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Coexpression of Multiple Sertoli Cell and Leydig Cell Marker Genes in the Spontaneous Testicular Tumor of F344 Rat: Evidence for Phenotypical Bifurcation of the Interstitial Cell Tumor

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The development of testicular tumor has been frequently observed in some laboratory rat strains. In the present study, we have further characterized the testicular tumor that spontaneously develops in the F344 rat (F344/Jcl). Tumor cells first appeared in the interstitium and developed into multifocal nodular lesions. In the later stage, the whole testes were occupied by tumor cells that consisted of three different types of cells in morphological appearance: large clear type, small eosinophilic type and intermediate type. To determine the character of these cells, we examined the expression of marker genes for Sertoli cells (e.g., transferrin) and Leydig cells (e.g., 3β-hydroxysteroid dehydrogenase 1 (3β-HSD 1)). Transferrin and 3β-HSD 1 mRNAs were found in all 8 tumor samples analyzed by northern blotting. By *in situ* hybridization, we observed a substantial amount of 3β-HSD 1 mRNA and little or no transferrin mRNA in the large clear cells. In contrast, the small eosinophilic cells showed little or no 3β-HSD 1 mRNA and a large amount of transferrin mRNA, suggesting that the tumor was a mixture of at least two types of cells. Other Sertoli cell marker genes, such as cyclic protein 2 and sulfated glycoprotein 2, were expressed in all 8 tumors analyzed, and testin and steel factor (SLF), the c-*kit* receptor ligand, were also expressed in some of the tumors (testin, 75%; SLF, 25%), while other Leydig cell markers, LH receptor and c-*kit*, were expressed in 87% and 80% of the tumors, respectively. These results indicate that the spontaneous testicular tumor of F344 rat is of interstitium origin, showing phenotypical bifurcation possibly via transdifferentiation.

Key words: F344 rat — Testicular tumor — Sertoli cell — Leydig cell — Transdifferentiation

The mammalian testis is composed of multiple cell types. Because of this, classification of tumorigenic lesions developing in this organ is rather complicated. The testicular tumor of germ cell origin is the most common in humans, whereas stromal and mesenchymal tumors are more frequent in rodents. The development of testicular tumor has been frequently observed in some laboratory rat strains, such as F344 and Wistar.[1–4] By 10 to 12 months of age, these strains show nodular interstitial hyperplasia of the testis. Around the age of 24 months, almost all male rats develop testicular tumors. As the tumors have been found to originate from the interstitium and to express various enzymes for androgen metabolism, previous authors have concluded that the tumors are of Leydig cell origin. However, there are also reports of some morphological variants in the tumor,[4,5] suggesting that the tumor is composed of multiple cell types.

In this study, the spontaneous testicular tumor of F344 rats bred in a breeder's colony was further characterized. Hyperplasia of the interstitial cells was first found and then developed into multifocal nodular lesions with uniform cells. In the later stage, the whole testis was occupied by three different morphological types of cells: large clear type, small eosinophilic type and intermediate type. To examine the origin of the tumor from the molecular viewpoint, we examined the expression of both Sertoli cell and Leydig cell marker genes. The results indicated phenotypical bifurcation of the interstitial tumor of the testis.

MATERIALS AND METHODS

Animals Male F344 rats (F344/Jcl) were purchased from Nihon Clea Inc. (Tokyo). Rats were killed at the age of 9 to 29 months to observe the development of the testicular tumor. The testes, seminal vesicles and prostate were excised, weighed and fixed in Bouin's fluid for histological examinations or quickly frozen in liquid nitrogen. More than 90 testes were examined.

Northern blot analyses RNA samples from different tissues were prepared by the method of Glisin *et al.*[6] A sample of 20 μg of total RNA was denatured and subjected to formalin-agarose gel electrophoresis. After

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Table I. Sertoli Cell and Leydig Cell Marker Genes Used in This Study

Marker gene[a)]	Cell specificity	(%) positivity in the tumor
CP-2	Sertoli	100
SGP-2	Sertoli	100
Transferrin	Sertoli	100
Testin	Sertoli	75
SLF	Sertoli	25
ABP	Sertoli	0
FSHR	Sertoli	0
Inhibin-α	Sertoli/Leydig[b)]	100
3β-HSD1	Leydig	100
LHR	Leydig	87
c-*kit*	Leydig	80

a) Abbreviations, see the legend to Fig. 4.
b) Expressed in immature Leydig cells.

transfer of the material to a nitrocellulose membrane, hybridization was carried out according to the procedure of Southern.[7] DNA probes used in this study are listed in Table I.[8–18] All probes were ^{32}P-radiolabeled with a multiprime labeling kit (Amersham, Tokyo) before use for hybridization. The filter was washed under a stringent condition and then examined with a Fujix bioimage analyzer (Fuji Film, Tokyo).

***In situ* hybridization** The testes of 22-month-old F344 rats with tumors at various stages were rinsed in phosphate-buffered saline, directly frozen in O. C. T. compound (Miles, Elkhart, IL) and sectioned. The resulting cryostat sections (7 μm) were placed on silane-coated slides for analysis. *In situ* hybridization reactions were carried out by utilizing a digoxygenin-alkaline phosphatase system (Boehringer-Mannheim, Tokyo), as described previously, with minor modifications.[19, 20] The 0.8 kb *Eco*R I/*Bgl* II fragment of a mouse 3β-HSD 1 cDNA clone (pBS3β1.10.3) was subcloned into pBluescript. This was used as a template for the anti-sense and sense RNA probes. The digoxigenin-labeled antisense 3β-HSD 1 RNA probe was generated by transcribing the linearized template with T7 RNA polymerase, and the labeled sense probe was generated with T3 RNA polymerase. The 0.7 kb *Pst* I/*Hin*c II fragment of a rat transferrin cDNA clone (pSP65Tf) was also used as a template for the antisense probe and the sense probe. After hybridization and alkaline phosphatase reaction with nitroblue tetrazolium salt, the slides were washed with water and counterstained with methyl-green.

RESULTS

The testicular tumor of F344/Jcl rat Testicular tissues were sectioned, stained with hematoxylin and eosin, and examined under a light microscope. First, hyperplasia of the interstitial cells appeared in the testes of 12-month-old rats, followed by development of multifocal nodular lesions with uniform cells having round nuclei, punctuated nucleoli and broad eosinophilic cytoplasm (Fig. 1, A). Around the age of 24 months, the whole testes were occupied by tumor cells in all samples examined. At this stage, marked elevation of the testis weight (2.59 g on average: 163% of 12-week-old adult rats) but reduction of the seminal vesicle weight (3.91 mg/g body weight on average: 20% of 12-week-old adult rats) were observed, suggesting a reduction of the serum androgen level due to destruction of the testicular architecture by tumor development. This also implies that the tumor does not produce significant amounts of androgen. The tumor was typically composed of three types of cells: large cells with mature nuclei, punctuated nucleoli and clear cytoplasm, small cells with relatively large immature nuclei, fine chromatin and narrow eosinophilic cytoplasm and an intermediate type having the characters of both types of cells (Fig. 1, B and C). Glandular structure, described in a previous report,[5] was also observed, though it represented only a small portion of the whole tumor (not shown).

Expression of transferrin and 3β-HSD 1 genes in the testicular tumor Transferrin is produced solely by Sertoli cells in the testis and has been accepted as a representative marker for this cell type, whereas 3β-HSD is the most commonly used Leydig cell marker.[9, 10] Messenger RNA was prepared from testes completely occupied by tumor cells and expression of the two marker genes was examined by northern blotting. Transferrin and 3β-HSD 1 mRNAs were found in all tumor samples examined (Fig. 2).

***In situ* distribution of transferrin and 3β-HSD 1 mRNAs in the testicular tumor** We prepared testis samples from 22-month-old rats and examined the expression patterns of transferrin and 3β-HSD 1 genes in these tumors by *in situ* hybridization analysis. In the early stage tumor, we could not detect any significant amount of transferrin or 3β-HSD 1 mRNA (Fig. 3, A and B). It appeared that expression levels of the genes were very low, since coloration was comparable to the control staining with transferrin and 3β-HSD 1 sense probes (data not shown). The advanced tumor consisted of three types of cells as described above. In the large cells, 3β-HSD 1 mRNA was clearly detected, but little or no transferrin mRNA was found. In contrast, transferrin mRNA was detected, but 3β-HSD 1 mRNA was not in the small type of cells (Fig. 3, C and D). The expression pattern of these two genes was not clear in the intermediate type cells. These data suggest that the large type cells have some properties of the Leydig cell and the small cells have some properties of the Sertoli cell.

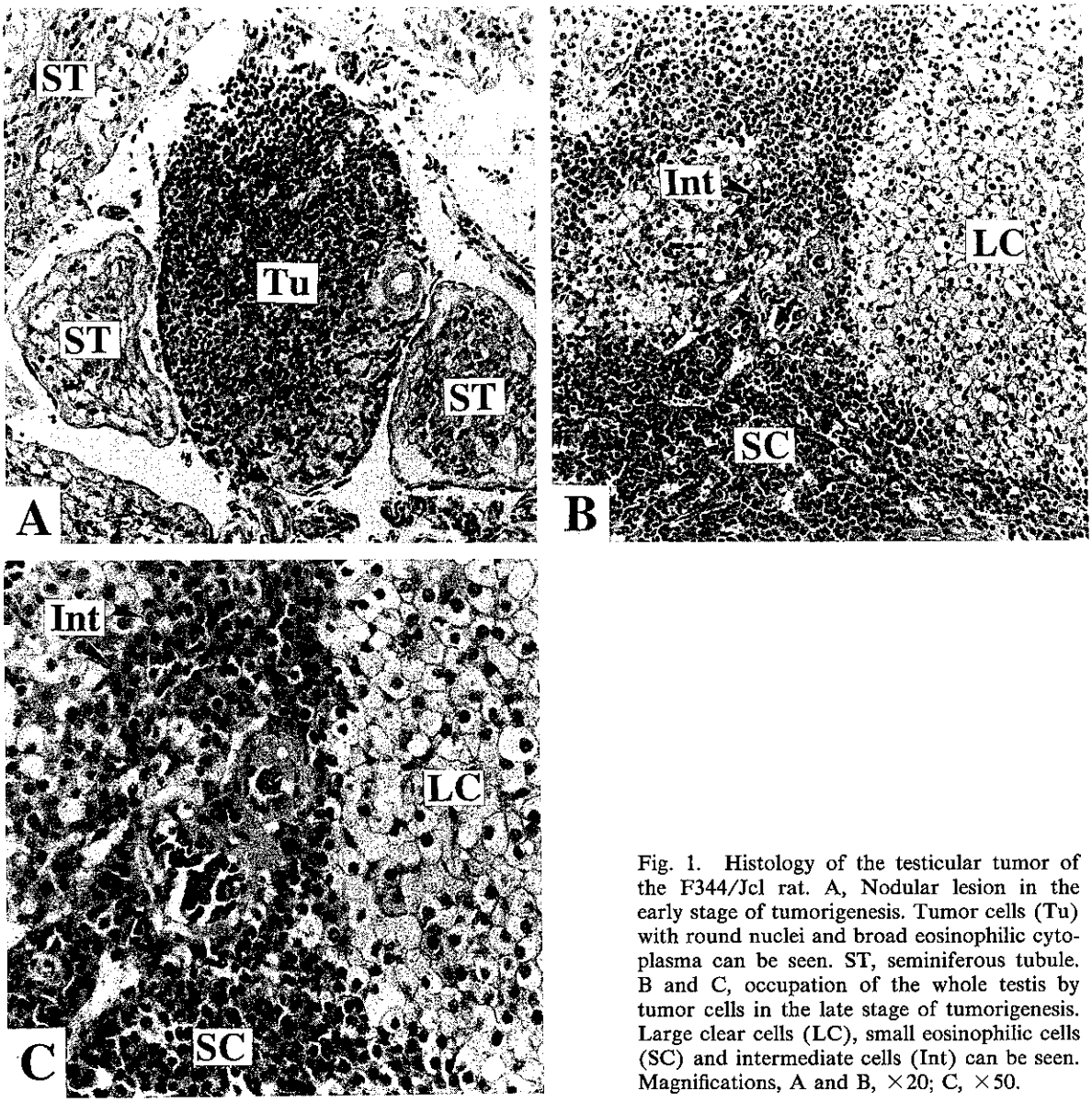

Fig. 1. Histology of the testicular tumor of the F344/Jcl rat. A, Nodular lesion in the early stage of tumorigenesis. Tumor cells (Tu) with round nuclei and broad eosinophilic cytoplasma can be seen. ST, seminiferous tubule. B and C, occupation of the whole testis by tumor cells in the late stage of tumorigenesis. Large clear cells (LC), small eosinophilic cells (SC) and intermediate cells (Int) can be seen. Magnifications, A and B, ×20; C, ×50.

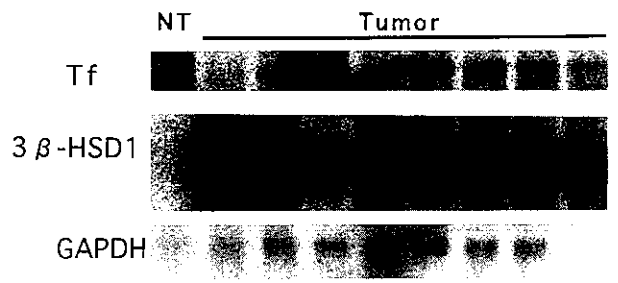

Fig. 2. Expressions of transferrin and 3β-HSD 1 genes in the testicular tumor. Twenty micrograms of cytoplasmic RNA from rat testes was analyzed by northern blotting. Transferrin (Tf) and 3β-HSD 1 mRNAs were detected in all tumors analyzed. GAPDH, glyceraldehyde 3-phosphate dehydrogenase mRNA, as a control. NT, normal testis of 12-week-old adult rat.

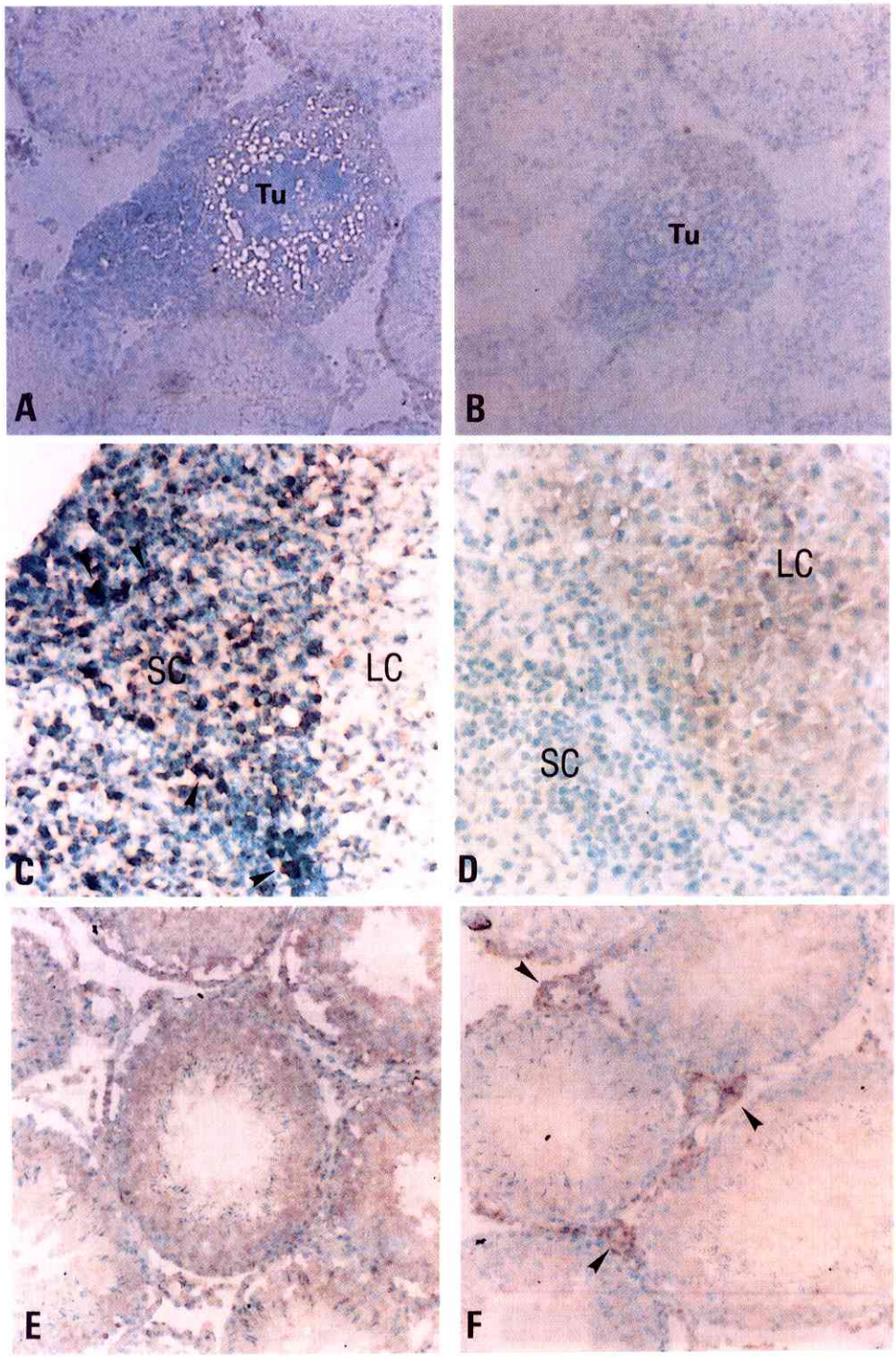

Fig. 3. *In situ* hybridization analysis of the tumors. The testicular tumors of 22-month-old rats were hybridized with transferrin antisense probe (A, C and E) and 3β-HSD 1 antisense probe (B, D and F). Nitroblue tetrazolium, which produces a dark blue stain, was used for detecting the gene-specific hybridization, while nuclei were counterstained with methyl green. LC and SC represent the large type of cells and the small type of cells, respectively. The detection of transferrin mRNA in the SC is difficult, because of the narrow cytoplasm. Representative stainings are indicated (arrowheads) in C. The localizations of intratubular transferrin mRNA (E) and interstitial 3β-HSD 1 mRNA (F, arrowheads) in the normal testis are also shown. Tu: tumor lesions at an early stage. Magnifications, A, B, E and F $\times$20; C and D $\times$50.

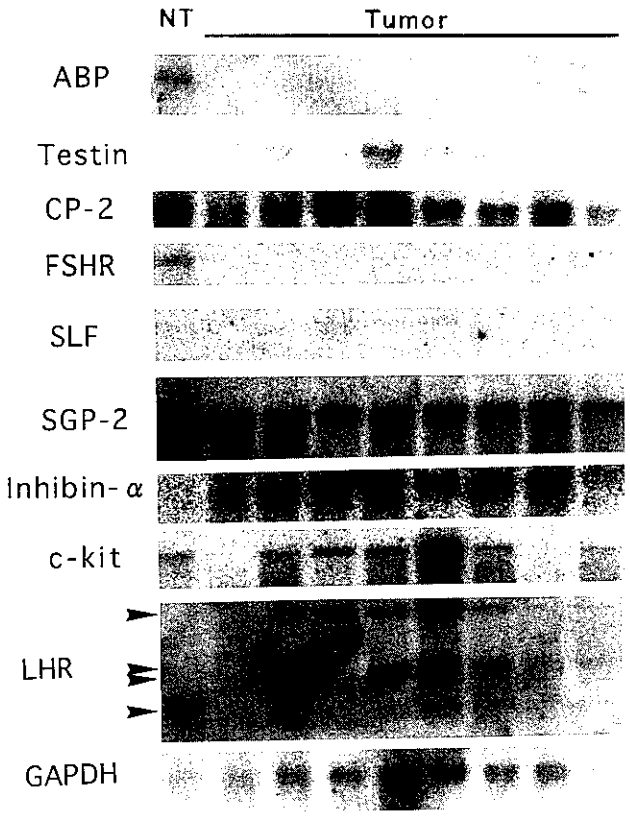

Fig. 4. Expression of multiple Sertoli cell and Leydig cell marker genes in the testicular tumor. Twenty micrograms of cytoplasmic RNA from rat testes was analyzed by northern blotting. The following mRNAs were examined: ABP, androgen binding protein; CP-2, cyclic protein 2; FSHR, follicular stimulating hormone receptor; SLF, steel factor (c-*kit* ligand); SGP-2, sulfated glycoprotein 2; LHR, luteinizing hormone receptor; and GAPDH, glyceraldehyde 3-phosphate dehydrogenase. NT, normal testis of 12-week-old adult rat.

Expression of other Sertoli cell and Leydig cell marker genes in the testicular tumor In order to confirm the presence of the Sertoli cell-like component in the testicular tumor, we examined the expression of other Sertoli cell marker genes (besides transferrin gene), in comparison with Leydig cell marker genes, by northern blotting. Four other (out of 6) Sertoli cell-specific genes were distinctly expressed, i.e., cyclic protein 2 (CP-2)[13] and sulfated glycoprotein 2 (SGP-2)[12] in all 8 tumors analyzed, and testin[11] and steel factor (SLF),[18] the c-*kit* receptor ligand, in some of the tumors (testin, 75%; SLF, 25%), while Leydig cell marker genes (LH receptor[16] and c-*kit*[17]) were also expressed in most of the tumors. Inhibin-α[8] mRNA, found in both Sertoli cells and immature Leydig cells, was also detected in all the tumors (Fig. 4). These observations indicate that the testicular tumor exhibits properties charateristic of Sertoli cells to various degrees.

DISCUSSION

In the testicular tumor of F344/Jcl rat, we found three types of tumor cells in terms of morphological appearance: large clear cells, small eosinophilic cells and an intermediate type. This observation suggested that the tumor was composed of cells of different lineages, prompting us to investigate the expression of Sertoli cell and Leydig cell marker genes in the tumor. F344 rat is a well known interstitial cell tumor-prone strain, though its testicular tumor has not yet been well characterized from the molecular viewpoint.

Our results, together with a possible explanation of the phenotypical bifurcation of the testicular tumor, are summarized in Fig. 5. In this study, the tumor cells were first found in the interstitium and therefore the origin of the tumor might be the Leydig cell, in which the 3β-HSD 1 gene but not the transferrin gene is known to be expressed. However, in the early stage of tumorigenesis, the tumor cells seemed not to express either the 3β-HSD 1 or the transferrin gene. These cells might not have been sufficiently differentiated. At the late stage, around 24 months of age, the tumor cells clearly show phenotypical divergence to large clear cells with high expression of the 3β-HSD 1 gene and small eosinophilic cells with high expression of the transferrin gene.

In a previous report on the development of Leydig cell tumor in a substrain of Wistar rat, Teerds *et al.* observed two types of tumor cells, in accordance with to our results.[3] They concluded that this tumor is of Leydig cell origin because the tumor cells had developed at the interstitium, showed 3β-HSD gene expression, and had some sensitivity to ethane dimethanesulfonate (EDS), which selectively kills Leydig cells. In the testes of EDS-treated rats bearing the tumors, they found some EDS-resistant cells, in addition to necrotized cells. Thus, they considered that the resistant cells were in a more immature state, though still of Leydig cell origin. Similarly, EDS sensitivity was also examined in the testicular tumor of our F344 rat. We found distinct expression of the transferrin gene, but disappearance of 3β-HSD 1 gene expression, in the tumor of EDS-treated animals, suggesting that the EDS-resistant cells were not immature undifferentiated Leydig cells, but were cells that had diverged from Leydig cell lineage (G. Kondoh, unpublished data). Since several Sertoli cell marker genes were expressed in late-stage testicular tumors, some cells in the tumor had apparently differentiated to the Sertoli cell lineage. It is possible that the testicular tumor in F344/Jcl rat might be a case of epithelial-mesenchymal transdifferentiation,[21,22] because the Leydig cell is known

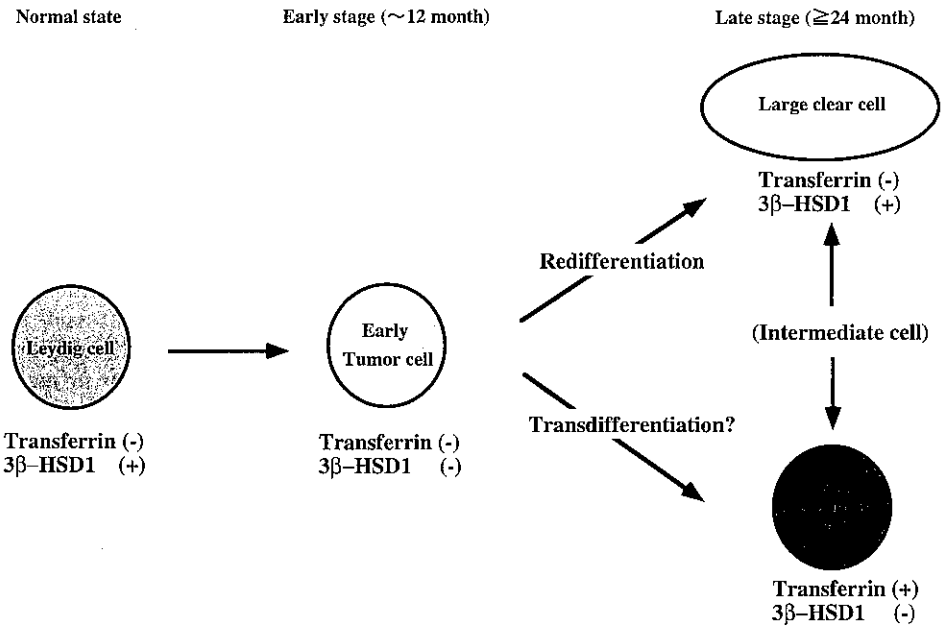

Fig. 5. A possible explanation of the phenotypical bifurcation of the testicular tumor of F344/Jcl rat.

to originate from the mesenchym, while the Sertoli cell is of epithelial origin (Fig. 5).

In the present study, we have characterized the spontaneous testicular tumor of the F344 rat bred in a breeder's colony. We observed expression of multiple Sertoli cell marker genes in the testicular tumor, in addition to Leydig cell marker genes. This rat model may be useful for investigating the mechanism of interstitial cell tumor development and its phenotypical bifurcation.

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