Spatial expression of germ cell markers during maturation of human fetal male gonads: an immunohistochemical study

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BACKGROUND: The aim of the present study was to examine fetal male germ cells for expression of proteins associated with differentiation and maturation and to compare them with morphologically defined subpopulations. METHODS: Testes of 61 fetuses from week 12 of gestation to the newborn period were selected. Immunohistochemistry was performed using antibodies to proteins associated with differentiation of germ cells (c-KIT, AP-2γ) or pluripotency (OCT3/4), oncofetal protein M2A and spermatogonial marker MAGE-A4. RESULTS: Two subtypes of fetal germ cells were detected by quantification and immunohistochemistry. Nearly all germ cells with morphological criteria of gonocytes and intermediate cells co-expressed OCT3/4, c-KIT, M2A and AP-2γ. Starting from week 12, their number increased up to week 18/19 and then declined continuously during further development. After week 25, pre-spermatogonia were predominant and expressed MAGE-A4 selectively. CONCLUSIONS: Fetal male germ cells are comprised of two major groups with distinct immunohistochemical phenotypes. Germ cells that are predominantly found before week 25 of gestation co-express oncofetal proteins OCT3/4, c-KIT, M2A and AP-2γ. After week 25, most germ cells have lost their pluripotent potential and acquire a spermatogonial phenotype defined by expression of MAGE-A4.

Key words: AP-2γ/MAGE-A4/M2A/gonocyte/pre-spermatogonia

Introduction

Primordial germ cells (PGC) are pluripotent cells that migrate to the genital ridge where they are called gonocytes in male and oogonia in female gonads. In female and male murine embryos, germ cells then first undergo several rounds of mitosis before they enter a pre-meiotic stage by 12.5 days post-conception by up-regulation of meiotic genes such as Sce3 (Di Carlo et al., 2000). In male mice, block of meiosis is accompanied by Sertoli cell differentiation and the occurrence of pre-spermatogonia (McLaren, 2003). Unfortunately, the maturation process of fetal germ cells in humans is not fully understood although previous studies indicate that, in contrast to rodents, human fetal germ cells are non-homogeneous in terms of morphology and marker expression (Fukuda et al., 1975; Wartenberg, 1976; Franke et al., 2004; Gaskell et al., 2004). For example, from an examination of 25 embryonic and fetal testes, Gaskell et al. (2004) detected three different germ cell populations with different combinations of immunohistochemical markers.

Detailed knowledge of the regular differentiation process is the prerequisite for identifying pathological changes in germ cell differentiation. For example, developmentally arrested fetal germ cells are supposed to be a source of carcinoma in situ or intratubular germ cell neoplasia unclassified (IGCNU) (Skakkebaek et al., 1987). The hypothesis of the embryonic/fetal origin of neoplastic germ cells has been substantiated mainly by phenotypic similarities between neoplastic and fetal germ cells (Honecker et al., 2004a) as well as by genomic studies (Almstrup et al., 2004; Hœi-Hansen et al., 2004) and epidemiological data (Moller and Skakkebaek, 1999; Jacobsen et al., 2000). In fact, previously published studies indicate that fetal germ cells express a number of markers also found in neoplastic germ cells including placental alkaline phosphatase (PLAP), glycosylated monomeric sialoglycoprotein M2A and other proteins that are believed to be involved in the maturation process of fetal germ cells including transcription factors OCT3/4 and AP-2γ and the receptor tyrosine kinase c-KIT (Franke et al., 2004; Hœi-Hansen et al., 2004b; Honecker et al., 2004b; Rajpert-De Meyts et al., 2004). Detailed studies of the expression of these markers during fetal development have not been done until now; thus the aim of the present work was to examine the physiological maturation process of fetal germ cells during the second and third gestational trimesters by detection of c-KIT, OCT3/4, MAGE-A4, AP-2γ and the oncofetal marker M2A. Therefore, we prepared tissue arrays from 61 testes of normally developed fetuses from gestational week 12 to the
full-term neonate. Sertoli cells were detected by antibodies to inhibin α and cytokeratin 18 (CK18). Other proteins were selected based on their involvement in regulation of the cell cycle (Ki-67) or their association with Sertoli–germ cell interaction and differentiation including neural cell adhesion molecule (NCAM) (Orth et al., 2000), E-cadherin (Di Carlo and De Felici, 2000; Honecker et al., 2004b), connexin43 (Perez-Arnemardiz et al., 2001) and androgen receptor (Zhou et al., 1996; Sharpe et al., 2003).

Because the expression of MAGE-A4 in IGCNU has been controversial in previous reports (Aubry et al., 2001) and in order to demonstrate whether melanoma-associated antigen (MAGE-A4) and other oncofetal proteins are co-localized in neoplastic germ cells of IGCNU, we also analysed 6 IGCNU samples from patients with invasive germ cell tumours.

**Materials and methods**

**Tissue samples**

Fetal tissue stored at room temperature was selected from the archives of the Section of Pediatric Pathology, Department of Pathology, University of Bonn Medical Center. Use of the tissue for scientific purposes was approved by an institutional ethics committee. The tissue samples included fetuses from weeks 12 (n = 2), 13 (n = 3), 14 (n = 3), 15 (n = 3), 16 (n = 4), 17 (n = 3), 18 (n = 3), 19 (n = 3), 20 (n = 3), 21 (n = 4), 22 (n = 2), 23 (n = 3), 24 (n = 2), 25 (n = 3), 26 (n = 2), 27 (n = 2), 32 (n = 2), 33 (n = 3), 34 (n = 3), 35 (n = 2), 36 (n = 2), 37 (n = 3) of pregnancy and one from a neonate that had died within 24 h after birth (n = 61). None of the fetuses had signs of maceration or autolysis at autopsy and sufficient tissue preservation was confirmed by haematoxylin–eosin staining. Causes of death were spontaneous abortion (mainly amnion infection or placental insufficiency), neontal death or induced legal terminations. All terminations were performed by the Department of Pathology, University of Bonn. The developmental age of the fetuses was determined by the date of the last menstrual bleeding. Weight and length measurements evaluated at the autopsy were used to assure proper gestational development.

In addition, six cases of IGCNU from patients with different germ cell tumours (one seminoma, three embryonal carcinomas, one yolk sac tumour and one choriocarcinoma) were retrieved from the tissue archives of the Department of Pathology, University of Bonn Medical Center. The ages of the patients were 33, 22, 20, 18, 17 and 19 years respectively.

**Immunohistochemical staining**

Testes were dissected, fixed in 4% phosphate-buffered formalin for 2 days at room temperature and processed in paraffin wax. Dewaxed, paraffin-embedded 4 μm thick tissue sections were microwave-pretreated in Tris–EDTA buffer (10 mmol/l tris base, 1 mmol/l EDTA solution, 0.05% Tween 20, pH 8.0). Primary antibodies to the following proteins were used: MAGE-A4, M2A-antigen, c-KIT, OCT3/4, AP-2γ, inhibin α, androgen receptor, connexin43, E-cadherin, NCAM, cytokeratin 18 and Ki-67. Details of antibodies and the dilutions for immunohistochemistry are given in Table I. Single immunohistochemistry was performed using the Dako EnVision-AEC Kit and manufacturer's protocol (Dako, Hamburg, Germany). Briefly, endogenous peroxidase was blocked for 5 min in 0.03% H2O2 (diluted in distilled water). Sections washed in Tris-buffered saline (TBS; 0.05 mol/l Tris and 0.85% NaCl, pH 7.6) were incubated with primary antibodies overnight at 4°C. Thereafter, a horseradish peroxidase (HRP)-labelled polymer conjugated with a secondary antibody was applied (Dako EnVision-AEC Kit). The staining was visualized with 3-amino-9-ethyl-carbazole and counterstained with haematoxylin. Negative controls were performed using buffer instead of the primary antibody and resulted in complete absence of signal. Additional negative controls were performed by incubation with pre-immune serum from each animal species (rabbit, mouse, goat) instead of a primary antibody and also resulted in complete absence of an immunohistochemical signal.

Double staining was performed with EnVision-Doublestain Kit (Dako) which included horseradish peroxidase and alkaline phosphatase labelled polymer and substrates DAB and Fast Red according to the manufacturer’s recommendation. The following antibody pairs were used for double staining: M2A and MAGE-A4, c-KIT and MAGE-A4, AP-2γ and MAGE-A4, OCT3/4 and MAGE-A4, Ki-67-1 and MAGE-A4, M2A and c-KIT, c-KIT and AP-2γ, c-KIT and OCT3/4, Ki-67 and c-KIT.

Immunofluorescence was performed employing antibody pairs M2A and c-KIT, c-KIT and MAGE-A4, M2A and MAGE-A4. Briefly, dewaxed formalin-fixed, paraffin-embedded 4-μm thick tissue sections were microwave-pretreated in Tris–EDTA buffer and then incubated with pre-immune serum from the species from which the secondary antibody is derived (for 20 min). Antibody pairs from different species (M2A and c-KIT, c-KIT and MAGE-A4) were simultaneously incubated at 4°C overnight, while the monoclonal antibodies for double staining of M2A and MAGE-A4 were applied in a sequential manner. The immune reaction was visualized by Cy5 (rabbit anti-mouse and goat anti-rabbit) and Cy3 (rabbit anti-mouse)-conjugated secondary antibodies (Novocastra Laboratories, Newcastle upon Tyne, UK).

**Tissue array preparation, assessment of immunohistochemical staining and image capture**

From each case, three 1.0 mm cores of testicular tissue were randomly taken to form a 20×30×10 mm recipient paraffin block. A total of 183

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<th>Table I. Antibodies used in the present study</th>
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cores were taken and placed in two array blocks in ascending order of gestational age. Semiquantitative assessment of the immunohistochemical staining with antibodies to M2A-antigen, c-KIT, OCT3/4, AP-2γ and Ki-67 was performed by counting the positively stained germ cells in five high power fields (HPF) in each case (area of a single HPF 0.125 mm², objective for HPF ×40). The total number of germ cells was obtained from counting in slides stained with inhibin α, which marked Sertoli cells and omitted germ cells. Whole tissue samples from each of the gestational weeks 14, 19, 22, 27, 33 and 37 and from the neonate were, in addition, selected for immunohistochemical detection of MAGE-A4, OCT3/4, M2A, AP-2γ, c-KIT and Ki-67. This was done to ensure that results obtained from tissue microarrays are representative of the whole testis. For this purpose, 15 randomly selected high power fields were counted in stained whole tissue sections. The counting was done independently by two different observers (K.P. and H.Z.). Non-immunofluorescence stainings obtained from single and double staining is shown in Figures 1, 2 Figure 4, and those from double staining in Figure 3.

Counting of germ cells and proliferation index

In the testes from the term neonate, germ cells were clearly distinguishable from Sertoli cells using haematoxylin and eosin staining, since germ cells at this stage have large-sized and round nuclei compared to the cylindrical shape of Sertoli cells. In contrast, during the second and the early third trimester, germ cells varied significantly with respect to size and nuclear morphology. Therefore, immunohistochemistry with an antibody to inhibin α was employed to distinguish germ cells from Sertoli cells reliably. Similar gestational weeks were grouped for calculations (Table II). The number of germ cells from each case was counted in five independent high power fields. In parallel, the number of Sertoli cells was determined and the ratio of germ to Sertoli cells was calculated (Table II). These investigations were done mainly to estimate the distribution of germ cells positive for selected markers MAGE-A4, M2A, c-KIT, AP-2γ, Ki-67 and OCT3/4 in relation to all germ cells at the respective developmental week.

As shown in Table II, at the 12th/13th week, an average of 46 germ cells per HPF was counted. The ratio of germ to Sertoli cells was 1:6.5 and the proliferation marker Ki-67 was expressed in 17% of germ cells. At gestational week 18/19, an increase in germ cells up to 70 per HPF was accompanied by high expression of the proliferation marker Ki-67 (21%) and a higher ratio of germ cells to Sertoli cells (1:6). Thereafter, the number of germ cells per HPF decreased again, and around the gestational week 36, 43 germ cells per HPF were counted. At the same time, the ratio of germ to Sertoli cells decreased to 1:12 (week 36/37) and again 21% of germ cells were positive for Ki-67.

**Immunohistochemical detection of c-KIT, M2A, AP-2γ, OCT3/4 and MAGE-A4 in single staining**

Immunohistochemistry with c-KIT and M2A antibodies showed the expected membranous staining of germ cells. MAGE-A4 was expressed in the cytoplasm of germ cells. Antibodies to OCT3/4 and AP-2γ produced distinct and strong nuclear staining in germ cells. The number of germ cells expressing each of the proteins was estimated from single-stained immunohistochemical slides. The average value in five

| Table II. Numbers of germ cells positive for M2A, c-KIT, AP-2γ, OCT3/4, MAGE-A4 and Ki-67 in fetal and newborn testes in relation to the total number of germ cells per high power field |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Gestational age (weeks)** | **12/13** | **14/15** | **16/17** | **18/19** | **20/21** | **22/23** | **24/25** | **26/27** | **32/33** | **34/35** | **36 (nb)** |
| No. of cases | 5 | 6 | 7 | 6 | 6 | 7 | 5 | 5 | 5 | 4 | 5 | 6 |
| Total no. of germ cells (per HPF) | 46 ± 5 | 51 ± 3 | 60 ± 6 | 70 ± 5 | 60 ± 7 | 55 ± 3 | 53 ± 4 | 52 ± 5 | 53 ± 3 | 48 ± 4 | 43 ± 5 |
| Ki-67 (%) | 8 ± 2 | 10 ± 2 | 9 ± 3 | 15 ± 1 | 5 ± 1 | 10 ± 1 | 10 ± 1 | 9 ± 3 | 11 ± 2 | 9 ± 2 |
| M2A (%) | 42 ± 3 | 46 ± 2 | 48 ± 3 | 67 ± 5 | 41 ± 3 | 33 ± 7 | 27 ± 5 | 12 ± 4 | 5 ± 1 | 6 ± 1 | 3 ± 1 |
| c-KIT (%) | 40 ± 8 | 42 ± 5 | 46 ± 5 | 65 ± 5 | 36 ± 4 | 34 ± 8 | 22 ± 4 | 10 ± 1 | 3 ± 1 | 3 ± 2 | 2 ± 1 |
| AP-2γ (%) | 35 ± 2 | 38 ± 4 | 39 ± 2 | 58 ± 5 | 33 ± 8 | 30 ± 6 | 24 ± 4 | 9 ± 5 | 1 ± 1 | 1 ± 0 | 0 |
| OCT3/4 (%) | 76 | 74 | 65 | 82 | 55 | 54 | 45 | 17 | 2 | 2 | 0 |
| MAGE-A4 (%) | 74 | 71 | 68 | 78 | 53 | 51 | 38 | 15 | 0 | 2 | 0 |

Means and SD were calculated from counting of positive cells in five independent high power fields (HPF; ×40, field of 0.125 mm²). Percentages result from deviation of average numbers of germ cells positive for the respective marker and total number of germ cells. GC/SC ratio results from relation of total germ cells (GC) to Sertoli cells (SC) per HPF.

nb = newborn.
As seen in Table II and Figure 5, roughly equal numbers of fetal germ cells expressed M2A, c-KIT, OCT3/4 and AP-2γ. Around week 12/13, most germ cells detected were positive for M2A (91%), c-KIT (87%), AP-2γ (76%) and OCT3/4 (74%). The number of positive germ cells increased further up to weeks 18/19 of gestation (M2A, 96%; c-KIT, 88%; AP-2γ, 82%; OCT3/4, 78%) and began to decrease gradually after week 19. For example, around week 24, the number of germ cells expressing c-KIT declined to 22/53 (41%) and decreased further at week 35 (2/43, 5%). During the last weeks of the third trimester, expression of M2A, c-KIT, OCT3/4 and AP-2γ was found only in loose apoptotic germ cells in the lumen of the tubules. In contrast to the decreasing number of germ cells positive for M2A, c-KIT, OCT3/4 and AP-2γ, a continuously increasing number of MAGE-A4-positive cells was observed. For example, at week 12/13, only a few germ cells expressed MAGE-A4 (2/46, 4%). MAGE-A4 expression increased continuously during further development, reaching 50% at week 24 and 93% in the testis of the neonate.

To verify the results obtained from immunohistochemical staining of tissue arrays, whole testes from weeks 14, 19, 22, 27, 33 and 37 and from a neonate were stained with antibodies to OCT3/4, M2A, c-KIT, MAGE-A4, Ap-2γ and Ki-67. The numbers of positive germ cells assessed in whole testes were identical with the results obtained from tissue arrays. Histological and immunohistochemical correlation analyses and co-expression studies of c-KIT, M2A, AP-2γ, OCT3/4, Ki-67 and MAGE-A4

Expression of the markers described above was correlated with conventionally defined types of germ cells as established by Fukuda et al. (1975). We found that expression of M2A, AP-2γ, c-KIT and OCT3/4 was mainly associated with gonocytes, which were identified by their round central nuclei, high nuclear to
cytoplasmic ratio and often prominent nucleoli (Figure 1A–D). All intermediate cells histologically defined by oval shape, enlarged cytoplasm and a round but slightly eccentric nucleus were also positive for these markers (Figure 1E). Gonocytes and intermediate cells were frequently located in the outer or middle layer of the tubule. In contrast, germ cells corresponding to pre-spermatogonia (Fukuda et al., 1975) expressed MAGE-A only (Figure 1F–H, Figures 2 and 3).

Co-localization studies showed that most gonocytes and intermediate cells co-expressed M2A, c-KIT, AP-2γ and OCT3/4 but not MAGE-A at every stage of testicular development. In particular, this co-expression was confirmed by immunohistochemistry using antibody pairs c-KIT and OCT3/4; M2A and c-KIT; c-KIT and AP-2γ; M2A and MAGE-A4, c-KIT and MAGE-A4, AP-2γ and MAGE-A4, OCT3/4 and MAGE-A4 and confirmed by immunofluorescence using antibody pairs c-KIT and M2A (Figures 2A, 3A and B). However, single intermediate cells co-expressed MAGE-A4 (Figure 2B, inset).

We also observed few germ cells that were positive for c-KIT or M2A but negative for AP-2γ and OCT3/4. Some of these cells had the appearance of typical gonocytes, but others had the form of intermediate cells. Furthermore, through examination of the antibodies used in our study, we found that M2A staining was extremely robust to different staining conditions and tissue preservation. Therefore, the highest yield of positively stained gonocytes and intermediate cells was obtained from staining with M2A antibody (compare Table II, Figure 5).

In contrast, none of MAGE-A4-positive pre-spermatogonia expressed markers found in gonocytes/intermediate cells (c-KIT, M2A, AP-2γ and OCT3/4) (Figures 2B, 3C and D). In the fetal testes from the second trimester, pre-spermatogonia were mostly located at the basement membrane and less frequently in the inner tubular layer. At the end of the third trimester and in the neonate, MAGE-A4-positive germ cells were found within the tubules in basal locations.

Co-localization studies with antibodies to Ki-67 and c-KIT revealed that most cells positive for Ki-67 expressed c-KIT on their surface. However, some MAGE-A4 positive germ cells were also positive for Ki-67 in their nucleus (not shown).

In addition, double staining of six adult testes with normal spermatogenesis and IGCNU was performed using antibodies to c-KIT and MAGE-A4, M2A and MAGE-A4. This was done to address the issue of whether neoplastic germ cells express markers of pre-spermatogonia. In each case, normal spermatogonia were consistently positive for MAGE-A4. In contrast, no staining could be demonstrated in neoplastic germ cells, which were marked by c-KIT and M2A (Figure 4).
**Immunohistochemical detection of Ki-67, E-cadherin, NCAM (CD56), cytokeratin 18, connexin43 and androgen receptor in fetal testes**

No expression of E-cadherin or androgen receptor was found in fetal testicular cords. Diffuse cytoplasmic staining for connexin43 was observed in Leydig cells at all gestational weeks examined but not in fetal testicular cords. NCAM and cytokeratin 18 expression was present in Sertoli cells in the testis in weeks 12–19 but not in later stages of testicular development (data not shown).

**Discussion**

The aim of the present study was to examine the expression of proteins associated with differentiation and maturation of germ cells in human fetal testes. We detected oncofetal markers including transcription factors OCT3/4 and AP-2γ, stem cell factor receptor c-KIT and antigen M2A in fetal male germ cells predominantly between gestational weeks 12 and 26. These results are in accordance with previous studies, which examined expression of respective markers during fetal development in normal male gonads (Jorgensen et al., 1995; Aubry et al., 2001; Franke et al., 2004; Hoei-Hansen et al., 2004a; Honecker et al., 2004a; Rajpert-De Meyts et al., 2004; Robinson et al., 2001). Furthermore, we found that two immunohistochemically distinct populations of fetal germ cells exist during the second and third trimesters. One of these populations comprises germ cells, consisting of gonocytes and intermediate cells, according to morphological characteristics established by Fukuda et al. (1975) and co-expresses c-KIT, M2A, OCT3/4 and AP-2γ. The second population of germ cells matches the morphological criteria of pre-spermatogonia (Fukuda et al., 1975) and expresses melanoma-associated antigen MAGE-A4, which is a specific marker for normal pre-meiotic germ cells (Aubry et al., 2001).

Our findings partially confirm the recent qualitative study published by Gaskell et al. (2004), who detected distinct expression of OCT3/4 together with c-KIT in a gonocytic population and MAGE-A4 in the population of pre-spermatogonia. Gaskell et al. also proposed the existence of a third germ cell population with the morphology of intermediate cells, which is devoid of c-KIT and MAGE-A4 expression. A similar third group was not found in our series. Differences between our study and previous reports might be the result of different tissue fixation (Bouin’s fluid versus formalin), different antigen retrieval techniques and application of a highly sensitive immunohistochemical detection method in the present study. According to our results, intermediate germ cells belong to the immunohistochemical group of germ cells positive for c-KIT, AP-2γ, OCT3/4 and M2A. However, single intermediate cells also showed co-expression of MAGE-A4. This finding is in accordance with the idea that intermediate cells represent a transition stage from gonocytes to pre-spermatogonia (Fukuda et al., 1975). Furthermore, compared to other markers used (c-KIT, AP-2γ, OCT3/4), D2-40 antibody to M2A antigen always resulted in sensitive and distinct staining independent of fixation artefacts or autolysis. We believe that the robustness of D2-40 was also the reason why slightly more germ cells were positive for M2A compared to c-KIT, AP-2γ and OCT3/4 in our study (Figure 5, Table II).

Furthermore, we found that the number of germ cells co-expressing oncofetal markers c-KIT, M2A, AP-2γ and OCT3/4 varied significantly depending on the developmental stage. In particular, their expression was strongly increased in week 18/19 (Figure 5). We believe that this peak reflects a burst of proliferation activity of gonocytes, as we found an increase in both Ki-67 expression and the overall germ cell number, while there was a transient decrease in the ratio of Sertoli cells to germ cells at this point of development. In general, the number of gonocytes and intermediate cells decreased continuously during the second and third trimesters, while the number of MAGE-A4-positive germ cells increased to the same degree. The differentiation process obviously progressed after the week 25, when >50% of germ cells expressed MAGE-A4 (Figure 5).

Understanding the process of pre-spermatogenesis and the role of proteins involved in germ cell differentiation may help to elucidate the pathogenesis of IGCNU and germ cell tumours as it is believed that malignant transformation of germ cells occurs in early fetal life (Skakkebaek et al., 1987; Rajpert-De Meyts et al., 2004). Expression of transcription factor AP-2γ has recently been detected in IGCNU and various germ cell tumours (Hoei-Hansen et al., 2004b; Pauls et al., 2005). Its cellular functions, particularly in germ cells, are not known in detail yet. Mice overexpressing AP-2γ in the mammary gland exhibited hyperproliferation and impaired differentiation of lactiferous ductules, suggesting a role of AP-2γ in the proliferation and maintenance of an undifferentiated state (Jager et al., 2003). Receptor tyrosine kinase c-KIT is crucial for germ cell migration, survival and proliferation in rodents and it is likely to be of the same importance in humans (Yamamoto et al., 1993; Yasuda et al., 1993; Robinson et al., 2001). KIT has been described as a target gene of AP-2 transcription factors (Yamamoto et al., 1993; Yasuda et al., 1993). Thus, prolonged AP-2γ expression and permanent activation of c-KIT might have effects upon malignant transformation by an increase in the survival of immature germ cells and arrest of germ cell differentiation. Another potential transforming mechanism involves OCT3/4, which is a POU-domain class 5 transcription factor and one of the candidate regulators in pluripotent and germline cells (Niwa et al., 2000). Disturbed regulation of OCT3/4 may also cause arrest of gonocytes at the pluripotent stage, consequently providing the basis for malignant germ cell transformation. We do not know the real biological pathway of the malignant transformation of germ cells, but we show here that putative factors involved in this process including OCT3/4, AP-2γ and c-KIT are strictly regulated in fetal germ cells. All of them are co-expressed during fetal development in the same germ cell population with morphological characteristics of gonocytes or intermediate cells. Before germ cells transit to pre-spermatogonia, as marked by expression of MAGE-A4, down-regulation of OCT3/4, AP-2γ and c-KIT occurs. We further show that fetal pre-spermatogonia are phenotypically close to mature spermatogonia because they share the expression of MAGE-A4, which has been found in pre-meiotic spermatogonia of adults in the present and in previous studies (Yakirevich et al., 2003). In contrast, neoplastic germ cells of
IGCNU were devoid of MAGE-A4 in our series. In conclusion, our findings further strengthen the hypothesis of the embryonic/fetal origin of germ cell neoplasia, although the latter have some phenotypic overlap with adult germ cells, as has been documented by previous studies using VASA gene product and Y-encoded testis-specific protein (TSPY) (Arnemann et al., 1991; Castrillon et al., 2000; Zeeman et al., 2002; Honecker et al., 2004).

At least in rodent testes, gonocytal maturation is associated with adherence to Sertoli cells and relocation to the basement membrane via intermediate filaments and connexin43 (Nagano et al., 2000; Orth et al., 2000; Perez-Armendariz et al., 2001). In our series, we found no expression of connexin43 and E-cadherin in fetal tubules. Neural cell adhesion molecule NCAM was expressed on the cell membrane of Sertoli cells in parallel to CK18 and both proteins were down-regulated after gestational week 19, reflecting the progress of Sertoli cell differentiation (Sharpe et al., 2003). Similar to the results obtained from mouse testes (Nagano et al., 2000), almost all of the pre-spermatogonia in our series were attached to the basement membrane of the tubules. In contrast, germ cells expressing OCT3/4, c-KIT or AP-2γ at the late stages of pregnancy were degenerated and located in the lumen of the tubules. This finding supports the idea that relocation of gonocytes is essential for their survival (Nagano et al., 2000). However, the factors and pathways involved in human germ cell differentiation remain largely unknown and further exploration of this process is necessary.

In summary, we show that two immunohistochemically distinct populations of fetal germ cells exist during the second and third trimesters. Proteins which are associated with pluripotency, survival and proliferation including OCT3/4, c-KIT and AP-2γ as well as oncofetal marker M2A2 antigen are present in fetal germ cells with the morphology of gonocytes and intermediate cells. Their number gradually decreases after week 20, paralleled by an increasing number of MAGE-A4-positive pre-spermatogonia. Results presented in our study provide evidence of functional and temporal regulation of the differentiation process in fetal testes. They may also serve as a reference for further analyses investigating abnormal fetal germ cell maturation in chromosomal aberrations (K.Pauls et al., unpublished data). In addition, we found here that the monoclonal antibody D2-40 to glycosylated monomorphic sialoglycoprotein M2A, which is expressed in fetal gonocytes and intermediate cells, is extremely robust to fixation artefacts and autofluorescence. In future, the use of M2A2 might also be extended to detection of IGCNU and metastatic seminoma in diagnostically challenging cases (K.Pauls et al., unpublished data).

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References


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