Distinct GAGE and MAGE-A expression during early human development indicate specific roles in lineage differentiation

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BACKGROUND: Expression of cancer/testis-associated proteins (CTAs) has traditionally been considered to be restricted to germ cells in normal tissues and to different types of malignancies. We have evaluated the potential role of CTAs in early human development. METHODS: Using immunohistochemistry and RT–PCR, we investigated the expression of CTAs in differentiated human embryonic stem cells (hESC) and in late embryos and early fetuses. RESULTS: We found that melanoma antigen A (MAGE-A) family members were expressed during differentiation of hESC to embryoid bodies and in teratomas, and overlapped with expression of the neuroectodermal markers beta-tubulin 3, Pax6 and nestin. A widespread expression of MAGE-A was also observed in neurons of the early developing central nervous system and peripheral nerves. G antigen (GAGE) expression was present in the early ectoderm of embryos, including cells of the ectodermal ring and apical epidermal ridge. Neuroectodermal cells in the floor plate and adjacent processes and endfeet of radial glial cells also expressed GAGE. In addition, GAGE family members were expressed in the peripheral adrenal cortex of 6–9-week-old embryos and fetuses, which specifically correlated with massive cellular proliferation and establishment of the definitive and fetal zones. Overlapping expression of MAGE-A and GAGE proteins occurred in migrating primordial germ cells. CONCLUSIONS: Our results show that CTAs, in addition to their role in germ cells, may be involved in early development of various types of somatic cells, and suggest that they are implicated in specific differentiation processes.

Keywords: GAGE; MAGE-A; human development; hESC; teratoma

Introduction

Cancer/testis-associated proteins (CTAs) are frequently expressed in many different types of cancer and have received considerable attention as potential targets for cancer immunotherapy (Simpson et al., 2005). In normal tissues, CTAs have traditionally been considered to be expressed only in early stages of germ cells and tropheoblasts of the placenta (Simpson et al., 2005; Gjerstorff et al., 2007). However, recent studies have shown that CTAs can also be found in fetal Leydig and Sertoli cells and in mesenchymal stem cells (Cronwright et al., 2005; Gjerstorff et al., 2007), suggesting a more general role of these proteins in immature somatic cells.

Most CTAs exist as highly identical members of large families encoded by gene clusters located on the X chromosome [e.g. melanoma antigen A (MAGE-A), G antigen (GAGE), synovial sarcoma X breakpoint (SSX)]. These genes are organized into repeats and have undergone rapid evolution, possibly due to positive selection (Stevenson et al., 2007). The biological functions of CTAs have not been fully studied, but emerging evidence suggests that they may direct the proliferation, differentiation and survival of human germ cells. The MAGE-A family members are among the most well characterized CTAs, and have been shown to be involved in regulation of apoptosis and cell cycle progression, both through direct molecular interactions and by regulation of gene expression (Peikert et al., 2006; Yang et al., 2007a, b, c). MAGE-A1 and -A4 were found to interact with the nuclear proteins SKIP and HDAC1 and thereby inhibit transcriptional activation mediated by Notch-IC (Laduron et al., 2004), while MAGE-A and -C members were observed to down-regulate p53 transactivation by recruiting histone deacetylase 3 and suppressing p53-dependent apoptosis (Monde et al., 2006; Yang et al., 2007a, b, c).
Similarly, GAGE proteins have been shown to possess anti-apoptotic properties (Cilensek et al., 2002). Recently, MAGE-C1 and NY-ESO-1 were found to interact physically and to be co-expressed in tumor cells, indicating that these, and perhaps other, CTAs function coordinately (Cho et al., 2006).

In this study, we examined the expression of CTAs during organogenesis and differentiation processes of early human development using specimens of human embryos and fetuses and differentiated human embryonic stem cells (hESC). hESC are derived from the pluripotent cells of the inner cell mass of the blastocyst and, when cultured in vitro as embryoid bodies (EB) or allowed to form teratomas in mice, can give rise to a multitude of cell types of endodermal, mesodermal or ectodermal derivation. Thus, hESC provide a model to study lineage-specific differentiation.

Our results demonstrate that CTAs are expressed in various types of somatic cells during early human development, and suggest that CTAs are involved in specific differentiation processes.

Materials and Methods

Ethical approval

KMEB (Clinic for Molecular Endocrinology) hESC were obtained from the IVF clinic, Department of Obstetrics and Gynaecology, Odense University Hospital and Odense IVF Clinic, Odense. Patients received oral and written information and gave signed and informed consent. Fetal tissue specimens were obtained from abortion specimens collected for diagnostic purposes and deposited in the tissue bank of Odense University Hospital. All experiments were approved by the ethical committee of Funen and Vejle County (VF20050069). Additional paraffin sections from the Developmental Biology Unit, ICMM, the Panum Institute and freshly dissected samples from legal 1. trimester abortions from the Laboratory of Reproductive Biology, Rigshospitalet, and the Department of Obstetrics and Gynaecology, Frederiksberg Hospital, both University of Copenhagen, were included. Oral and written information was given and informed consent was obtained from all contributing women, according to and approved by The Regional Committee on Biomedical Research Ethics Copenhagen and Frederiksberg Counties (KF (01) 258206).

Embryonic and fetal specimens

Embryonic and fetal specimens were selected to include a broad range of different tissues and ages. Sections stained for GAGE, MAGE-A and NY-ESO-1 were obtained from 5- to 8-week-old whole embryos and from various organs and tissues blocks dissected from twenty-one 9- to 14-week-old male and female fetuses. Tissues from 17- and 23-week-old male and 28-week-old female fetuses were also investigated. The organs studied included the central nervous system (CNS) (cerebrum, brain stem, cerebellum and spinal cord), lung, heart, thyroid, thymus, pancreas, liver, intestine, spleen, adrenal gland, gonad, kidney and bladder. Ages of all embryos and fetuses are weeks post-conception.

Human embryonic stem cells

The hESC line HUES-9 was obtained from the Howard Hughes Medical Institute, Harvard University (Cowan et al., 2004), and hESC lines KMEB1-5 were established and characterized by the KMEB laboratory, Odense University Hospital, Odense, Denmark (Prokhorova et al., 2008). Undifferentiated hESC were maintained on feeder layers of mitotically inactivated mouse embryonic fibroblasts (MEF) feeders in Knockout Dulbecco’s Modified Eagle’s Medium (Invitrogen, Tastrup, Denmark) supplemented with 15% Knockout Serum Replacement (Invitrogen), 0.5% human serum albumin (ZLB Behring, Kgs. Lyngby, Denmark), 0.1 mM beta-mercaptopetanol (Sigma, Brondby, Denmark), 1% non-essential amino acids, 2 mM Glutamax-I (Invitrogen), 50 U/ml penicillin (Invitrogen), 50 μg/ml streptomycin (Invitrogen) and 8 ng/ml recombinant human basic fibroblast growth factor (bFGF) (Invitrogen). hESC cultures were passaged at a 1:3–1:6 split ratio every 4–7 days by trypsinization.

Differentiation of hESC through EB or teratoma formation

EB formation was induced by seeding hESC in growth media (specified above) without bFGF and beta-mercaptopetanol in ultra low adhesion culture dishes. After 48 h, EBs were separated from single cells by gravity sedimentation and cultured for up to 30 days. Teratomas were formed by injecting hESC under the kidney capsule as previously described (Frandsen et al., 2007) or subcutaneously with 4.5–6 million cells in the dorsolateral area in the side of NOD-SCID mice. After 8 (kidney capsule) or 4–6 weeks (subcutaneous), the mice were sacrificed and teratomas were removed, fixed in 4% normal buffered formalin and embedded in paraffin.

Antibodies

Antibodies used in this study were: GAGE mAb M3 [expected to recognize all members of the GAGE family; produced in-house (Gjerstorff et al., 2006)], MAGE-A mAb (recognizes MAGE-A1, -A2, -A3, -A4, -A6, -A10 and -A12; Zymed Laboratories Inc., San Francisco, CA, USA), NY-ESO-1 mAb (Zymed Laboratories Inc.), MAGE-C1 (Dako, Glostrup, Denmark), OCT3/4 mAb (Clone C10, Santa Cruz Biotechnology, Heidelberg, Germany), HNF4-alpha (Santa Cruz Biotechnology), Pax6 (Covance, USA), beta-tubulin 3 (R&D Systems, Minneapolis, MN, USA), Nkx6.1 (Developmental Studies Hybridoma Bank, Iowa City, IA), alpha fetoprotein (Dako), fetal antigen 1 and CD31 (Dako).

Immunohistochemistry

All embryonic, fetal tissue and teratoma specimens were fixed in 4% formaldehyde (pH 7.4) for 24 h and embedded in paraffin. EBs were fixed in 4% formaldehyde for 10 min and prepared as cell blocks using Shandon Cytoblock (Thermo Electron Corporation, Pittsburg, PA, USA). Procedures for immunohistochemical single and double-staining has been described previously (Gjerstorff et al., 2007). Immunohistochemical controls are included in Supplement data 1.

Real-time-polymerase chain reaction

Total RNA from undifferentiated and differentiated EBs and from freshly dissected embryonic adrenal glands and mesonephros was isolated using the Trizol reagent (Invitrogen) according to manufacturer’s recommendations, and RevertAid H minus First Strand cDNA synthesis kit (Fermentas) was used for cDNA synthesis. Semi-quantitative PCR was carried out with TMAPease DNA polymerase (Ampliqon, Rodovre, Denmark). QuantiTect primer assays MAGE-A1 (QTO0364847), -A2 (QTO0008778), -A6 (QTO0059129), -A10 (QTO005376), -B3 (QTO0205058) and beta-actin (QTO0095431) were purchased from Qiagen, and primers recognizing all known members of the GAGE family (GAGE-pan) were: 5’-CTG AGA TTC ATC TGT GTG AAA TAT GA-3’ and 5’-AGG CTT CGG CCC TTG A-3’. Relative quantification using real-time PCR was performed in triplicate using SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer’s instructions. The median relative expression levels were normalized with endogenous beta-actin levels. Primers were as above.
Results

CTA expression in hESC EBs

The expression of MAGE-A, GAGE and NY-ESO-1 in cultures of hESC lines HUES-9 and KMEB1-5 was examined by RT-PCR and showed that undifferentiated cells (Day 0) maintained on layers of MEF feeders did not express any of the CTAs studied (Fig. 1A). In contrast, MAGE-A2 and MAGE-A6 were present in differentiated cells of 20-day-old EBs derived from KMEB2, while weak expression of only MAGE-A2 was observed in KMEB1 EBs. No CTA expression was detected in KMEB5 EBs.

Immunostaining of 10- and 20-day-old EBs confirmed the expression of MAGE-A proteins in KMEB2 EBs (Fig. 1D and E), whereas no MAGE-A staining was detected in KMEB1 EBs (Fig. 1F and G). In addition, 20-day-old EBs derived from HUES-9 were also MAGE-A-positive (Fig. 1C). Positive cells were localized both in vacuolated and solid EBs. GAGE and NY-ESO-1 were not detected by RT-PCR (Fig. 1A) or by immunostaining in the hESC lines (data not shown).

CTA expression in hESC teratomas

Immunostaining of teratomas showed that MAGE-A proteins were expressed in teratomas formed from subcutaneous transplanted KMEB1 and KMEB2 cells (Fig. 2 and 3), while teratomas of subcutaneously transplanted KMEB5 and kidney transplanted HUES-9 exhibited no MAGE-A staining. In teratomas of both KMEB1 and KMEB2, the MAGE-A-positive cells primarily formed large cell-dense structures (Fig. 2A, C and D). However, MAGE-A-expressing cells were also found in association with epithelial structures (Fig. 2B and F). Serial stainings of parallel sections of KMEB1 teratomas showed a potential co-localization of MAGE-A, GAGE and OCT4 in cells associated with HNF4 alpha-positive epithelium (Fig. 2 E–H). GAGE expression in teratomas was limited to these cells and no NY-ESO-1 expression was observed.

Expression of MAGE-A family members in neuroectodermal cells of EBs and teratomas

Double-staining of HUES-9 EBs with antibodies recognizing well-established lineage markers and MAGE-A showed that a subset of cells positive for neuroectodermal markers betatubulin 3 and Pax6 were also MAGE-A-positive (Fig. 3A and C). However, beta-tubulin 3-negative and Pax6-negative MAGE-A-positive cells were also observed (Fig. 3B and D). In accordance with the EB double-stainings, staining of parallel sections of KMEB2 and HUES-9 teratomas showed overlap between MAGE-A expression and expression of neuroectodermal markers beta-tubulin 3, Pax6 and nestin; again, only a subset of beta-tubulin 3-, Pax6- and nestin-positive structures was also MAGE-A-positive (Fig. 3E–G, I and J). Some overlap between MAGE-A and the late neuronal marker tyrosine hydroxylase was also observed (Fig. 3E and H), while there was no overlap between MAGE-A and HNF4-alpha, Nkx6.1, alpha fetoprotein, fetal antigen 1 or CD31.

MAGE-A expression in the developing CNS

MAGE-A reactivity was widely observed in the spinal cord and brain stem of the early developing CNS (Fig. 4A and B). Reactivity was also seen in the ventral roots of the spinal nerves and in cranial nerves, e.g. nervus facialis (Fig. 4B) and nervus trochlearis, and in motor neurons of the spinal cord (Fig. 4A). Staining of neurons was confined to the cytoplasm and extended out into the axons. MAGE-A expression was also seen in the myoblasts of the myotomes, which were innervated by MAGE-A-positive spinal nerves (Fig. 4A). No MAGE-A reactivity was observed in the CNS, e.g. cerebrum, brain stem, cerebellum and spinal cord, of a 17- and a 23-week-old fetus (data not shown), or in the adult brain (Supplement data 1).

GAGE expression in the early ectoderm and neuroectoderm

A weak but distinct cytoplasmic reactivity for GAGE was observed in the early developing ectoderm and neuroectoderm (Fig. 5). The thickened ectoderm forming the ectodermal ring including the apical epidermal ridge showed a positive reaction in the earliest embryos investigated, i.e. from 5 (Fig. 5A and C) to 7 weeks. Neuroectodermal cells in the floor plate and adjacent processes and endfeet of radial glial cells also showed distinct reactivity (Fig. 5B). A minor fraction of the early neurons

Figure 1: CTA expression in hESC EBs.

(A) RT–PCR analysis of expression of MAGE-A, GAGE and NY-ESO-1 transcripts in undifferentiated and differentiated EBs of KMEB1, KMEB2 and KMEB5 hESC lines. Immunostaining of MAGE-A members in EBs of HUES-9 (B and C), KMEB2 (D and E) and KMEB1 (F and G) differentiated for 10 or 20 days, respectively. Magnification: ×20 (B and C), ×10 (D–G).
in the sensory ganglia exhibited positive cytoplasmic staining for GAGE (Fig. 5A).

**CTA expression in migrating PGCs**

CTA expression in primordial germ cells (PGCs) and gonocytes of the human gonadal primordium is well established (Gjerstorff et al., 2007; Nelson et al., 2007), but whether CTAs are also expressed in earlier stages of germ cells has not been examined. PGCs migrate from the yolk sac to the gonadal primordium during Week 4 and 5, and the tissues that they pass on their migratorial pathway include the dorsal mesentery, mesonephros, adrenal gland and pancreas. Examination of 5- to 9-week-old embryos and fetuses revealed that GAGE and MAGE-A were highly expressed in PGCs migrating in the dorsal mesentery or under the mesonephric epithelium and proceeding into the gonadal primordium (Fig. 6A). In addition, GAGE- and MAGE-A-positive PGCs were observed in the adrenal cortex of early fetuses (and in the adrenocortical cells) (Fig. 6B). The intensity of the staining varied and both nuclear and cytoplasmic staining was present. In older fetuses (up until Week 17), we also observed GAGE- and MAGE-A-positive PGCs in the adrenal cortex (Fig. 6C) and pancreas (Fig. 6D), the former located just beneath the capsule, whereas the latter were observed near the autonomic nerve fibers. No NY-ESO-1 expression was detected in PGCs (Supplement data 1).

**Expression of GAGE family members in the embryonic and fetal adrenal cortex**

Staining of embryos and fetuses revealed that GAGE proteins were expressed in mesenchymal cells of the adrenal cortex (Fig. 7). Positive cells were observed from Week 6 (Fig. 7A) and increased in intensity until Week 9 (Fig. 7B–D), when expression abruptly ended. The staining of the cortex cells varied both in intensity and in nuclear and cytoplasmic localization. Highest expression of GAGE was observed in cells of the outer part of the cortex, and expression gradually faded towards the center. At the end of the expression period, this radial arrangement of GAGE-positive cells became more irregular (Fig. 7D).

Semi-quantitative and quantitative RT–PCR confirmed that GAGE was expressed in the fetal adrenal gland from Week 7 to around Week 9 with a peak in expression around Week 8 (Fig. 7E and F). Notably, although outnumbered by cortex cells, PGCs were also present in the adrenal gland at this time and might have been an additional source of GAGE transcripts. In accordance, GAGE transcripts were also amplified from the mesonephros of a 5-week-old embryo that also contained migrating PGCs.

**Discussion**

To investigate the role of CTAs in organogenesis and tissue differentiation, we examined the expression of these proteins in differentiated hESC and in human embryos and fetuses.

None of the examined CTAs were expressed in undifferentiated cells of several hESC lines. In contrast, when hESC were allowed to spontaneously differentiate either as EBs or teratomas, we found that MAGE-A expression was induced in a subset of cells. Double immunostaining and parallel staining of serial sections with established lineage markers revealed that MAGE-A-positive cells also expressed markers of neuronal progenitors and post-mitotic neurons, suggesting that MAGE-A proteins may have a role in neuronal development. In agreement, we found that MAGE-A expression was present in neurons of the early developing nervous system. Expression was observed in both the CNS (e.g. the spinal cord and brain stem) and in peripheral nerves (e.g. nervus facialis and nervus trochlearis). Our results show that MAGE-A proteins are widely expressed in early post-mitotic neurons and indicate that MAGE-A proteins may have a role in neuronal differentiation. Interestingly, MAGE-A members have been shown to be inhibitors of Notch-mediated transcriptional activation (Laduron et al., 2004), while Notch itself can repress neuronal differentiation (Louvi and Artavanis-Tsakonas, 2006). This suggests that MAGE-A expression plays an important role in neuronal differentiation.

Other MAGE homology domain proteins are involved in neuronal development. A necdin-homologous protein is...
expressed during neurogenesis in *Drosophila*, and neddin and NRAGE (MAGE-D1) expression is present in the developing human and mouse CNS (Salehi et al., 2000). Neddin is present in most of the post-mitotic neurons throughout the CNS and peripheral nervous system, and strongly suppresses cell proliferation, promotes neuronal differentiation, and inhibits death of several cell lines and primary neurons (Maruyama et al., 1991; Aizawa et al., 1992; Hayashi et al., 1995; Taniura et al., 1999; Kobayashi et al., 2002; Takazaki et al., 2002). Whether MAGE-A proteins play a similar role remains to be determined.

MAGE-A expression was found in *in vitro* differentiated HUES-9 hESC, but not in HUES-9 teratomas, while KMEB1 and KMEB2 cells were MAGE-A-positive both as EBs and teratomas. This divergence may be a matter of graft site rather than cell line characteristics, since HUES-9 was implanted under the kidney capsule, whereas KMEB1 and KMEB2 teratomas were formed subcutaneously. Although recent results from our laboratory have show that graft site did not influence HUES-9 teratoma composition at the histological level (Prokhorova et al., 2008). KMEB3, KMEB4 and KMEB5 did not express MAGE-A during differentiation, although these cell lines have been shown to give rise to cells of neuroectodermal lineage (M.K. unpublished data).

**Figure 3:** MAGE-A expression in neuroectodermal cells of EBs and teratomas.

Double-staining for MAGE-A and beta-tubulin 3 (A and B) or Pax6 (C and D) in EBs of HUES-9 showed co-localization in some (arrows), but not all, cells. Co-expression of MAGE-A (E), Pax6 (F), nestin (G) and tyrosine hydroxylase (TH) (H) in a segment of a KMEB2 teratoma, and co-expression of beta-tubulin 3 (I) and MAGE-A (J) in a segment of a KMEB1 teratoma. Magnification: ×40 (A–D), ×10 (E–H), ×5 (I and J).

**Figure 4:** MAGE-A expression in the developing nervous system.

(A) Distinct reactivity for MAGE-A in a longitudinal section of a 38 days old embryo. The neurons of the spinal cord (sc) and the spinal nerve fibers (sf) that innervate the myotomes (m) exhibit cytoplasmic staining. The myoblasts of the myotomes are also positive. (B) A transverse section of an 8-week-old fetus with MAGE-A staining in the lower brain stem (lbs), nervus facialis (nf) and the trigeminal ganglion (tg). Magnification: ×20.

**Figure 5:** GAGE expression in the developing ectoderm and neuroectoderm of a 5-week-old embryo.

Transverse section of the dorsal surface showing GAGE staining of the ectoderm (ecto) (A), a minor fraction of the neurons of the spinal ganglia (sg) (A) and the endfeet of radial glial cells (ge) (B). Parasagittal section with GAGE-positive ectoderm (C). sc, spinal cord. Magnification: ×2.5 (A), ×40 (B and C).
This suggests that MAGE-A proteins may be useful markers for selection of differentiated neurons from hESC cultures. Immunostaining of fetal tissues showed that GAGE proteins were expressed in early ectodermal and neuroectodermal tissues, but were not present in more differentiated cells of these lineages. Interestingly, the GAGE expression in neuroectodermal cells did not correlate with MAGE-A, as seen in germ cells. GAGE expression was no longer present in neuroectodermal cells when MAGE-A expression was first detected. In addition, GAGE proteins were limited to a subset of neuroectodermal cells, while MAGE-A seemed to be ubiquitously expressed among all cells of this lineage.

GAGE expression was also observed in the periphery of the fetal adrenal cortex in the brief period from Week 6 to Week 9 of development. During the same period, cell proliferation can be observed in the outer part of the adrenal cortex, and cortex cells form radial cords and increase in size from the periphery to the central region of the gland (Nussdorfer, 1986; Bocian-Sobkowska et al., 1997; Mesiano and Jaffe, 1997; Else and Hammer, 2005). This leads to a massive expansion of the cortex and the establishment of the outer definitive zone and the inner fetal zone. A peak in GAGE expression was seen around Week 7–8, when cell proliferation is at its highest. Furthermore, expression abruptly disappeared during Week 9 as the two adrenal cortex zones were formed. These results indicate that GAGE proteins may have a role in proliferation and differentiation of cells of the adrenocortical primordium and in the formation of the definitive and fetal zones.

We earlier reported that GAGE proteins are expressed in Sertoli and Leydig cells of the fetal testis for a limited period of time (Gjerstorff et al., 2007). The origin of these cell types is unresolved. Both gonadal endocrine cell types and the adrenocortical primordium have been suggested to be derived from either the coelomic epithelium (Mesiano and Jaffe, 1997; Karl and Capel, 1998; Else and Hammer, 2005; Payne and Hardy, 2007) or from the intermediate mesoderm (Else and Hammer, 2005). However, the currently prevailing
concept is that cell clusters in the intermediate mesoderm derived from the cranial part of mesonephros fuse during Week 5 to form the adrenocortical anlage, whereas the most caudal portions give rise to the gonadal rete blastema, from whence the gonadal endocrine cells are derived (Wrobel and Suss, 1999). The expression of GAGE during the development of adrenal cortex, Leydig and Sertoli cells may further indicate a common origin of these cells.

We and others have shown that CTAs are expressed in both male and female germ cells when they have settled in the fetal gonad (Gjerstorff et al., 2007; Nelson et al., 2007). In the current study, we examined whether CTAs are also expressed in earlier stages of germ line cells (e.g. PGCs). We analyzed tissues along the migratory pathway of PGCs, including dorsal mesentery, mesonephros, adrenal gland and pancreas, and found that PGCs of these tissues were MAGE-A- and GAGE-positive. Thus, MAGE-A- and GAGE-expression in the germ line may be initiated during PGC migration or earlier, suggesting that these CTAs are not specifically involved in the final differentiation steps of human germ cell development, but rather fulfill general roles in these cells. The tyrosine receptor kinase KIT has been shown to play an important role in germ cells during migration towards the gonadal anlage, and throughout the further development of these cells in the gonad (Mauduit et al., 1999; Hoyer et al., 2005). This fits with our data showing that MAGE-A, like KIT, is expressed in germ cells from the PGC stage to meiosis, since it has been demonstrated that MAGE-A expression is positively regulated by tyrosine receptor kinase KIT (Yang et al., 2007a, b, c). In this study, NY-ESO-1 expression was not seen in PGCs, in accordance with earlier results showing that NY-ESO-1 was first detected in germ cells at Week 9 (Gjerstorff et al., 2007). In the pancreas and adrenal cortex, CTA-positive PGCs were present up to Week 17 of fetal development, long after the migration of PGCs to the gonad has stopped. These cells have probably become misplaced during migration and perish soon after.

Several studies have shown that hESC are capable of forming germ cell progenitors during spontaneous in vitro differentiation (Toyooka et al., 2003; Clark et al., 2004; Geijsen et al., 2004; Aflatoonian and Moore, 2006; West et al., 2006). Based on our observations that PGCs express both MAGE-A and GAGE, we would expect that these CTAs would also be expressed in hESC-derived germ cell progenitors. However, although MAGE-A-positive cells were present in hESC EB cultures, GAGE proteins were not detected by RT–PCR or immunocytochemical analysis of several hESC lines. In contrast, a small number of scattered MAGE-A- and GAGE-positive cells were found in KMB1 teratomas. These cells appeared to express OCT4, which is normally expressed only in undifferentiated ES cells and PGCs (Hay et al., 2004; Rajpert-De Meyts et al., 2004). In addition, the cells were located beneath HNF4 alpha-positive epithelium. HNF4 is normally present in hepatocytes, but is also expressed in cells of the yolk sac, where early PGCs reside (Duncan et al., 1994; Watt et al., 2003). Therefore, we speculate that the MAGE-A- and GAGE-positive cells of teratomas may be germ cell progenitors influenced by these HNF4 alpha-positive epithelial cells.

In conclusion, we have shown that CTAs, in addition to their role in cells of the germ line, are involved in early development of various types of somatic cells. Furthermore, their time restricted and tissue-specific expression during early differentiation suggest that CTAs may be implicated in specialization of these cell types. On a more specific note our results suggest that MAGE-A proteins may be useful markers in stem cell research for selection of differentiated neurons from hESC cultures.

Supplementary material
Supplementary material is available at HUMREP Journal online.

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