Chromosome X-encoded cancer/testis antigens show distinctive expression patterns in developing gonads and in testicular seminoma

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BACKGROUND: Cancer/testis (CT) antigens are cancer antigens normally expressed in adult testicular germ cells. The expression of chromosome X-encoded CT antigens (CT-X antigens) in human fetal gonads and in testicular seminomas was examined.

METHODS: The expression of 10 CT-X antigens (MAGEA, NY-ESO-1, GAGE, CT7/MAGEC1, CT10/MAGEC2, CT45, SAGE1, SSX2, NXF2 and SPANX) was studied immunohistochemically.

RESULTS: In adult human testis, SPANX is expressed in late spermatids and spermatozoa, whereas all other CT-X antigens are predominantly expressed in spermatogonia or primary spermatocytes. All CT-X antigens except SPANX are expressed in human fetal germ cells. CT-X-positive germ cells appear as early as 13 weeks after gestation, increase with age and reach a plateau at around 22 weeks. In the fetal ovary, CT-X-positive oogonia are most abundant at around 24 weeks and sharply decrease subsequently. CT-X antigens are almost exclusively expressed in OCT3/4-negative gonocytes and their expression appears to coincide with the loss of pluripotency. Spermatocytic seminoma, a neoplasm derived from adult pre-meiotic germ cells, showed uniform expression of all CT-X antigens except SPANX. In contrast, most seminomas (>80%) express CT7, CT45, GAGE and CT10 but express MAGEA, NXF2 and NY-ESO-1 at lower frequency, and very rarely express SSX2 and SAGE1.

CONCLUSIONS: Most CT-X antigens are expressed in human fetal germ cells after they have lost stem cell characteristics, with predominant expression in pre-meiotic germ cells. Spermatocytic seminomas showed expression of all CT-X antigens except SPANX, whereas classical seminomas only express some CT-X antigens, reflecting their different origins from adult versus fetal germ cells.

Key words: tumor antigen / cancer vaccine target / germ cell tumor / seminoma

Introduction

Cancer/testis (CT) antigens were initially identified during the search for immunogenic tumor antigens capable of eliciting spontaneous immune responses in patients with cancer. A study of MAGE, BAGE and GAGE antigens (Boon et al., 1997), the first group of tumor antigens shown to elicit cell-mediated immune responses in melanoma patients, showed mRNA expression limited to testis and no expression in any other normal adult tissue. Subsequent serological cloning of antigens that elicited antibody responses in patients with cancer identified SSX, NY-ESO-1 and CT7, all of which also shared this distinctive characteristic of testis-restricted expression and aberrant activation in various types of human cancer. This unique feature led us to designate this group of antigens as CT antigens, recognizing them as attractive targets for immunotherapy, particularly for therapeutic cancer vaccines (Simpson et al., 2005; Caballero and Chen, 2009).

The distinctive CT mRNA expression pattern also provided in silico analytic tools and led to subsequent identification of many novel genes with similar characteristics, and the number of CT and CT-like genes in the literature increased from 20 in 2002 (Scanlan et al., 2002), 44 in 2004 (Scanlan et al., 2004), to more than 110 in the most recent version of CTpedia, a CT-database established by the Ludwig Institute for Cancer Research (http://www.cta.lncc.br/; Hofmann et al., 2008; Almeida et al., 2009). Of these genes, around 30 were found to be on chromosome X, many of them existing as multigene families as a result...
of recent gene duplication. These CT genes on chromosome X, referred to as CT-X genes (versus non-X CT genes; Simpson et al., 2005; Hofmann et al., 2008), included most of the CT antigens with documented spontaneous immunogenicity in human, e.g. MAGEA, NY-ESO-1, SSX, GAGE, CT7, CT10, as well as other newer members for which the immunogenicity was less well studied, including SAGE1, CT45, SPANX and NXF2.

Although testicular expression is the defining characteristic of CT antigens, this criterion has only been met based on mRNA analysis for many CT genes and the presence of protein in testis and/or cancer has only been confirmed for a small subset of CT antigens, e.g. MAGEA, NY-ESO-1, GAGE, CT7, CT10, CT45 and SPANX. Among these, SPANX is involved in the morphogenesis of the mature spermatozoa (Zendman et al., 2003) and is expressed in the post-meiotic germ cells, mainly in spermatids and spermatozoa. In contrast, the functions of other CT-X genes are mostly unknown, and most of them are expressed in the pre-meiotic germ cells, mainly in the spermatogonia stage. Even within the group expressed in pre-meiotic germ cells, however, different expression patterns have been observed; CT45, for instance, is most prominently expressed in primary spermatocytes (Chen et al., 2009), and CT7, unlike most other CT-X antigens that are nuclear proteins, is located exclusively as a cytoplasmic protein in testis (Jungbluth et al., 2002). These differences implicate that CT-X antigens, despite their shared chromosomal localization and characteristic CT expression, are functionally heterogeneous.

To investigate the possible function of CT-X antigens, the protein expression of MAGEA, NY-ESO-1, CT7 and GAGE has been examined in developing gonads in two previous studies (Gjerstorff et al., 2007, Nelson et al., 2007). In the fetal ovary, CT7 was shown to be expressed in oogonia but not in the primary oocytes in the primordial follicles (Nelson et al., 2007). Similarly, MAGEA, NY-ESO-1 and GAGE expression has been detected in both fetal testis and ovary and the differences in their temporal and spatial distributions were described (Gjerstorff et al., 2007). The Gjerstorff et al. (2007) study, however, was limited to the analysis of MAGEA, NY-ESO-1 and GAGE, and Nelson et al. (2007) evaluated only human fetal ovarian expression, but not testicular expression. To systematically analyze the expression of CT-X antigens in adult human testis and also during human fetal gonad development, we have established a large panel of anti-CT antibodies and the expression of 10 CT-X proteins was evaluated by immunohistochemical (IHC) analysis. In addition, the expression of 10 CT-X proteins was analyzed in testicular seminoma. Based on these data, we found CT-X antigens to be a heterogeneous group of proteins with individual, distinctive patterns of expression during germ cell development of both testis and ovary, and this difference is also reflected in their distinct expression patterns in spermatocytic and classical seminomas.

### Materials and Methods

#### Tissues

Archival formalin-fixed paraffin-embedded tissue blocks of normal and tumor tissues, including paraffin blocks of human fetal tissues from stillbirth and fetuses aborted for various medical indications, were obtained from Department of Pathology and Laboratory Medicine at New York Presbyterian Hospital-Weill Cornell Medical Center following approval by the Institutional Review Boards. All hematoxylin-and-eosin stained tissue sections were evaluated and only specimens that showed normal histology and no or minimal autolysis were used for the study. For adult testis, tissue blocks from eight male individuals were used. For fetal CT-X antigen expression, 12 fetal testes and 12 fetal ovaries with gestational ages ranging from 13 weeks to full term (40 weeks) were identified and used for this study.

#### Monoclonal and polyclonal antibodies

The antibodies used are summarized in Table I. Antibodies against GAGE, SAGE1, MAGEA, SPANX and OCT3/4 were purchased commercially. GAGE antibody, produced against GAGE-7, is expected to react with all GAGE gene products owing to the extreme high sequence homology among the GAGE proteins. MAGE-A monoclonal antibody 6C1, produced against MAGE-A1, has been shown to be broad reactive for gene products of MAGEA multigene family, including MAGE-A1, A2, A3, A4, A6, A10 and A12 proteins (Rimoldi et al., 2000). SPANX antibody was produced against SPANX-C. However, because of the polyclonal nature of this antibody, it is highly likely that this antibody would also recognize SPANX-A and SPANX-B proteins.

Antibodies against the other CT-X antigens NY-ESO-1, SSX2, CT7, CT10, CT45 and NXF2 were produced and characterized in our

### Table 1 Antibodies used in the study of CT antigens in human gonads.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Origin</th>
<th>Code</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAGEA</td>
<td>Mouse</td>
<td>6C1</td>
<td>1 µg/ml</td>
<td>Santa-Cruz Biotech, Santa Cruz, CA, USA</td>
</tr>
<tr>
<td>NY-ESO-1</td>
<td>Mouse</td>
<td>E978</td>
<td>1 µg/ml</td>
<td>Jungbluth et al., 2001*</td>
</tr>
<tr>
<td>GAGE</td>
<td>Mouse</td>
<td>Clone 26</td>
<td>0.1 µg/ml</td>
<td>BD Biosciences, San Jose, CA, USA</td>
</tr>
<tr>
<td>CT7</td>
<td>Mouse</td>
<td>CT7-33</td>
<td>0.1 µg/ml</td>
<td>Jungbluth et al., 2002*</td>
</tr>
<tr>
<td>CT10</td>
<td>Mouse</td>
<td>LX-CT10-5</td>
<td>3.0 µg/ml</td>
<td>Zhuang et al., 2006*</td>
</tr>
<tr>
<td>CT45</td>
<td>Mouse</td>
<td>LX-CT45-10</td>
<td>1:5000 (ascites)</td>
<td>Chen et al., 2009*</td>
</tr>
<tr>
<td>SAGE1</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>1.5 µg/ml</td>
<td>Sigma-Aldrich, St. Louis, MO, USA</td>
</tr>
<tr>
<td>SSX2</td>
<td>Mouse</td>
<td>LS-SSX2-4</td>
<td>10 µg/ml</td>
<td>*</td>
</tr>
<tr>
<td>NXF2</td>
<td>Mouse</td>
<td>LX-NXF2-1</td>
<td>1:500 (ascites)</td>
<td>*</td>
</tr>
<tr>
<td>SPANX</td>
<td>Mouse</td>
<td>Polyclonal</td>
<td>2.5 µg/ml</td>
<td>Rockland Imm., Gilbertsville, PA, USA</td>
</tr>
<tr>
<td>OCT3/4</td>
<td>Mouse</td>
<td>sc-5279</td>
<td>1.0 µg/ml</td>
<td>Santa-Cruz Biotech, Santa Cruz, CA, USA</td>
</tr>
</tbody>
</table>

*Research antibodies produced in our laboratory.
laboratory. Antibodies against NY-ESO-1, CT7, CT10 and CT45 have been previously described (Jungbluth et al., 2001, 2002; Zhuang et al., 2006; Chen et al., 2009). For generating SSX2 and NXF2 monoclonal antibodies, full-length SSX2 and NXF2 cDNA sequences were cloned into a prokaryotic expression vector pQE30 (Qiagen) and subsequent induction of recombinant protein synthesis and purification by Ni²⁺ affinity chromatography were performed as previously described (Chen et al., 1994). Mouse monoclonal antibodies were then produced and characterized following previously described protocols (Chen et al., 2009). The specificity of the SSX2 and NXF2 monoclonal antibodies was confirmed by positive IHC staining in testicular germ cells (see text below) and negative staining in a panel of normal adult tissues that included the esophagus, stomach, colon, pancreas, liver, bladder, breast, prostate, uterine cervix, uterus (proliferative and secretory endometrium), kidney, lung, thyroid and skin. SSX2 is a member of the SSX multigene family and the possibility that the anti-SSX2 antibody might cross-react with other SSX proteins for example SSX1 and SSX4 cannot be excluded.

IHC analysis

IHC analysis was performed using formalin-fixed paraffin-embedded tissues. Whole sections were used for fetal gonad study and protein in seminoma was initially evaluated using a tissue microarray (TMA) in which each case was represented by three 0.6-mm tissue cores. The TMA included 72 seminomas and a single case of spermatocytic seminoma. An additional case of spermatocytic seminoma was subsequently identified and analyzed in whole sections. No intratubular germ cell neoplasia was included in the seminoma tissue cores. Tissue sections (5 μm) on coated slides were deparaffinized, rehydrated and treated in H₂O₂ to block the endogenous peroxidase activity. The sections were then subjected to antigen retrieval by autoclaving for 15 min in 10-mM citrate buffer, pH 6.0. The sections were incubated with the primary antibody (or pertinent negative controls, i.e. mouse IgG control or pre-immune rabbit serum) for 1 h at room temperature, followed by detection using DAKO Envision + horse-radish peroxidase mouse (or rabbit) detection system (DakoCytomation) and 3,3’-diaminobenzidine (DAB) as the chromogen. The slides were counterstained with hematoxylin and evaluated. In testis, the number of positive gonocytes was counted in 20 cross-sectional tubules and the expression was scaled as + (≤2 positive cells/tubule), ++ (3–5/tubule) or ++++ (>5/tubule). Tubules that were sectioned longitudinally or tangentially were not included for the grading and the tubules with visibly smaller diameters than most other tubules were assumed to be sections from the tip of the tubules and not included. In ovary, the extent of expression was estimated based on the average number of positive cells per 400× high-power field (HPF) and scaled as +/− (rare positive cells, <1/HPF), + (<10/HPF), ++ (10–30/HPF) and ++++ (>30/HPF).

For double immunostaining, the first antibody staining was performed as above, using DAB as chromogen. After DAB chromogen reaction, the slides were washed with Tris-buffered saline pH 7.0, blocked by Dual Endogenous Enzyme Block (DakoCytomation) and incubated with the second antibody for 30 min at room temperature, followed by detection using LSAB 2 System-Alkaline Phosphatase (DakoCytomation) and developed by Permanent Red chromogen. The slides were then counterstained and scored as described above.

Results

CT-X antigens in human fetal testis

The presence of CT-X antigens was evaluated in fetal testis of 13-, 18-, 21-, 22-, 32- and 40-week gestation. The results are summarized in Table II and examples of the IHC staining are shown in Fig. 2. At 13 weeks, immature germ cells positive for MAGEA, NY-ESO-1, GAGE, CT7, CT10, CT45 or NXF2 were identified in some but not all testicular cords. The number of germ cells positive for these CT-X antigens increased in later gestation and germ cells positive for these CT-X antigens were noted in almost every testicular cord in testis from 22-week gestation onwards. The number appeared to plateau after 22 weeks and no difference was noted between testes at 32- and 40-week gestation. In comparison to MAGEA, NY-ESO-1, GAGE, CT7, CT10, CT45 and NXF2, however, SPANX expression was not detected in any fetal testis, and SAGE1 and SSX2-positive cells were rare in 13-week testis (<5 cells on the entire section of testis). These SAGE1 and SSX2-positive germ cells, SSX2 in particular, increased in number in later gestation but remained fewer than those positive for other CT-X antigens.

Besides SAGE1 and SSX2, the relative abundance of germ cells positive for other CT-X also differs in the developing testis cords, particularly before 20-week gestation. During early gestation, GAGE-, CT7-, CT45- and NXF2-positive cells were most abundant, followed by MAGEA, CT10 and NY-ESO-1 (in that approximate order). CT7- and GAGE-positive germ cells consistently outnumbered MAGEA- or NY-ESO-1-positive cells throughout fetal development and these findings were confirmed by double-staining experiments (Fig. 3A and B). Similarly, MAGEA-positive/NY-ESO-1-negative cells were easily identified in fetal testis (Fig. 3C).

In addition to the expected expression in developing germ cells, expression of GAGE and CT7, but not other CT-X antigens, was detected in non-germ cells in developing testis. Pre-Leydig cells, the stromal cells in the testicular interstitium between seminiferous tubules, showed strong expression of GAGE in the 18-week testis, also positive but weaker in 13 weeks, and became mostly negative
in testis 21-week gestation and later (Fig. 2). Pre-Sertoli cells, located in the seminiferous tubules between germ cells, also expressed GAGE up to 22-week gestation but were negative in the 32-week and full-term testis (Fig. 2). In addition to GAGE, pre-Sertoli cells in the 21- and 22-week testis showed weak staining of CT7 but were CT7 negative in 13-, 18-, 32- or 40-week testis. Unlike GAGE, pre-Leydig cells did not express CT7 at any gestational age (Fig. 2).

**CT-X antigens in human fetal ovary**

The localization of CT-X antigens in fetal ovaries of 16-, 19-, 20-, 24-, 34- and 39-week gestation was analyzed and is summarized in Table III. Examples of immunostaining are shown in Fig. 4. As in the fetal testis, different temporal and spatial distributions were observed, separating these CT-X antigens into a few groups. No SPANX or SSX2 protein was detected in any fetal ovary examined. In contrast, all other eight CT-X antigens were detectable in oogonia (and possibly early oocytes) at 16-week gestation and could be identified in ovarian cortex throughout gestation. However, while CT-X-positive gonocytes in the fetal testis were highest in number during second and third trimesters, these antigens were most abundant in the fetal ovary at around 24 weeks of gestation, with a major decrease observed at 34-week gestation, and many of them, including...
Table II Expression of CT antigens in human fetal and adult testis.

<table>
<thead>
<tr>
<th>Gestational age (weeks)</th>
<th>13</th>
<th>18</th>
<th>22</th>
<th>32</th>
<th>40</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-meiotic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT7/GAGE</td>
<td>+++a</td>
<td>+++a</td>
<td>+++a</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>CT45/NXF2</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>NY-ESO-1/MAGEA/CT10</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>SAGE1/SSX2</td>
<td>+/-b</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>SPANX</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Post-meiotic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CT7 and GAGE are also expressed in pre-Sertoli and/or pre-Leydig cells in 13-, 18- and 22-week testes (see Fig. 2).

b+/−, very rare positive cells, <10 positive cells on entire section; + to +++, weak to strong expression, based on percentage of tubules with positive gonocytes and number of positive gonocytes per tubule.

Figure 2 CT-X antigens in human fetal testis, detected by immunohistochemistry. The expression patterns of MAGEA, CT7 and GAGE are illustrated. Positive staining was observed for all three antigens in gonocytes at 13-week gestation and reached a plateau after 22 weeks. CT7 and GAGE were more abundant than MAGEA in 13- and 18-week testes. GAGE was also present in pre-Sertoli cells (in tubules between gonocytes) up to 22-week gestation and in pre-Leydig cells (between the tubules) up to 18-week gestation. Transient expression of CT7 was also observed in pre-Sertoli cells at 22 weeks, but not in pre-Leydig cells. The 100-μm scale bar shown in the last panel is applicable to all panels.
MAGEA, NY-ESO-1, CT10 and CT45, were almost absent in the ovary of 39-week gestation.

These eight CT-X antigens with oogonia expression (MAGEA, GAGE, NY-ESO-1, CT7, CT10, CT45, SAGE1 and NXF2) could be further separated into two groups based on their expression in the oocytes in the primordial follicles. MAGEA, NY-ESO-1, CT10, CT45 and SAGE1 were expressed in oogonia (and likely early oocytes) but not in oocytes in primordial follicles, whereas GAGE, CT7 and NXF2 were expressed both in oogonia and in oocytes (Fig. 4, bottom row). GAGE, while detected as a nuclear protein in a very small subset of oogonia, was present in large amounts in a proportion of oocytes in the 34- and 39-week ovaries but as a cytoplasmic protein (Fig. 4, inset). CT7, in addition to abundant expression in oogonia, also showed expression in oocytes but in a smaller subset than the GAGE-positive population. NXF2 was the most abundantly expressed CT-X gene in the oocytes, detectable as a nuclear protein in almost all oocytes in primordial follicles at 39-week gestation (Fig. 4, inset).

Table III Expression of CT antigens in human fetal ovary.

<table>
<thead>
<tr>
<th>Gestational age (weeks)</th>
<th>16</th>
<th>19</th>
<th>20</th>
<th>24</th>
<th>34</th>
<th>39 oogonia</th>
<th>39 oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT7</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MAGEA/CT10/CT45</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>+/-b</td>
<td>–</td>
</tr>
<tr>
<td>NXF2</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>GAGE</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
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<td>+++</td>
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<td>NY-ESO-1</td>
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<td>+/−</td>
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<td>−</td>
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<td>SAGE1</td>
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<td>+/-</td>
<td>++</td>
<td>−</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SSX2/SPANX</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

*aCT7 also expressed in supporting cells in the sex cords (see Fig. 4).

b+/−, very rare positive cells; + to ++++, weak to strong expression, based on number of positive oogonia; expression in oocytes in primordial follicles listed for 39-week ovary.
The number of developing oogonia with positive staining for individual CT-X was highly variable, as we observed in testicular gonocytes. CT7 was the most abundant, followed by MAGEA, NXF2, CT45 and CT10, while GAGE, NY-ESO-1 and SAGE1-positive oogonia were least abundant. The differential expression was also confirmed by double staining of CT7/MAGEA, CT7/NY-ESO-1 or CT7/GAGE, as illustrated by many CT7-positive/MAGEA-negative oogonia in Fig. 3D. Of interest, GAGE, while more prominently expressed than MAGEA, CT10 and CT45 etc. in the fetal testis (see above), was expressed in much fewer germ cells than those antigens in the fetal ovary.

In addition to their localization in germ cells, CT7 was detected in the pre-granulosa cells that surround the oogonia in the sex cords and this was seen throughout ovaries from 16-, 20- and 24-week gestation (Fig. 4, left column). Interstitial stromal cells between the sex cords did not contain CT7 or any other CT-X antigens at any stage of gestation. This non-germ cell expression of CT-X in the fetal ovary was also observed for GAGE (low abundance in pre-granulosa cells at 24 weeks, see Fig. 4), but not for any other CT-X antigens tested.

CT-X antigens in human testicular seminoma

The presence of CT-X antigens in germ cell tumors was examined in 74 testicular seminomas, including 72 classical seminomas and two spermatocytic seminomas (Fig. 5). The classical seminomas were
tested in a tissue array, whereas whole sections were examined in the two spermatocytic seminomas.

Scoring of the classical seminoma tissue array revealed four different levels of CT-X expression frequency: (i) positive in the vast majority (>80%) of cases: CT7 (67/73), CT45 (67/73), GAGE (63/73) and CT10 (62/73); (ii) positive in >10% but <80% of cases: MAGEA (44/73), NXF2 (17/73), NY-ESO-1 (12/73); (iii) weak, focal positive in very few (<10%) cases of classical seminomas but strongly positive in spermatocytic seminoma: SSX2 (5/73) and SAGE1 (3/73) and (iv) negative in all cases: SPANX.

For spermatocytic seminoma, both cases examined showed diffuse (>90%) strong staining for 8 of 10 CT-X evaluated, moderate expression of SSX2 in 50–70% of tumor cells and no expression of SPANX. In comparison, most classical seminomas, even when CT-X positive, often showed less intense and more heterogeneous staining patterns (Fig. 5).

Co-staining of OCT3/4 and CT-X in human fetal gonads and in seminoma

Germ cells in early fetal development express the pluripotent stem cell marker OCT3/4 in the nuclei and the relationship between OCT3/4 and CT-X expression in fetal gonads was examined by double staining for OCT3/4 and CT-X antigens, including MAGEA, GAGE, CT7, CT10, CT45 and NY-ESO-1 (Fig. 6). The results showed that the vast majority of CT-X-positive gonocytes showed no OCT3/4 expression, therefore showing a mutually exclusive pattern. However, GAGE, although present in a mutually exclusive manner with OCT3/4 in the fetal ovary, showed co-expression with OCT3/4 in a small subset of germ cells in all four fetal testes examined (Fig. 6C inset).

Reflecting their expression in different cell populations, OCT3/4-positive cells and CT-X-positive cells also had different spatial distributions anatomically. In testicular cords, CT-X-positive germ cells were located more peripherally than OCT3/4-positive cells and most of the CT-X-positive cells but not OCT3/4-positive cells were anchored to the basal lamina, as illustrated in Fig. 6A–C. In the fetal ovary, OCT3/4-positive cells had the highest concentration in superficial cortex, immediately beneath the coelomic epithelium. In contrast, CT-X-positive oogonia and oocytes were more abundant in the inner part of the ovarian cortex and in medulla (Fig. 6D).

This mostly mutually exclusive expression of OCT3/4 and CT-X antigens in developing germ cells, however, was not observed in classical seminoma. All classical seminomas expressed OCT3/4 and CT-X antigens in developing germ cells, however, was not observed in classical seminoma. All classical seminomas expressed OCT3/4 and double staining of the seminoma tissue array for OCT3/4 and CT7 (or OCT3/4 and MAGEA) showed tumor cells to be diffusely positive for both markers in many cases. Both cases of spermatocytic seminomas, expressing many CT-X antigens (see above), did not express OCT3/4.

Discussion

In the present study, we have utilized a panel of antibodies to evaluate the protein expression and localization of CT-X antigens in fetal gonads during development. Immunofluorescent techniques, although could potentially be more superior to IHC analysis, particularly in the double-labeling experiments, was found to generate high background
on the formalin-fixed archival materials for most antibodies used in the study. Because of this technical difficulty, the entire study was carried out using IHC methodologies. We found that all CT-X antigens except SPANX were switched on in prenatal gonads after stem cell marker OCT3/4 was switched off and many of them were detectable by 13-week gestation. The CT-X antigens are mainly expressed in pre-meiotic germ cells in the fetal testis and ovary and appear to be switched off before meiosis occurs. Exceptions to this do occur, and oocytes in primordial follicles can express NXF2, CT7 and GAGE. Despite the overall similar expression pattern of most CT-X antigens in fetal gonads, the frequency of germ cells expressing individual CT-X antigens is highly variable and this difference is reflected in their frequency of detection in testicular classical seminomas. In contrast, spermatocytic seminoma expresses all CT-X antigens (except SPANX), reflecting their origin from adult testicular germ cells.

Previous analysis of CT-X protein expression in adult testis showed that most CT-X antigens, including MAGEA, NY-ESO-1, GAGE, SAGE1, CT7 and CT10, are mainly expressed in spermatogonia, the self-renewing progenitor cell population in adult testis (Jungbluth et al., 2000, 2001, 2002; Gjerstorff et al., 2006; Zhuang et al., 2006). This has led to the hypothesis that most CT-X antigens in testicular germ cells may be activated in a co-ordinated manner by a single ‘master switch’, such as transcriptional factor CTCF/BORIS (Vatolin et al., 2005) or by regulatory events that are known to activate CT-X genes, e.g. hypomethylation. This, however, turned out to be an oversimplified assumption, as our findings have demonstrated CT-X antigens as a heterogeneous group of germ cell differentiation antigens, with individually distinctive expression patterns in developing gonads, and some of these differences are also reflected in their expression in seminoma.

In the fetal testis, all germ cells are derived from OCT3/4-positive primordial germ cells (PGCs) that have migrated to the gonads from the dorsal yolk sac at around 5 weeks of gestation. As these OCT3/4-positive pluripotent cells settle into gonocytes and develop into germ cells, the expression of OCT3/4 is lost (Looijenga et al., 2003; Rajpert-De Meyts et al., 2004). In the current study, we found that most CT-X antigens are expressed in the OCT3/4-negative germ cells, indicating that the switch-on of CT-X antigen expression likely follows the loss of pluripotency in these stem cells and signals a commitment to germ cell lineage. Our data confirmed the previously described mutually exclusive expression between OCT3/4 and MAGEA4, OCT3/4 and NY-ESO-1 (Gjerstorff et al., 2007), and we found this sequential expression of OCT3/4 and CT-X also to be true for CT7, CT10 and CT45 and presumably also other CT-X genes. Based on this sequential expression of OCT3/4 and MAGEA4, Gaskell et al. (2004) have previously suggested that germ cell precursors in the fetal testis go through three stages of maturation from OCT3/4+ MAGEA4− to OCT3/4-MAGEA4− to OCT3/4-MAGEA4+. In our study, we found many MAGEA-negative/CT7-positive germ cells in early fetal testis but only
MAGEA-positive/CT7-positive spermatogonia in adult testis. This would support the notion that MAGEA-negative pre-spermatogonia in prenatal life eventually mature into MAGEA-positive spermatogonia in the post-natal testis and CT7 (and/or GAGE) positivity could be the marker of this OCT3/4 and MAGEA4 dual negative, intermediate stage pre-spermatogonia population. In comparison with CT7 and MAGEA, however, not all CT-X antigens with a predominant expression in spermatogonia are expressed by all spermatogonia in the adult testis. For instance, MAGEA-positive/NY-ESO-1-negative spermatogonia can be easily identified in both the fetal and adult testis. This may indicate that NY-ESO-1 is only expressed in a subset of germ cells both in fetus and in adult, or that adult spermatogonia, while perpetually positive for CT7 and MAGEA, go through phases that are NY-ESO-1 positive and NY-ESO-1 negative.

Our findings indicate that CT-X antigens can be separated into several groups based on their temporal emergence during testicular germ cell development. GAGE-positive germ cells likely appear first in the fetal testis, present in a small subset of OCT3/4-positive cells in all fetal testes examined. This was followed by the expression of CT7, CT10, CT45, MAGEA, SAGE1 and NY-ESO-1, all of them detectable in 13-week fetal testis but at different levels of abundance. The SSX gene family members, including SSX2 and SSX4 (data not shown), showed very limited expression in fetal germ cells, in contrast to their significant expression in adult spermatogonia. The SPANX family, a group of genes involved in the morphogenesis of mature sperm cells (Zendman et al., 2003), is not expressed in prenatal testis or ovary.

CT-X antigens not only emerge at different times during germ cell development but also are switched off at different times and this was best observed in the fetal ovary. While the prenatal testis is populated exclusively by pre-meiotic germ cells, the fetal ovary consists of both pre-meiotic oogonia and primary oocytes that can be pre-meiotic in meiosis, arrested at the diplotene phase of meiosis (in which they will remain into adult life), or are undergoing atresia (Konishi et al., 1986; Mueller, 2001). The PGCs and oogonia are located in the superficial ovarian cortex and they actively proliferate and migrate toward medulla as they mature into primary oocytes. This is confirmed by our finding that OCT3/4-positive cells are mostly located in the peripheral zone in the fetal ovary, as was previously observed (Stoop et al., 2005). In contrast, CT-X-positive oogonia and oocytes are OCT3/4 negative and are more centrally located. In the fetal testis, the CT-X-positive germ cell population reaches a plateau at around 22 weeks and this population would persist until birth. In comparison, oogonia that are positive for MAGEA, NY-ESO-1, CT7, CT10, SAGE1 and/or CT45 peaked in ovaries at around 24 weeks of gestation, largely coinciding with the peak of germ cells in ovary (Mueller, 2001), and then decreased precipitously, and very few cells were positive to this group of CT-X antigens at 39 weeks. This finding suggests that these CT-X genes are mainly, some likely exclusively, expressed in pre-meiotic oogonia and early oocytes. This is also consistent with the finding in adult testis, in which expression of these antigens is restricted to pre-meiotic sperm cells. An exception to this notion, however, was the prominent expression of GAGE and NXF2 (and CT7 to a lesser extent) in oocytes in the primordial follicles, suggesting that these three CT antigens may have distinctive functions in oocytes.

An additional piece of evidence for heterogeneous CT-X gene expression was the presence of GAGE and CT7 in the non-germ cell population of fetal gonads, albeit transiently. Confirming the findings of Gjerstorff et al. (2007), we found GAGE to be expressed in pre-Sertoli and pre-Leydig cells in the fetal testis between 13 and 22-week gestation but disappeared in later gestation. In addition, we also detected CT7 transiently in pre-Sertoli cells in testis and in the pre-granulosa cells in the developing ovigerous cords in the fetal ovary but not in pre-Leydig cells or interstitial ovarian stromal cells. None of the CT-X antigens were detected by IHC outside the gonads in a panel of normal fetal tissues that were 13 weeks and older (data not shown) and the presence of CT7 and GAGE in the non-germ cell population of fetal gonads is the only known example of somatic expression of this group of CT-X antigens to date. Although Gjerstorff et al. (2008) reported GAGE expression in neuroectodermal cells and adrenal cortex of 6–9-week-old embryos, we were not able to evaluate embryonic tissues in this study and we did not detect GAGE expression in the adrenal cortex and brain from fetuses 13 weeks and older.

As germ cell lineage-specific antigens, one might expect CT-X antigens to be expressed at high frequency in their neoplastic counterparts and this hypothesis was investigated in testicular seminoma. Seminomas can be separated into classical seminoma and spermatocytic seminoma, and these two entities are clinically and histologically distinctive. Spermatocytic seminoma is clinically a benign neoplasm of older adults, with the peak incidence three decades later than the classical seminoma, which is a malignant germ cell tumor that occurs mostly in young adults during their second and third decades of life (Eble, 2004). PGCs, also referred to as gonocytes once they migrate to gonads, are believed to be the cells of origin in classical seminoma. In contrast, spermatocytic seminoma originates from adult spermatogonia and/or spermatocytes (Looijenga et al., 2007). This biological difference between classical and spermatocytic seminoma was confirmed by cDNA microarray analysis in which spermatocytic seminomas were clearly separated from classical seminomas by unsupervised clustering (Looijenga et al., 2006). Interestingly, spermatocytic seminomas showed higher expression of several CT antigens than classical seminoma in the microarray study by Looijenga et al. (2007) and the list included GAGE, SSX, SAGE1 and MAGEA family genes. In agreement with these data, previous IHC analyses have also shown ubiquitous expression of SSX (Stoop et al., 2001) and MAGEA (Rajpert-De Meyts et al., 2003) in spermatocytic seminoma. This observation is now further expanded by our protein expression data that showed diffuse strong expression of all CT-X antigens except SPANX in spermatocytic seminoma. In contrast, classical seminomas showed a highly variable frequency of expression of individual CT-Xs, ranging from 80 to 90% of cases for CT7, GAGE, CT45 and CT10, 60% for MAGEA, to ~20% for NY-ESO-1 and weak focal expression of SAGE1 and SSX2 in <10% of the cases. This range of CT-X frequency in classical seminoma roughly reflected the relative abundance of these CT-X antigens in fetal germ cells. One major difference between the fetal germ cells and their neoplastic counterparts, however, is the co-expression of OCT3/4 and CT-X antigens. Since OCT3/4 is switched off before the emergence of almost all CT-X antigens, co-expression of OCT3/4 and CT-X antigens, such as CT7 and MAGEA, is an abnormal phenotype only observed in neoplastic germ cells. It is possible that germ cell tumors may be derived from OCT3/4-positive and CT-X-negative gonocytes but these tumor precursor cells then return to their normal differentiation pathway and...
acquire the CT-X-positive phenotype, while retaining OCT3/4 expression. Alternatively, it is possible that germ cell tumors may be derived from the more mature CT-X-positive germ cells, with aberrant re-activation of the silenced OCT3/4 gene. Examination of CT-X expression in non-seminomatous germ cell tumors and in their in situ components, the intratubular germ cell neoplasia, should help to distinguish these two possibilities and these experiments are ongoing.

**Authors’ roles**

Y.-T.C. involved in the study design and data interpretation; drafting, revising and final approval of manuscript R.C. and P.L. involved in the data acquisition and analysis; critical reading, revising and final approval of manuscript. D.B. and B.J. contributed in providing material/reagent critical for data acquisition; critical reading, revising and final approval of manuscript. L.J.O. involved in the study design and data interpretation; critical reading, revising and final approval of manuscript.

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