1 negative. From this stage onwards the embryos were decapitated and the trunk sectioned and stained. At stage 20 (H&H) long after neural tube closure, SSEA-1 expression was restricted to the alar plate and the ventricle in the dorsal region of the spinal cord. The roof plate, floor plate and basal plate were negative (Fig. 3C). At stage 24 (H&H) the alar plate of the spinal cord continued to be SSEA-1 positive. In addition, SSEA-1 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).

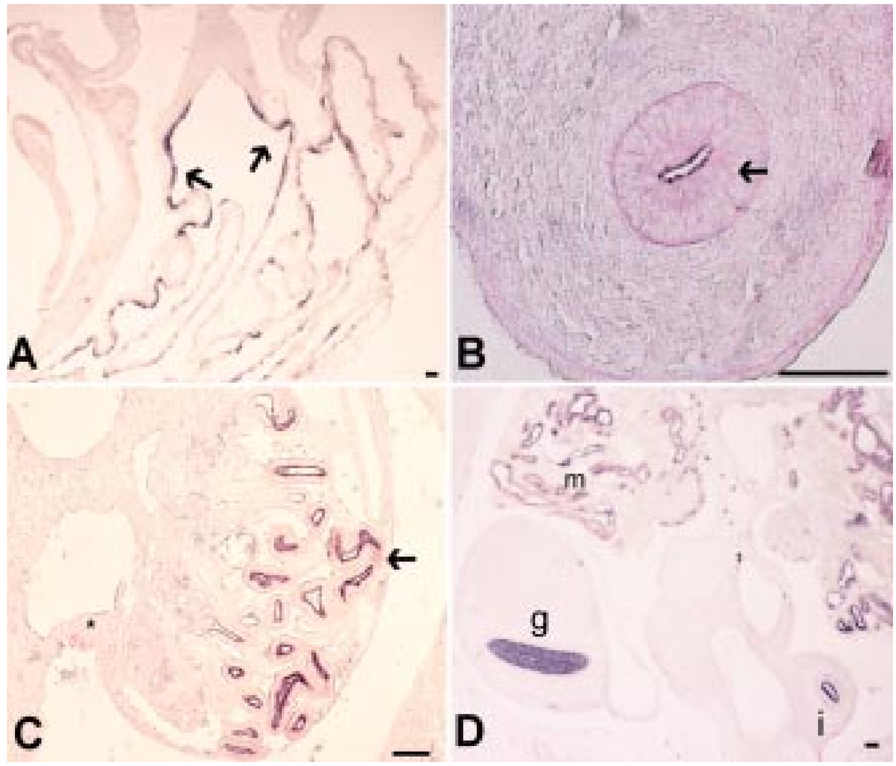

Fig. 4. SSEA-1 expression in turkey (A,B,C) and chicken (D) somatic tissues. (A) *At stage 20 (H&H) SSEA-1 expression is localized in the turkey yolk sac endoderm.* **(B** *and* **C)** *Stage 24 (H&H) turkey embryo sections immunostained with SSEA-1 and counterstained with PAS stain. SSEA-1 staining is observed in the luminal surface of the developing gut* **(B)** *and in the mesonephric tubules* **(C)**. *Note the SSEA-1 negative, PAS positive germ cells in the turkey gonad (asterisk).* **(D)** *At stage 27/28 (H&H) SSEA-1 is expressed on the luminal surfaces of the chicken gizzard (g) and intestine (i) and in the mesonephric tubules (m). Bar, 50 μm.*

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* *al.*, 1996) and, hence, could not be used to study early germ cell development.

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^x. 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^x 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-endothelium 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 Gupta and Bakst (1993). Older embryos from stages 2-30 (day 1 to day 9 of incubation) were staged according to criteria established by 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 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 6.5 (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) (Parosaniline HCl 1%, sodium bisulfite 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 Permount.

Immunohistochemistry with SSEA-1 antibody

Immunohistochemical studies were carried out using the Vectastain ABC-AP kit (Vector Laboratories, Burlingame, California). Blastoderms, epiblast, hypoblast, blood smears or cryosections were rinsed thrice in PBS for a total time of 30-90 min, depending on the thickness of the tissue. They were 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, blastoderms, 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 counter-stained 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 $CaCl_2$) according to the procedure described by Cho *et al.* (1996). Approximately $5x10^5$ 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.

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