Down-Regulation of Stem Cell Genes, Including Those in a 200-kb Gene Cluster at 12p13.31, Is Associated with In vivo Differentiation of Human Male Germ Cell Tumors


Abstract

Adult male germ cell tumors (GCTs) comprise distinct groups: seminomas and nonseminomas, which include pluripotent embryonal carcinomas as well as other histologic subtypes exhibiting various stages of differentiation. Almost all GCTs show 12p gain, but the target genes have not been clearly defined. To identify 12p target genes, we examined Affymetrix (Santa Clara, CA) U133A+B microarray (~83% coverage of 12p genes) expression profiles of 17 seminomas, 84 nonseminoma GCTs, and 5 normal testis samples. Seventy-three genes on 12p were significantly overexpressed, including GLUT3 and RE1 (overexpressed in all GCTs) and CCND2 and FLJ22028 (overexpressed in all GCTs, except choriocarcinomas). We characterized a 200-kb gene cluster at 12p13.31 that exhibited coordinated overexpression in embryonal carcinomas and seminomas, which included the known stem cell genes NANOG, STELLA, and GDF3 and two previously uncharacterized genes. A search for other coordinately regulated genomic clusters of stem cell genes did not reveal any genomic regions similar to that at 12p13.31. Comparison of embryonal carcinoma with seminomas revealed relative overexpression of several stem cell–associated genes in embryonal carcinoma, including several core “stemness” genes (EBF1, TDGF1, and Sox2) and several downstream targets of WNT, NODAL, and FGF signaling (FGF4, NODAL, and ZFP42). Our results indicate that 12p gain is a functionally relevant change leading to activation of proliferation and reestablishment/maintenance of stem cell function through activation of key stem cell genes. Furthermore, the differential expression of core stem cell genes may explain the differences in pluripotency between embryonal carcinomas and seminomas. (Cancer Res 2006; 66(2): 820-7)

Introduction

Adult male germ cell tumors (GCTs) are the most commonly diagnosed solid tumors in men ages 18 to 35 years (1). These tumors are thought to originate from transformed germ cells, and most commonly present as a testicular mass, although ~10% present at extragonadal sites (2). GCTs are unique in that they form distinct histologic subtypes that mimic different lineages of the developing human embryo. GCTs can be divided into two main groups based on the degree and type of differentiation (i.e., seminomas, which resemble undifferentiated primitive germ cells, and nonseminomas, which show varying degrees of embryonic and extraembryonic patterns of differentiation; refs. 2, 3). Nonseminomatous GCTs are further subdivided into embryonal carcinomas, which show early zygotic or embryonal-like differentiation, yolk sac tumors and choriocarcinomas, which exhibit extraembryonal forms of differentiation, and teratomas, which show somatic differentiation along multiple lineages (3). Both seminomas and embryonal carcinoma are known to express stem cell markers, such as POU5F1 (4) and NANOG (5).

Cytogenetic analysis has identified 12p gain as the most frequent change in GCTs, occurring in ~100% of cases (6), regardless of histology. Gain of 12p is usually manifested as one or more copies of an isochromosome 12p [i(12p); ref. 7], with tandem duplication of 12p material occurring at a lower frequency (8). We and others have also observed the presence of high-level subregional amplifications of 12p (9–11). Given the frequency of 12p gain in all types of GCT and the presence of extra copies of 12p in some premalignant intratubular germ cell neoplasia (ITGCN; refs. 12, 13), it has been suggested that 12p gain may be involved in the early stages of germ cell tumorigenesis or may perhaps even be the initiating event (14). In contrast, others have reported that 12p gain does not occur frequently in pure ITGCN and instead is associated with progression (15, 16). Regardless, gain of 12p remains a genetic hallmark of adult male GCTs.

The 12p region has been studied extensively, and we have proposed that the proto-oncogene CCND2 may be (one of) the targets of 12p gain (17). Other genes on 12p are also known to be overexpressed in GCT, including genes associated with stem cells (18, 19), but their importance in tumorigenesis is not well established. Interestingly, the formation of i(12p) in cultured human embryonic stem cells has been reported recently, which may give proliferative or self-renewal advantages to these cells (20). Amplification of 12p is commonly found in other human neoplasms, including esophageal (21) and ovarian (22) carcinomas, indicating that 12p target genes may have broader implications in oncogenesis.

We sought to identify target genes on 12p and infer their significance in germ cell tumorigenesis and to identify genes that are associated with the pluripotent phenotype of embryonal carcinoma tumors. We recently reported expression profiling on a group of 84 nonseminomatous GCT samples that were stratified into distinct histologic subtypes according to an expression-based classifier (23). We have analyzed these samples along with 17 seminoma samples to identify differentially expressed genes on 12p. We identified a 200-kb coordinately regulated region at 12p13.31 that was overexpressed in embryonal carcinomas and seminomas, which consisted of several stem cell–associated genes. No other

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: R.S.K. Chaganti, Cell Biology Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021. Phone: 212-639-8121; Fax: 212-717-3541; E-mail: chaganti@mskcc.org.

© 2006 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-05-2445
coordinately regulated genomic regions of stem cell gene clusters were evident. Comparison of embryonal carcinomas to seminomas identified overexpression of several core stem cell genes in embryonal carcinoma and differential activation of WNT, NODAL, BMP, and FGF pathways between the subtypes. These results suggest that 12p gain may play dual roles in GCT tumorigenesis: establishment or maintenance of a stem cell phenotype through gain of a cluster of 12p13.31 genes and conferring a proliferative advantage. Our results further suggest that differential expression of core stem cell genes may account for the differences in pluripotency of embryonal carcinoma and seminoma tumors.

Materials and Methods

Tumor specimens. Tumor tissues were collected under institutional review board–approved protocols at the Memorial Sloan-Kettering Cancer Center (New York, NY) between 1987 and 1999. The tumor samples consisted of 17 seminomas, 15 pure embryonal carcinomas, 15 pure teratomas, 10 pure yolk sac tumors, 2 pure choriocarcinomas, and 42 nonseminomatous GCTs with mixed histologies. Profiling of the nonseminomatous GCT specimens was reported recently (23). Using the histologic classifier that we developed recently (23), the mixed nonseminomatous GCT specimens were classified according to their predominant histology. For controls, five normal testis specimens from similarly aged patients with no evidence of testicular cancer were used. A pooled sample of all five normal specimens was also profiled and included in all figures but was not used in identifying differentially expressed genes.

Cell culture. The pluripotent embryonal carcinoma cell lines 27X-1 and NT2/D1 were maintained as described previously (24). The 27X-1 cells were exposed to 10 μmol/L all-trans-retinoic acid (RA; Sigma, St. Louis, MO) to induce differentiation. Cells were reseeded every 2 to 3 days. Cells were harvested at 0, 2, 4, 8, 12, 18, and 24 hours and 3, 6, 8, and 10 days after treatment for isolation of RNA using RNAeasy columns (Qiagen, Valencia, CA) according to the manufacturer’s protocol. The time course was done in triplicate.

RNA isolation from tumor tissues. Fresh tumor tissue was grossly dissected to remove normal contamination with no further microdissection. Tumor tissue was homogenized using a Brinkman homogenizer, and RNA was isolated using RNAeasy Midi columns (Qiagen) according to the manufacturer’s protocol. RNA was quantified by UV absorbance and quality was verified by A260/A280 ratios and integrity of 28S and 18S bands on denaturing agarose gels. Samples that showed evidence of DNA contamination were digested with RNase-free DNase (Qiagen) followed by purification through RNAeasy Mini columns (Qiagen).

cDNA synthesis, labeling, and hybridization. The cDNA synthesis and cRNA labeling for hybridization to U133A and U133B microarrays (Affymetrix, Santa Clara, CA) was done as described previously (23, 24). The size of the labeled cRNA was evaluated on an Agilent Bioanalyzer (Agilent, Foster City, CA) to ensure probe quality for hybridization. Hybridization, washing, and imaging were done as described previously (23).

Reverse transcription-PCR expression analysis. Total RNA (1 μg) was reverse transcribed using 300 ng random hexamers (Invitrogen, Carlsbad, CA), deoxynucleotide triphosphates at a final concentration of 500 μmol/L, and 100 units SuperScript II reverse transcriptase (Invitrogen) at 42°C. The genes tested were NANOG, STELLA, AY115139 (the putative homeobox C14 gene, HOXC14), CLEFT7, GDF3, FLJ40451 (NANOG2), HS.129302, ERF, TDGF1, SOX2, SOX17, GIP, and CD24, with ACTB as a control. PCR was carried out with 30 pmol of each primer in a total volume of 30 μL using 20 to 50 ng of target cDNA using 2.5 units Pfu Turbo polymerase (Stratagene, La Jolla, CA) or 1 unit HotStarTaq (Qiagen). The product was run on 1.4% agarose gels and stained with ethidium bromide for visualization. Primers were designed to span introns to distinguish between mRNA and DNA for all genes, except HS.129302, which has no introns, and CLEFT7. Primer sequences and PCR conditions are available as Supplementary Table S1.

Immunohistochemistry. Formalin-fixed, paraffin-embedded mixed GCT specimens were sectioned at 4 μm, baked on Superfrost slides for 30 minutes at 60°C, deparaffinized using xylenes, and then rehydrated through an ethanol series. For SOX2 staining, the sections were pretreated with 3% hydrogen peroxide and blocked using conventional methods. SOX2 antibody (R&D Systems, Minneapolis, MN; diluted 1:100) was incubated on the section overnight at 4°C. Secondary antibody and streptavidin were applied each for 1 hour at room temperature. 3',3'-Diaminobenzidine tetrahydrochloride (Sigma) was used as the chromagen for detection with hematoxylin counterstain. To detect GDF3, the same protocol was used, except that sections were pretreated with trypsin for 30 minutes at 37°C. Primary antibody for GDF3 staining was a goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:100).

Data analysis. Background subtraction, normalization, and log2 transformation of the array data were done using the Robust Multi-Array Average (RMA) method (25) within the Bioconductor for R software package (26). Genes were annotated according to the June 23, 2004 Affymetrix annotation. In accordance with Minimum Information About a Microarray Experiment guidelines (27), both RMA-processed and raw data files (CEL and EXP) for all 101 GCTs and the normal testis specimens have been deposited in the Gene Expression Omnibus public repository (GEO accession no. GSE 3218). To identify significantly differentially expressed transcripts, the maxT function in multtest in bioconductor for R was used. This is a permutation-based method to adjust P values to correct for multiple comparisons (28). We compared expression in each histologic subtype to the five normal testis samples and used 10,000 permutations within maxT (adjusted P < 0.05 is considered significant). Fisher’s exact test was used to test whether overexpressed clones from 12p were overrepresented compared with clones from other chromosome arms. For validation of stem cell–associated genes in the 27X-1 cell lines, a one-sided paired t test was used to test for differences in expression between the baseline time point and 10-day time point. Visualization of the data was done using the Cluster/Treeview software package (29).

Results

Identification of target genes on 12p. We have recently published results from expression profiling of 84 nonseminomatous GCT tumor specimens (23). We analyzed these specimens along with 17 new seminoma profiles to identify differentially expressed genes between tumors of each of the histologic subtypes and normal testes specimens. There were 6,038 transcripts that showed a significant change in expression in at least one histologic subtype relative to normal, with 2,613 of those transcripts being overexpressed (Supplementary Table S2). Seminomas displayed the most changes, whereas choriocarcinoma had the least. Specifically for 12p, there were 107 transcripts (89 genes) that showed a significant change in at least one of the tumor types relative to normal testis, with 86 transcripts being overexpressed. We currently estimate that there are ~400 genes on 12p, consistent with our previous estimates (9). We identified 555 transcripts representing 331 genes on the U133A+B microarrays for an estimated coverage of 83%. The complete list of transcripts from 12p showing significantly different expression is included as Supplementary Table S3. Although genes from other chromosomes clearly play a role in GCT etiology and progression, the importance of the gain of 12p is further exemplified by the overrepresentation of overexpressed transcripts from 12p compared with overexpressed transcripts from all other chromosomes by Fisher’s exact test (P < 0.001), consistent with previous findings (30).

There were 21 transcripts (16 genes) on 12p that were significantly down-regulated in at least one of the histologic subtypes relative to normal testes. These included several genes...
related to meiosis, sperm function, and/or spermatogenesis, such as PLCZ1, CAPZA3, GSG1, SOX5, and ACRBP, which had significantly lower levels of expression in all GCTs relative to normal. Other down-regulated genes included H2A/ and C12ORF2. There were also some genes that showed lower levels of expression in only a subset of the tumor types, including the uncharacterized IMAGE clone 5295478 (down-regulated in seminomas) and CSDA, which was significant only in yolk sac tumor specimens.

The remaining 86 significant transcripts (73 genes) on 12p were overexpressed in at least one of the tumor subtypes compared with normal testis specimens. There were two genes that were up-regulated in all tumor subtypes regardless of histology. These were SLC2A3 (GLUT3), which has been described previously in GCTs (19), and REA. Four genes showed significantly higher expression in all but one histologic subtype, including the previously identified CCND2 (refs. 17, 19; which was high in all but choriocarcinoma), FLJ22028 (high in all but choriocarcinoma), and the metabolic genes GAPDH and TPI1 (which were high in all subtypes, except teratoma). Eight genes were overexpressed in three subtypes, including CD9, FLJ22662 (high in all but teratoma and yolk sac tumors) and TEAD4, LRP, TERA, and PTPN6 (high in all but teratoma and choriocarcinoma). The remaining genes were overexpressed in two or fewer histologic subtypes.

Particularly interesting was the overexpression of 14 transcripts on 12p seen only in embryonal carcinomas and seminomas, including several genes implicated in stem cell and GCT biology (5, 30). These included AK3 and GABARAPL1. Most importantly, there were two previously identified stem cell–associated genes that mapped within 100 kb of each other on 12p13.31 [i.e., STELLA (DPPA3) and NANOG]. A previously unreported gene, Hs.129302, located close to STELLA and NANOG (Fig. 1A), also displayed comparable overexpression in embryonal carcinomas and seminomas, confirming the existence of a coordinately regulated region of stem cell–associated genes that had been suggested previously (18). Further examination revealed that the pluripotency-associated gene GDF3 (18), which also maps to this region, was overexpressed only in embryonal carcinoma specimens (Fig. 1A). In contrast, SLC2A14, a gene that lies between NANOG and Hs.129302 within this region, was highly expressed in all tumor samples (data not shown). The coordinately regulated 200-kb gene cluster seemed to be flanked terminally by APOBEC1 and proximally by SLC2A3 and FOXJ2 (not shown in Fig. 1A), none of which were overexpressed only in embryonal carcinomas and seminomas.

Detailed mapping of the region from the May 2004 freeze6 showed that several other genes that were not on the microarrays also mapped to this 200-kb region, including A1Y151139 (HOXC14), a C-type lectin (CLECSF7), and a NANOG pseudogene FLJ40515 (NANOG2) as shown in Fig. 1A. We did reverse transcription-PCR (RT-PCR) analysis on a set of tumors consisting of four pure subtypes of each of embryonal carcinoma, teratoma, yolk sac tumor, and seminoma and four choriocarcinoma specimens (two pure, two mixed) as an internal validation of the expression of genes from the array that mapped between GDF3 and Hs.129302 (excluding SLC2A14) and to test expression for the three genes that were not included on the arrays. NANOG2 and CLECSF7 were not

---

6 http://genome.ucsc.edu.
highly expressed in the samples (Fig. 1B). STELLA, NANOG, and Hs.129302 shared a similar expression pattern: high in embryonal carcinomas and seminomas and low in teratoma, yolk sac tumors, and pure choriocarcinoma. GDF3 and HOXC14 exhibited high levels of expression primarily in the embryonal carcinoma samples, with some seminoma specimens showing detectable expression. The expression levels from the microarray data for GDF3, STELLA, NANOG, and Hs.129302 in pure embryonal carcinoma, teratoma, yolk sac tumor, choriocarcinoma, and seminoma samples relative to normal testes are shown in Fig. 2.

Expression patterns of additional genes associated with stem cell phenotypes. Given the expression of known stem cell markers, such as POU5F1, within embryonal carcinomas and seminomas and the described cluster of genes on 12p with a similar expression pattern, we sought to evaluate all other chromosomes to determine if any additional stem cell–associated clusters existed. For the initial analysis, transcripts that were strongly correlated with the core stem cell gene POU5F1 were selected. We found 41 such transcripts (36 genes), all with a correlation value of ≥0.80 (Supplementary Table S4). Of these 41 transcripts, all but 3 were also found to be significantly differentially expressed between embryonal carcinomas or seminomas and normal testes specimens, including all of the 12p genes. Several of the genes were reported previously to be targets of POU5F1, including UPPI, UTF1, REX1 (ZFP42), and NANOG (31, 32). Considering chromosomal location, 12p was the most highly represented and, not surprisingly, included three of the genes described above (STELLA, NANOG, and Hs.129302) plus TEAD4 and PHC1, which were located ∼5.0 and ∼1.0 Mb away from the 12p13.31 cluster, respectively. In addition, four genes on 10q shared this expression pattern, consisting of SLC25A16, FRAT2, UTF1, and VENTX2. These last two genes were located close to one another at 10q26.3, separated by 6.6 kb. We also observed three genes that shared the stem cell–like expression pattern on each of 1p and 7q, but none of these genes were located close to one another on the chromosomes. Genes representative of this type of expression pattern are shown in Supplementary Fig. S1.

To further validate if these genes behaved in a manner consistent with stem cell function, we examined the changes in their expression in the pluripotent 27X-1 embryonal carcinoma cell line on induction of differentiation with RA along a parietal yolk sac tumor lineage. Only 8 of the 36 genes that correlated with POU5F1 did not show significant decreases in expression on treatment with the morphogen, consistent with the hypothesis that they may be involved in stem cell maintenance or markers of an undifferentiated state (Fig. 3). One of these 8 genes, PTPAF3 (NALP7), showed a significant increase in expression following differentiation. The remaining 7 genes were not significantly altered, including the known stem cell markers ZFP42 and STELLA. Our results confirm that a large number of stem cell–associated genes are highly expressed in embryonal carcinomas and seminomas as reported previously (33).
Differential gene expression in embryonal carcinoma versus seminomas. To identify genes that account for the differences in pluripotency between embryonal carcinomas and seminomas, we examined these specimens for differentially expressed genes. There were 777 transcripts (~660 genes) that were differentially expressed between the two tumor types by maxT analysis. We focused on the 31 transcripts that showed 24-fold expression in seminomas compared with embryonal carcinoma samples, and the 40 and 120 transcripts that were expressed at least 4- or 2-fold higher, respectively, in embryonal carcinomas compared with seminomas. The 25 most differentially expressed transcripts for each tumor class are shown in Supplementary Table S5A and B. Several of these genes have not been identified in previous screens of embryonal carcinomas compared with seminomas. Compared with embryonal carcinomas, seminomas showed high levels of expression of GIP, TCL1A, and XIST. Interestingly, among the genes more highly expressed in embryonal carcinomas compared with seminomas were SOX2, which has been reported previously (30), as well as TDGF1 and FGFR1. All of these genes have been identified as being essential genes that define “stemness” (34). We examined the expression levels of these three genes along with the other four defining markers of “stemness” (ZFP42, POU5F1, EBAF, and THY1) in embryonal carcinomas and seminomas and found that all were highly expressed in embryonal carcinoma but only ZFP42, POU5F1, and THY1 showed high levels of expression in seminomas (Supplementary Fig. S2). We validated several of the differentially expressed genes (EBAF, SOX2, TDGF1, CD24, GIP, SOX17, and KIT) by RT-PCR analysis in a subset of the tumors that had been used for expression profiling. As expected, all genes showed an expression pattern that was consistent with the data from the microarray analysis (Supplementary Fig. S3). We also did immunohistochemical staining for GDF3 and SOX2 protein in ITGCN, seminoma, and embryonal carcinoma specimens that were not used for expression profiling. As expected, SOX2 was highly expressed in the nucleus of embryonal carcinoma samples (Fig. 4C) but not in seminomas (Fig. 4B) or ITGCN (Fig. 4A). Interestingly, SOX2 was expressed in in situ embryonal carcinoma lesions (data not shown). GDF3 showed strong cytoplasmatic staining in embryonal carcinoma, with weaker staining in seminomas and ITGCN (Supplementary Fig. S4A-C). There was also some evidence of staining of spermatogonia but not in Sertoli cells in these specimens.

Activation of WNT signaling in embryonal carcinoma versus seminomas. Many of the differentially expressed genes between embryonal carcinomas and seminomas have been implicated in developmental signaling pathways (Fig. 5). We observed differential overexpression of genes involved in NODAL, FGF, BMP, and WNT signaling between embryonal carcinoma and seminoma samples. In particular, the WNT signaling pathway was highly represented as reported previously (30, 35). In embryonal carcinoma, WNT5A and FZD7 were overexpressed relative to seminomas as reported previously (30) as well as the TCF-like transcription factor TCF7L and several previously identified downstream targets of WNT signaling, including versican (36), connexin 43 (36), JUN (37), and FGFR4 (38). In contrast, seminomas showed overexpression of a different WNT ligand (WNT2B) and several known downstream WNT targets, including the homeobox gene CDX1 (39) and NRRCAM (40). We also did staining for β-catenin, which is normally translocated to the nucleus on activation of WNT signaling. This revealed intense staining in the membrane and some staining in the cytoplasm of embryonal carcinoma specimens but no nuclear staining (Fig. 6B), whereas in seminomas staining was limited to the membranes (Fig. 6A). We also examined β-catenin localization in the pluripotent embryonal carcinoma cell line NT2/D1 by immunocytochemistry, which revealed that staining was limited to the membranes and cytoplasm (data not shown).

Discussion

GCTs are characterized by gain of 12p in ~100% of cases (41). Gain of 12p seems to occur early during GCT tumorigenesis, as some ITGCN lesions show evidence of 12p gain (13) and overexpression of 12p genes, such as NAGOG (5). However, whether gain is associated with initiation or progression is still unclear. Several candidate genes on 12p have been suggested, but...
we observed on 12p13.31. Other than to together in the genome and be coordinately expressed (45, 46) as is also a homeobox gene, which have been found to cluster only 6.6 kb, shared this expression pattern. Interestingly, the stem cell gene

other such genomic regions. However, we did observe that these have not been based on comprehensive expression screens. We analyzed expression profiles from a panel of 101 GCT specimens and 5 normal testis samples using Affymetrix U133A-B microarrays, which provide ~83% coverage of 12p transcripts. We identified differentially expressed genes within histologic subtypes of GCT, including 107 transcripts on 12p. Several genes were altered in all or all but one histologic subtypes. These included CCND2, which we have identified previously as potentially important in GCT etiology by reinitiating cell cycle and providing a proliferative advantage for the tumor (2). The REA (repressor of estrogen activation) gene, which regulates transcriptional activity, was also highly expressed in all tumor subtypes. The GLUT3 glucose transporter was highly expressed in all tumor types relative to normal testis as reported previously (19). This gene also maps to the 12p13.13 region that harbors the stem cell cluster that we have defined but does not share the stem cell–related expression pattern of the other genes in this region. Perhaps most interesting is the overexpression in three tumor types (excluding yolk sac tumor and teratoma) of CD9, a tetraspanin receptor. Tetraspanin proteins have been implicated in cell adhesion, migration, and proliferation (42). CD9 has been implicated in the maintenance of undifferentiated embryonic stem cells in the mouse (43). Furthermore, the murine form of CD9 is the receptor for pregnancy-specific glycoprotein 17 (44). We have shown recently that high levels of expression of pregnancy-specific glycoproteins are predictive of choriocarcinoma histology (23). At this time, it is unclear as to what role many of these genes play in GCT tumorigenesis.

We observed that several genes that map to 12p13.31 and have been implicated in stem cell maintenance (18) were coordinately overexpressed in the seminomas and/or pluripotent embryonal carcinoma samples. These included GDF3, STELLA, Hs.129302 (a potential pseudogene designated as FEEENE by AceView), and the homeobox genes HOXC14 and NANOGEN. A search for other coordinately regulated stem cell–associated regions failed to find any other such genomic regions. However, we did observe that the stem cell gene UTF1 and VENTX2 on 10q26.3, separated by only 6.6 kb, shared this expression pattern. Interestingly, VENTX2 is also a homeobox gene, which have been found to cluster together in the genome and be coordinately expressed (45, 46) as we observed on 12p13.31. Other than UTF1, the closest proximal or distal genes to VENTX2 that were present on the array did not show a stem cell pattern of expression. Furthermore, this region has not been reported to be frequently gained in GCTs, suggesting that there is not a coordinately regulated cluster of stem cell genes at 10q26.3. Our results thus confirm the presence of a 200-kb region on 12p that is coordinately regulated in pluripotent GCTs, such as embryonal carcinoma, and identifies two additional genes (Hs.129302 and HOXC14) that map to this region.

Our results also suggest that the stem cell–associated genes within the 12p13.31 region may be coregulated to maintain expression only in "stem cell–like" cells. Elevated expression of genes mapped to this region have been reported previously in seminoma and embryonal carcinoma (30) and in cultured human embryonic stem cells (20). We have further observed that these and other stem cell–related genes are down-regulated in response to differentiation in cultured embryonal carcinoma cells and that expression levels are lower in the differentiated yolk sac tumor, choriocarcinoma, and teratoma samples. Together, these results suggest multiple roles for the selection for 12p gain during GCT tumorigenesis (i.e., providing a proliferative advantage and reestablishment and/or maintenance of a stem cell phenotype). This would be most consistent with 12p gain occurring early, perhaps at tumor initiation. In support of this notion, i(12p) has been detected in ITGCN (13), the precursor to invasive GCT. Furthermore, overexpression of genes on 12p, such as NANOGEN and TEAD4, have been observed in ITGCN lesions (5).

Based largely on immunohistochemical and morphologic criteria, seminomas have been suggested to resemble undifferentiated germ cells. In contrast, embryonal carcinoma resembles pluripotent embryonic stem cells and has the capacity to differentiate. In support of this notion, another study identified genes that are differentially expressed between the two tumor types and, based on differential gene expression, postulated that seminomas more closely resemble primordial germ cells, whereas embryonal carcinoma represents a more primitive ectoderm-like cell (30). A recent study also examined differentially expressed genes in embryonal carcinomas and seminomas and identified 169 genes (35), of which 56 transcripts (40 genes) overlapped with genes identified in our study, including ones overexpressed in seminomas [KIT, which was known previously (47), GAL, CPEB1, and SOX17] and genes overexpressed in embryonal carcinoma (BCAT1, GDF3, and DNM7B). Our results indicated that embryonal carcinoma specimens show expression of genes that

---

have been implicated in stem cell function to a much greater extent than seminoma samples. Indeed, of the seven genes that have been proposed as unequivocal markers of human embryonic stem cells (34), only POU5F1, THY1, and ZFP42 were highly expressed in seminoma samples, whereas all seven were expressed in embryonal carcinoma samples. The four additional genes expressed in embryonal carcinoma only (TDGF1, EBAF, SOX2, and FGF4) have all been implicated as targets of WNT, TGFβ, FGF, BMP, and NODAL signaling pathways during embryogenesis (48), suggesting that these signaling pathways may play an important role in germ cell tumorigenesis as well. EBAF, TDGF1, and STELLA have all recently been identified as genes that are highly expressed in nonseminoma GCTs (49), although our results indicate that expression of these genes is limited to embryonal carcinoma samples. WNT signaling has been suggested previously to explain the differences between embryonal carcinoma and seminoma (30), although in the present study we saw evidence of WNT signaling in both, albeit with different ligands and downstream targets. β-Catenin, which is normally found in cell membranes, can be translocated to the nucleus on activation of canonical WNT signaling and thus is a marker for active WNT signaling. Staining for nuclear β-catenin was negative in both embryonal carcinoma tumors and cell lines. However, in the pluripotent murine teratocarcinoma cell line F9, RA-induced differentiation results in stabilization of β-catenin (50). Furthermore, in this study, it was found that inhibition of WNT signaling by introduction of a dominant-negative Tcf4 molecule attenuates the response of F9 cells to RA (50). Our results suggest that highly active WNT signaling may not be important for maintenance of an undifferentiated, pluripotent phenotype in embryonal carcinoma cells, but activation of WNT signaling may be required for a response to a differentiation signal in these cells. Our results further suggest that other signaling pathways may also be important in maintaining pluripotency in embryonal carcinoma cells.

This study represents the largest expression profiling of GCTs to date, allowing the identification of previously unrecognized differentially expressed genes, including several novel candidate

Figure 6. β-Catenin protein expression in seminoma and embryonal carcinoma. A, staining of seminoma for β-catenin is limited to membranes. B, staining of embryonal carcinoma for β-catenin reveals intense membrane staining, with some cytoplasmic staining also present.
target genes on 12p and stem cell–associated genes. Genetic changes represented by consistent gain or loss of chromosomes and chromosomal arms have been documented in many tumor types, although their biological significance remains unclear. Our results indicate that gain of the entire 12p arm of chromosome 12 may play multiple roles, driving the selection of this large genetic alteration during GCT tumorigenesis. Our study also raises several important questions that need to be addressed, such as the timing of overexpression of POU5F1 and the homeobox/stem cell–related genes on 12p, the mechanism of silencing of stem cell genes on differentiation into yolk sac tumor, teratoma, and choriocarcinoma lineages, and the roles of WNT, BMP and FGF pathways and additional stem cell genes, such as SOX2, TDGF1, and FGFR4, in the reestablishment of pluripotency and subsequent development of embryonal carcinoma tumors.

References

Down-Regulation of Stem Cell Genes, Including Those in a 200-kb Gene Cluster at 12p13.31, Is Associated with In vivo Differentiation of Human Male Germ Cell Tumors

James E. Korkola, Jane Houldsworth, Rajendrakumar S.V. Chadalavada, et al.