Progenitor cells of the testosterone-producing Leydig cells revealed

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The cells responsible for production of the male sex hormone testosterone, the Leydig cells of the testis, are post-mitotic cells with neuroendocrine characteristics. Their origin during ontogeny and regeneration processes is still a matter of debate. Here, we show that cells of testicular blood vessels, namely vascular smooth muscle cells and pericytes, are the progenitors of Leydig cells. Resembling stem cells of the nervous system, the Leydig cell progenitors are characterized by the expression of nestin. Using an in vivo model to induce and monitor the synchronized generation of a completely new Leydig cell population in adult rats, we demonstrate specific proliferation of vascular progenitors and their subsequent transdifferentiation into steroidogenic Leydig cells which, in addition, rapidly acquire neuronal and glial properties. These findings, shown to be representative also for ontogenetic Leydig cell formation and for the human testis, provide further evidence that cellular components of blood vessels can act as progenitor cells for organogenesis and repair.

Introduction

The temporally regulated emergence during ontogeny of the testosterone-producing Leydig cells is essential for the proper development of male individuals. This includes the presence/activity of distinct phenotypes arising prenatally ("fetal" Leydig cells) or during puberty ("adult" Leydig cells), respectively (for reviews see Ge et al., 1996; Habert et al., 2001; Mendis-Handagama and Ariyaratne, 2001). Despite considerable efforts, the origin of the Leydig cells is still a matter of debate (Benton et al., 1995; Ariyaratne et al., 2000; Habert et al., 2001; Brennan and Capel, 2004). Based on the realization of neural characteristics of Leydig cells (Schulze et al., 1987; Davidoff et al., 1993, 1999, 2002), we asked whether cells distinguished by the expression of the intermediate filament protein nestin, which have been established to act as stem/progenitor cells in the nervous system (Lendahl et al., 1990; Rietze et al., 2001; Sahlgren et al., 2001), could be involved also in the generation of Leydig cells. To address this issue, we made use of an experimental model where a single injection of the cytotoxic compound ethane dimethane sulphonate (EDS) into adult rats initially eliminates the existing Leydig cell population, which is followed by a synchronized and complete Leydig cell regeneration (Teerds, 1996). By this approach, we identified vascular smooth muscle cells (VSMCs) and pericytes (PCs) as the progenitors of all Leydig cell phenotypes, indicating that vascular cell types, acting like adult stem cells, play a critical role in organ formation.

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Abbreviations used in this paper: CNS, central nervous system; EDS, ethane dimethane sulphonate; GFAP, glial fibrillary acidic protein; NF-H, neurofilament-H; PC, pericyte; PDGFR, PDGF receptor; SMA, smooth muscle α-actin.

Results

The time period of Leydig cell disappearance is characterized by elevated nestin expression

To prove and document reliably the known fate of Leydig cells in the rat testis after a single i.p. EDS injection, we monitored the expression of a Leydig cell marker protein, cytochrome P450 side chain cleavage enzyme (CytP450), representing the rate-limiting enzyme of steroidogenesis. CytP450-immunoreactive cells completely disappeared 3 d after EDS treatment and began to reappear ~14 d after EDS treatment, detectable primarily as cell clusters located in the vicinity of intertubular vessels (Fig. 1 a) and in form of single, peritubularly distributed spindle-shaped cells (Fig. 1 b). At this time, the total amount of Leydig cells is still very low (Fig. 1 c), and the expression of CytP450 is not yet detectable by immunoblotting (see Fig. 2 a).

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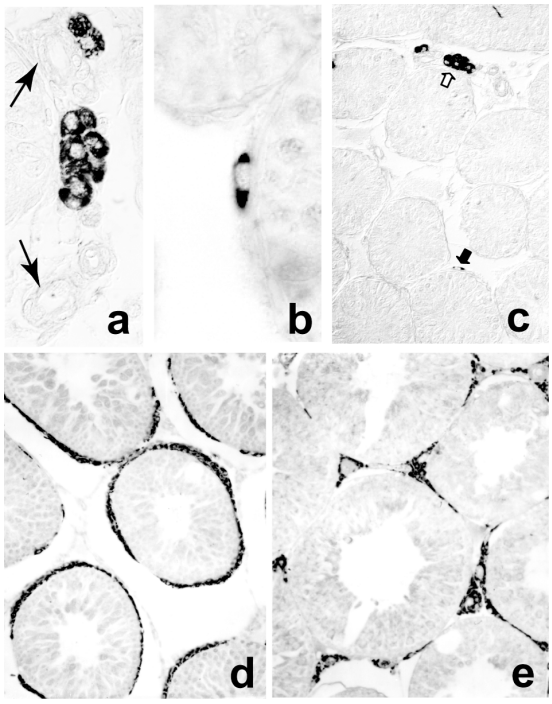

Figure 1. **Reappearance of Leydig cells after EDS treatment.** Leydig (CytP450-immunoreactive) cells are first detectable near blood vessels (arrows) in the form of clusters (a) or as single spindle-shaped cells (b) in testes 14 d after EDS. The cluster (white arrow) and spindle-shaped cell (black arrow) magnified in a and b, respectively, are indicated in c. The accumulation of Leydig cells at d 21 (d) and d 30 (e) after EDS, distributed around (d) and between (e) seminiferous tubules, is shown.

At d 21 after EDS, the number of CytP450-immunoreactive Leydig cells has increased vigorously, and its overwhelming majority is now peritubularly localized. (Fig. 1 d). By d 30 after EDS administration, the number of peritubular Leydig cells has decreased, and the cells are now accumulated preferentially within the intertubular connective tissue (Fig. 1 e), resembling the localization of Leydig cells in normal adult testes.

To investigate whether nestin is expressed in testis, we initially performed immunoblot analyses. These studies were capable of demonstrating low levels of nestin, detectable as a protein of ~440 kD (Kachinsky et al., 1995), in normal (untreated) 3-mo-old animals. However, the expression in testis of nestin was rapidly and massively enhanced after EDS treatment (Fig. 2 a), and the most significant elevation was observed precisely until the onset of massive Leydig cell regeneration (see the analogous immunoblot for CytP450). Thus, Leydig cell disappearance clearly coincides with elevation, and Leydig cell reappearance with diminution of nestin expression.

VSMCs and PCs are the sites of nestin expression and show pronounced cell division activity

Immunohistochemical analyses showed that nestin is localized to the testicular microvasculature. There is clear evidence that VSMCs are the sites of nestin immunoreactivity, whereas endothelial cells are nestin negative (Fig. 2, b and c). Note in addition (see Fig. 2 c) the occurrence in some larger blood vessels of either nestin$^-$ (arrowhead) or nestin$^+$ (arrow) VSMCs. Selected sections displaying the transition from precapillaries to capillaries demonstrate that PCs, in addition to VSMCs, are the sites of nestin expression (Fig. 2 d). Such sections served also to demonstrate coexpression of nestin (Fig. 2 d) and smooth muscle α-actin (SMA; Fig. 2 e) in these cells. In addition, 2 d after EDS administration, only these vascular wall cell types (Fig. 2 f) display nuclear labeling by BrdU (to examine dividing cells, the animals had been injected with BrdU 2 h before decapitation and organ dissection), indicating their proliferative activity. The principal distribution pattern of nestin immunoreactivity in testis at d 2 after EDS, localized to the intertubular and peritubular microvasculature (Fig. 2 g), and the pronounced mitotic activity at that time of VSMCs and PCs at a peritubular position (Fig. 2 h) are illustrated. We note that the contractile peritubular myoid cells neither express nestin nor show mitotic activity. Comprehensive immunohistochemical analyses proved that the increase in nestin expression during the first week after EDS injection (as assessed by immunoblotting) is based on an increase in the number of nestin-immunoreactive VSMCs and PCs.

Nestin-expressing VSMCs and PCs protrude from vessel walls and are transformed into Leydig cells

14 d after EDS, nestin-positive cells begin to protrude from the vessel walls (Fig. 3, a–c) and to accumulate in their vicinity in form of clusters (Fig. 3 d), strikingly similar to the (CytP450 immunoreactive) Leydig cell clusters observed at that time (Fig. 1 a). Conditions where these cell clusters are still in contact with the blood vessel walls they derive from are detectable (Fig. 3 c). As revealed by double staining, the clusters consist of cells expressing either both nestin and CytP450, or only CytP450 (Fig. 3, e and f), indicating a process in which nestin-immunoreactive cells, during and/or after their displacement from vessel walls, first acquire steroidogenic properties (expression of CytP450) and then loose nestin, finally resulting in typical Leydig cells. This conversion into Leydig cells is accompanied also by a loss of SMA immunoreactivity (not depicted), providing further evidence for a transdifferentiation phenomenon. Approximately 1 wk later, when newly formed Leydig cells are abundant and localized primarily around the tubular compartments, nestin expression is generally reduced but still clearly detectable at larger blood vessels (Fig. 3 g). Newly formed Leydig cells that still display nestin immunoreactivity are only rarely to be found at d 20 (Fig. 3 h) and are nearly undetectable at d 21 (Fig. 3 i), indicating a faster transformation process at that time. In contrast to the situation early after EDS treatment, the rapid and abundant transformation is not accompanied by a pronounced cell division activity. Note that the number of BrdU-labeled nuclei of stem cell–like progenitors is several fold higher at d 2 (Fig. 3 j) than at d 21 after EDS (Fig. 3 k). Thus, proliferation of the Leydig cell progenitors predominates during the first week after EDS, whereas their transformation begins at d 14 and accelerates 1 wk later.

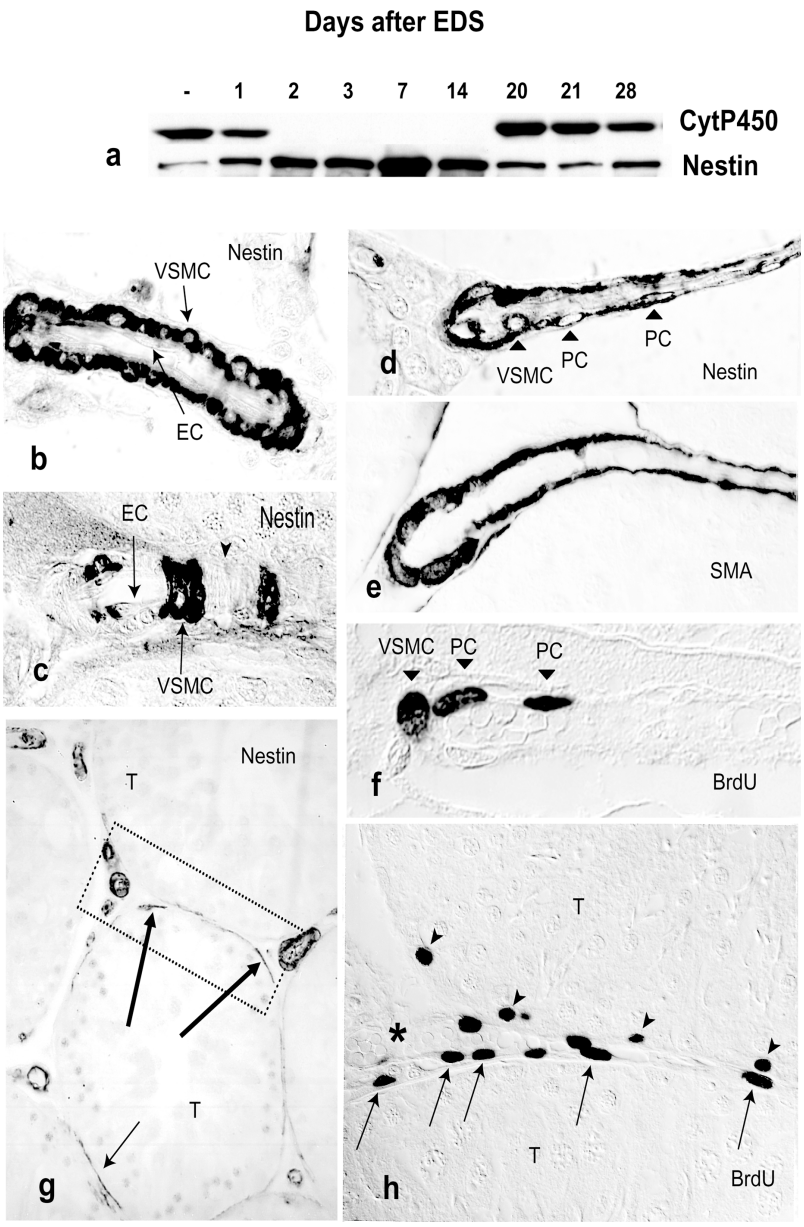

Figure 2. **EDS exposure induces testicular nestin expression, localized to VSMCs and PCs.** (a) Immunoblots demonstrate increased nestin expression during the period of Leydig cell (see levels of CytP450) depletion. (b–d) Immunohistochemical analyses show that nestin is localized to vascular smooth muscle cells (VSMC) and pericytes (PC) (d), but not to endothelial cells (EC; see b and c, with longitudinal orientation) of testicular blood vessels. (e) VSMCs and PCs are stained for smooth muscle α-actin (SMA). (f–h) At d 2 after EDS, VSMCs and PCs proliferate as indicated by nuclear incorporation of BrdU (f and h). The testicular distribution of nestin immunoreactivity at that time, localized to large intertubular as well as to peritubular (arrows) vessels, is shown in g. Higher magnification of an area corresponding to the box marked in g demonstrates proliferating (BrdU labeled) cells of the peritubular microvasculature (h, arrows). Note that some spermatogonia (arrowheads) are also detectable. The lumen of a blood vessel filled with erythrocytes is marked by an asterisk. T, seminiferous tubules.

Leydig cell progenitors and Leydig cells are characterized by the distinct expression of neuronal/glial markers

Next, we examined intermediate filament proteins that are expressed subsequent to nestin in cells of the nervous system during their differentiation into the neuronal/glial lineage (Dahlstrand et al., 1995; Doetsch, 2003). These studies revealed a conspicuous regulation of neurofilament-H (NF-H). Its expression in testis increases (first detectable at d 7) later than that of nestin (d 1, see Fig. 2 a) and is sustained at high levels for a relatively short time (around d 14) after EDS exposure (Fig. 4 a). Thus, transiently elevated expression of NF-H, localized to the vascular Leydig cell progenitors (Fig. 4 b), coincides with their transition from proliferative to transformation activity.

In contrast to NF-H, which is detectable only at low levels also in Leydig cells (Davidoff et al., 1999), the glial fibrillary acidic protein (GFAP) was found to be predominantly expressed in these cells. Consistently, GFAP levels are scarcely detectable by immunoblotting during the period of Leydig cell depletion (Fig. 4 a). The Leydig cell–specific expression of GFAP was confirmed by immunohistochemical analyses, demonstrating localization to Leydig cells (Fig. 4 c). Further studies revealed that newly generated Leydig cells, independent on their intertubular (Fig. 4, d, e, and h; 14 d after EDS) or peritubular (Fig. 4, f and g; 21 d after EDS) site of generation, rapidly become immunoreactive for diverse neuronal markers such as growth-associated protein 43 (Fig. 4 d), tyrosine kinase A (Fig. 4, e and f), glial cell line–derived neurotrophic factor (Fig. 4 g), neural cell adhesion molecule (Fig. 4 h), neuron-specific enolase, microtubule-associated protein-2, synaptophysin, and NeuroD (not depicted), as well as for GFAP (Fig. 4 c), 2′,3′-cyclic nucleotide 3′-phosphodiesterase, and A2B5-antigen (not depicted), known to be expressed by astrocytes, oligodendrocytes, and glial progenitor cells, respectively (Davidoff et al.,

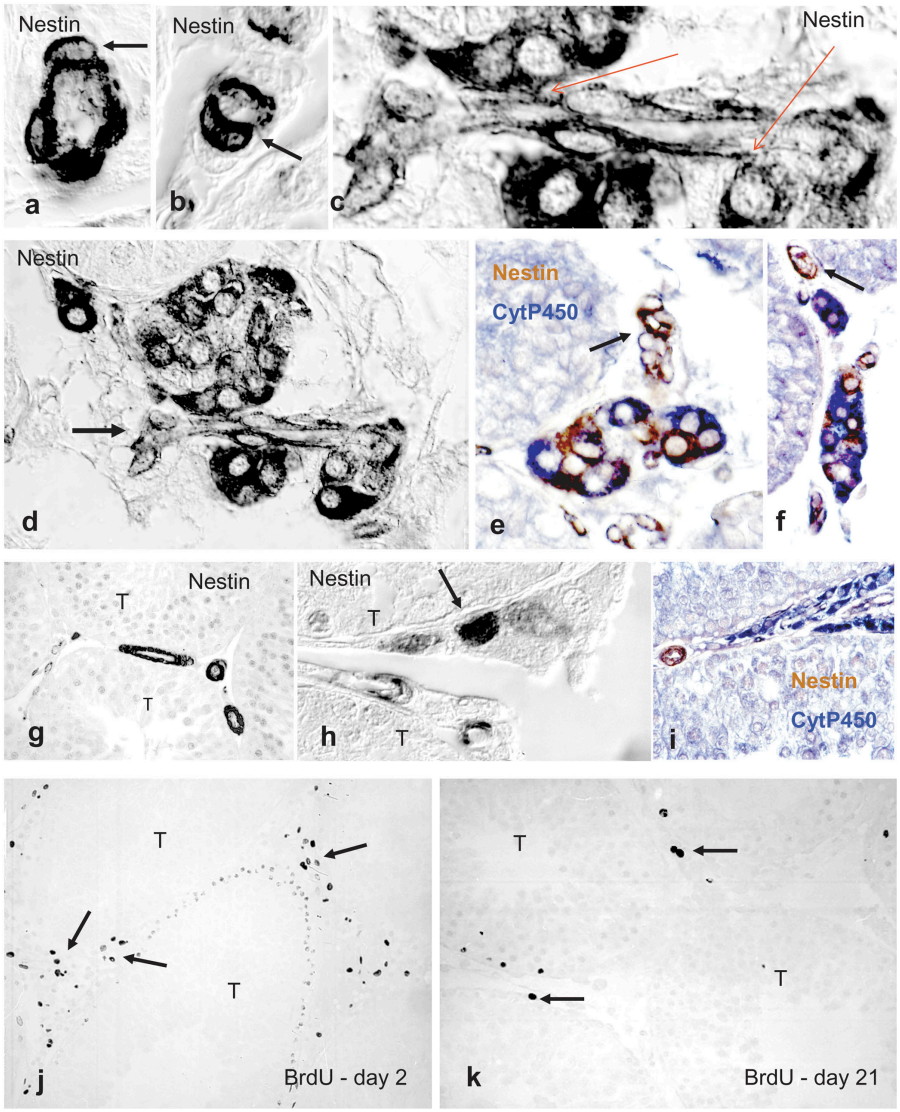

Figure 3. **Nestin-expressing cells protrude from blood vessel walls and transform into Leydig cells.** 14 d after EDS, nestin-immunoreactive cells begin to protrude (arrows) from intertubular vessel walls (a and b) and begin to form cell clusters in their direct vicinity (c and d). The orientation of such a vessel between newly formed cell clusters (d, indicated by an arrow) and sites where transdifferentiating progenitor cells are still in contact with the vessel they derive from (c, red arrows) are shown. These clusters consist of cells that either coexpress nestin (brown) and CytP450 (blue), or express Cyt P450 only (e and f). 1 wk later, nestin$^+$ cells are visible at a peritubular position (g and h), and newly formed Leydig cells are only occasionally (h, day 20) or not at all (i, day 21) nestin positive. At this time (k, day 21), the number of proliferating (BrdU labeled) vascular wall cells (arrows) is much lower than at d 2 after EDS (j). T, seminiferous tubules.

2002). Thus, after transformation from vascular progenitors, the Leydig cells are distinguished by the expression of several neuronal/glial markers, indicative of a remarkable pluripotent neural phenotype.

Next, we examined the proteoglycan NG2, known to be expressed in neuronal and glial cell progenitors of the central and peripheral nervous system (Schneider et al., 2001; Chekenya et al., 2002; Belachew et al., 2003) and previously suggested as a marker for PCs (Ozerdem et al., 2001). Like nestin (see Fig. 2 and Fig. 3), NG2 immunoreactivity was found both in VSMCs and PCs (Fig. 4, i–k), as well as in newly generated Leydig cells (Fig. 4 k), further indicating a relationship between these cell types. Finally, the selective identification of PDGF receptor-β (PDGFR-β; Fig. 4, l and m), known to be involved in development of VSMCs and PCs (Lindahl et al., 1997; Lindahl and Betsholtz, 1998; Hellström et al., 2001; Hoch and Soriano, 2003; Lindblom et al., 2003), and myelin/oligodendrocyte-specific protein (not depicted) immunoreactivity in both newly generated (at d 14 after EDS) Leydig cells and their vascular progenitors, strongly supported their lineage relationship.

Leydig cell generation during postnatal development shows similarities to the Leydig cell repopulation process after EDS treatment

Next, we investigated whether the regeneration of Leydig cells after EDS treatment has similarities to the formation of a mature Leydig cell population in rats during normal postnatal development. Immunoblot analyses of Cyt P450scc revealed a biphasic expression pattern (Fig. 5 a): protein levels most significantly increase during puberty (d 24 and 27) to attain maximum (i.e., adult) values, but there is also an elevated expression at the earliest (postnatal day 5) stage examined. This developmental pattern is consistent with a gradual decrease in the number of fetal-type Leydig cells during initial postnatal development followed by the appearance and plentiful accumulation of adult-type Leydig cells (Ge et al., 1996). Comparative analyses of nestin and NF-H revealed striking similarities to the Leydig cell repopulation process after EDS treatment. Nestin expression is initially high and declines in time with the abundant emergence of (adult) Leydig cells, whereas the postnatal expression of NF-H

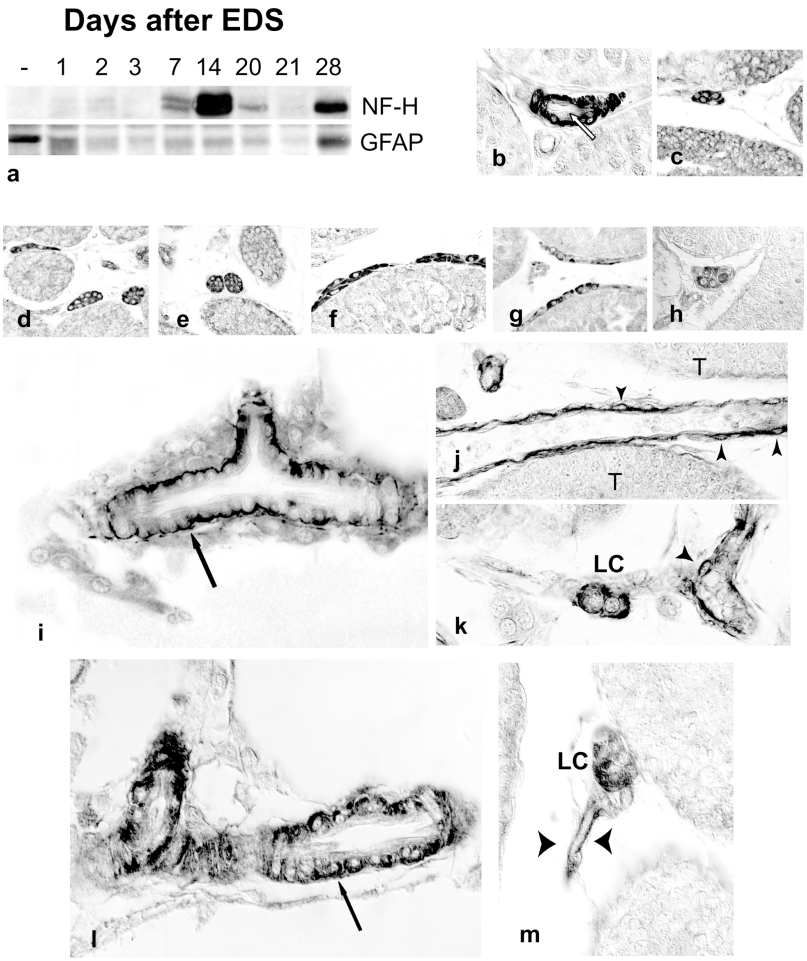

Figure 4. **Neural characteristics of Leydig cells and their vascular progenitors.** (a) Immunoblots show transiently elevated levels of NF-H and a reduced expression of GFAP in testis during the period of Leydig cell depletion after EDS treatment. VSMCs, but not endothelial cells (EC, marked by an arrow), are the sites of NF-H expression (b). In contrast, newly formed Leydig cells are immunoreactive for GFAP (c), GAP43 (d), TrkA (e and f), GDNF (g), and N-CAM (h). Both VSMCs (i, arrow) and PCs (j and k, arrowheads), as well as newly formed Leydig cells (k, LC), are characterized by the expression of NG2, and Leydig cells (LC) together with their vascular progenitors (VSMC, arrow; PCs, arrowheads) are sites of PDGFR-β expression (l and m). T, seminiferous tubules.

commences later (between d 5 and 10) and peaks (around d 15) before the switch from nestin to CytP450 expression (Fig. 5 a).

At the cellular level, VSMCs and PCs as well as fetal-type Leydig cells are the major sites of nestin expression during early (d 5) postnatal development (Fig. 5 b), and the formation of Leydig cell clusters at blood vessels is detectable similar to the situation 14 d after EDS treatment (Fig. 5 b). During puberty (postnatal day 27; Fig. 5 c), nestin-positive vascular cells are visible in close proximity to seminiferous tubules and large intertubular vessels. Nestin$^+$ VSMCs are traceable also in testes of adult rats (Fig. 5 d). Their distinct localization to blood vessels within Leydig cell groups is indicative of a continuous generation of new Leydig cells by transformation from nestin$^+$ progenitors in the mature testis.

Analyses of nestin-GFP transgenic mice and human testes

As in rats, a similar pattern of nestin-immunoreactive cells was found in testes of adult mice (unpublished data). To verify the sites of nestin expression by a different approach, we made use of nestin-GFP transgenic mice. These studies, performed with animals expressing GFP under the control of the central nervous system (CNS)–specific nestin second intronic enhancer (Lothian and Lendahl, 1997; Mignone et al., 2004), confirmed the expression of nestin in Leydig cells and their vascular progenitors (Fig. 5, e–g).

Finally, comparative analyses revealed that this is also valid for the human testis (Fig. 5 h). Remarkably, numerous (still nestin-immunoreactive) newly formed Leydig cells are detectable within areas of Leydig cell hyperplasia in testes of elderly men (Fig. 5 i). Thus, transformation of stem cell–like progenitors into Leydig cells can explain (hitherto incomprehensible) increases in the number of Leydig cells (Clegg et al., 1997) despite their lack of mitotic activity (Teerds et al., 1989; Benton et al., 1995).

Discussion

Identification of vascular Leydig cell progenitors

This paper identifies VSMCs and PCs as the progenitors of Leydig cells (summarized in Fig. 6). We show that the Leydig cell progenitor cells are characterized by (1) the expression of nestin, a marker protein for stem cells of the nervous system (Lendahl et al., 1990; Rietze et al., 2001; Sahlgren et al., 2001); (2) a transient up-regulation in these cells of NF-H, known to be expressed in the nervous system during the differentiation of stem cells into the neuronal lineage (Dahlstrand et al., 1995); and (3) that the newly formed Leydig cells rapidly become immunoreactive for diverse neuronal and glial markers. Thus, three independent lines of evidence point to a similarity be-

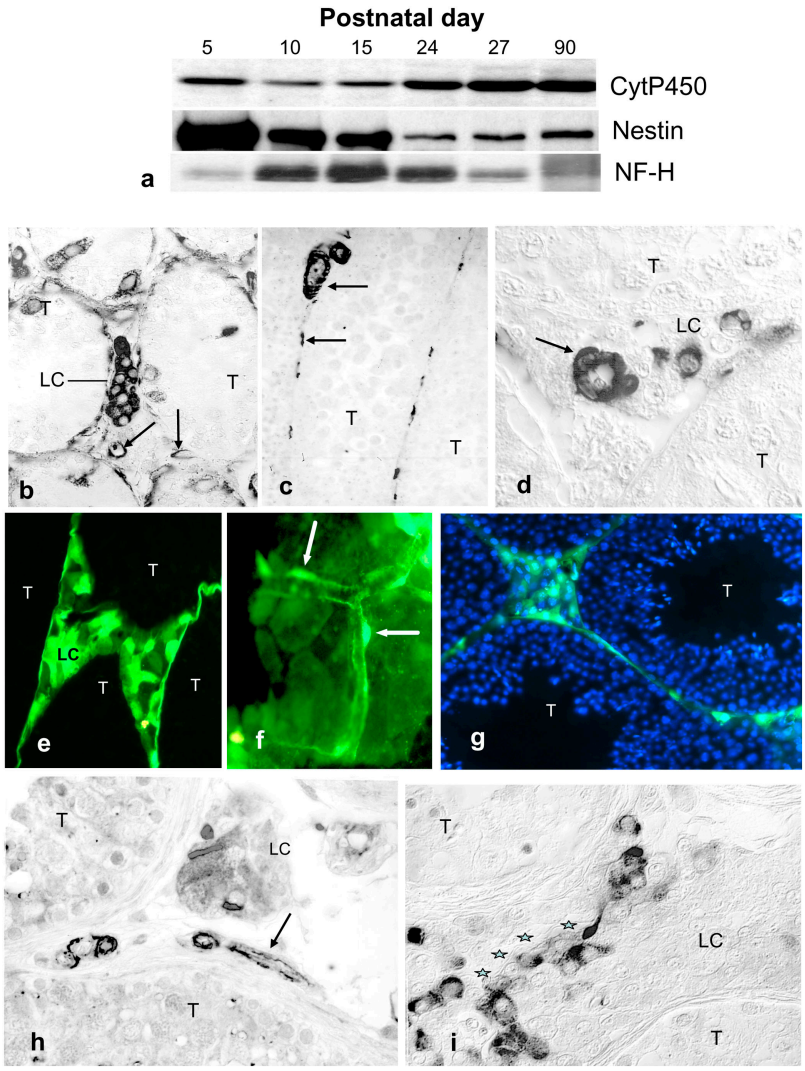

Figure 5. **Nestin expression in testis during rat ontogenetic development, in nestin-GFP transgenic mice, and adult humans.** (a) Immunoblots show the regulation in rat testis of CytP450, nestin, and NF-H during postnatal development. (b–d) The distribution of nestin-immunoreactive structures in rat testis at postnatal days 5 (b), 27 (c) and 90 (d) is shown. (e–g) GFP expression in testes of transgenic mice expressing GFP under the control of the nestin second-intron enhancer (green fluorescence) is detectable in Leydig cells and their vascular progenitors based on morphology (arrows point to PCs). In g, nuclei are additionally marked by DAPI (blue). (h and i) Immunohistochemical staining for nestin on adult human testis sections from individuals without (h) or with (i) Leydig cell hyperplasia. Tubular compartments (T), Leydig cells (LC), and nestin-positive vessels (arrows) are indicated. In i, stars mark the lumen of a small vessel.

tween the vascular Leydig cell progenitors and stem cells/progenitors of the nervous system, consistent with and supporting findings (for review see Carmeliet, 2003) that neural and vascular systems use common genetic pathways. In this context, the recent identification of a factor (Arx), implicated in both brain development and Leydig cell differentiation (Kitamura et al., 2002), has to be noted.

The in vivo model used in this study was crucial for the identification of the Leydig cell progenitors. We revealed that after EDS treatment, Leydig cell regeneration is preceded by a specifically induced proliferation of nestin-expressing VSMCs and PCs, and we were capable of demonstrating the dynamic process of their conversion into steroidogenic Leydig cells. These findings were substantiated by immunoblot analyses that clearly demonstrated, both in the EDS model and during postnatal development, the functional interrelation between the quantity of progenitor cells (indicated by nestin levels) and the quantity of Leydig cells (indicated by CytP450 levels). It will be of interest to identify the signaling pathways implicated in the activation of progenitor proliferation as well as in triggering the transdifferentiation processes in (pre)pubertal and adult animals. Data obtained by analyzing fetal Leydig cell development refer to PDGFs, acting via PDGFRs α/β (Gnessi et al., 2000; Basciani et al., 2002; Brennan et al., 2003; Hoch and Soriano, 2003) and Desert hedgehog (Clark et al., 2000; Yao et al., 2002) as possible candidate molecules. In addition, factors such as bone morphogenetic proteins, known to regulate cell fate choice and differentiation of CNS stem cells (Rajan et al., 2003), have to be considered.

Nestin expression in testis

Although a functional role for nestin is still poorly understood, its specific expression in activated/dynamic cells (e.g., cells that proliferate, migrate, or rapidly change their phenotype) is well established (Sahlgren et al., 2001). Originally identified as a marker for neural progenitor cells during early stages of CNS development (Lendahl et al., 1990), it was meanwhile demonstrated that nestin is also expressed in a variety of tissues outside the CNS (Vaittinen et al., 2001; Zulewski et al., 2001; Li et al., 2003) and in the adult organism during regeneration of injured tissue (Frisén et al., 1995; Okano, 2002). Our present findings demonstrate elevated nestin expression in testis both during development as well as during regeneration after experimentally induced Leydig cell elimination in adult animals.

Consistent with observations in other tissues (Lendahl et al., 1990; Sejersen and Lendahl, 1993), nestin was found to be down-regulated and replaced by other intermediate filament proteins upon terminal differentiation. Nestin expression was clearly localized to PCs/VSMCs and newly generated Leydig cells in rat, mouse, and human testes. This characteristic pattern was additionally verified by the analysis of nestin-GFP transgenic animals. It has to be noted that the nestin-GFP mice used in our study direct transgene expression to cell types of neural origin (Mignone et al., 2004), and that GFP was found to be expressed selectively in the adult brain in areas related to continuous neurogenesis (Mignone et al., 2004). Thus, the identification in these animals of GFP in testicular vascular wall (PCs, VSMCs) cells and Leydig cells essentially supports similarity to properties of neural stem/progenitor cells. It is of interest that the mean number of nestin-expressing Leydig cells in mouse (detectable both in the GFP model and by immunohistochemistry in wild-type mice; unpublished data) as well as in human testes is significantly higher than in rat testes, suggesting species-specific differences in the velocity of nestin down-regulation after terminal differentiation.

Lineage relationship between PCs, VSMCs, and Leydig cells

The present paper reveals a close relationship between PCs and VSMCs of the testis microvasculature. Both cell types express SMA and nestin and proliferate in response to EDS treatment. The identification of NG2 and PDGFR-β immunoreactivity in both cell types corroborates their relationship, and in addition demonstrates properties similar to those of neural progenitors of the CNS (Belachew et al., 2003). These findings are consistent with several previous reports, indicating functional (Allt and Lawrenson, 2001; Hoofnagle et al., 2004; Hu et al., 2004) or lineage (Alliot et al., 1999; Korn et al., 2002) relationship between PCs and VSMCs. Evidence for their transdifferentiation into Leydig cells is based on clear coincidence of marker expression, i.e., transient coexpression of nestin with CytP450 in newly generated Leydig cells. Moreover, the observed connection of newly formed Leydig cell clusters with the blood vessel walls further demonstrates their origin, and previously detected basement membrane fragments at the cell surface of certain Leydig cells (Kuopio and Pelliniemi, 1989; Haider et al., 1995) probably represent remnants of the basal lamina known to surround PCs (Sims, 1991; Nehls and Drenckhahn, 1993). Consistently, immunoreactivity for NG2 and for PDGFR-β, recently established as reliable markers for activated PCs (Ozerdem and Stallcup, 2003) as well as for myelin/oligodendrocyte-specific protein, was identified in both newly generated Leydig cells and their vascular progenitors. Finally, lineage relationship was significantly supported by analyses of nestin-GFP transgenic mice, showing specific (and selective) expression of the transgene in Leydig cells and their progenitors.

The potential involvement of circulating cells in the process of Leydig cell (re)generation requires attention. However, several lines of evidence argue against such a possibility. The observed immediate induction of proliferation/nestin expression in PCs and VSMCs during the EDS-elicited Leydig cell destruction strongly refers to resident cells, rapidly responding to newly generated and/or disappearing (testosterone) signaling molecules. Furthermore, immunohistochemical analyses, performed with antibodies against the mesodermal/hematopoietic stem cell marker CD34, did not reveal any marker expression in PCs or SMVCs of the testis microvasculature, whereas staining was detectable in endothelial and intertubular connective tissue cells (unpublished data). These findings, indicating an involvement of resident rather than circulating cells, are consistent with cellular activities responsible for increased nestin expression after injury in other tissues (Frisén et al., 1995; Amoh et al., 2004). Remarkably, a recent investigation, identifying so-called side population (SP) cells in testis (Kubota et al., 2003),

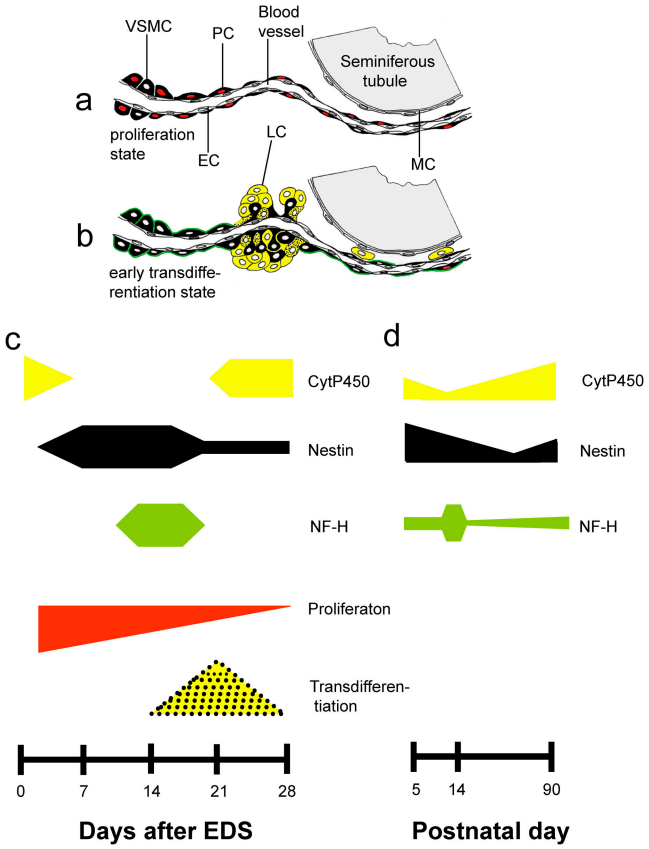

Figure 6. **Schematic representation of transdifferentiation of vascular progenitors into Leydig cells.** (a) Nestin-expressing (black) vascular smooth muscle cells (VSMC) and pericytes (PC) of testicular blood vessels, but not endothelial cells (EC) or peritubular myoid cells (MC), vigorously proliferate (indicated by red-labeled nuclei) during the first week after EDS-induced Leydig cell depletion. (b) Around d 14 after EDS, nestin-expressing VSMCs and PCs, now marked also by a transient expression of NF-H (green contour line), begin to protrude from intertubular vessels and begin to form cell clusters. By transdifferentiation, the cells first acquire steroidogenic properties (yellow, black dotted) and finally loose nestin, resulting in typical (yellow) Leydig cells (LC). (c and d) Time scale of Leydig cell (re)generation after EDS treatment (c) and during ontogenetic development (d). (c) Leydig cell depletion, marked by the absence of CytP450 (yellow), rapidly induces nestin expression (black) and proliferation activity (red). The conversion of proliferative into transformation activity (yellow, black dotted) is preceded by a transient expression of NF-H (green). (d) A similar correlation between CytP450, nestin, and NF-H expression is detectable during postnatal testis development.

showed that testis SP cells do not derive from or contain circulating blood cells, but represent resident cells in this tissue.

Considering that many of the properties of Leydig cells and their progenitors are compatible with a neural crest origin (Davidoff et al., 1993, 1999, 2002; Lobo et al., 2004), we started to address this issue experimentally. However, investigations of mice with targeted mutations (see Materials and methods) in genes of the neural crest–specific neuregulin receptors, ErbB2 and ErbB3 (Britsch et al., 1998), did not yet reveal clear-cut results. In this context, it might be of general interest that we recognized during these studies endogenous β-galactosidase expression in different testicular cell types (unpublished data), which precludes a reliable characterization of testes of transgenic animals expressing β-galactosidase fusion proteins.

Leydig cell heterogeneity

Furthermore, our observations regarding the different Leydig cell phenotypes are worth mentioning. We noted that the hypertrophied cells arising first in the form of clusters during the Leydig cell repopulation process resemble typical fetal-type cells, whereas the second-arising spindle-shaped cells are similar to the so-called intermediate-type cells that are generated as early stages of adult Leydig cells. Although all phenotypes derive from (the same) nestin-positive vascular progenitor cells, the fetal-type cells strictly emerge at intertubular and the intermediate-/adult-type cells at peritubular sites. This strongly suggests local environmental effects (Kerr et al., 1987; Teerds, 1996; Gnessi et al., 2000), which are assumed to be responsible also for the observed differences in the extent of Leydig cell regeneration between tubules with either high or low recovery of spermatogenesis.

In conclusion, the conversion of VSMCs into neuroendocrine Leydig cells represents a novel example of transdifferentiation (Echeverri and Tanaka, 2002; Tosh and Slack, 2002) in vivo and demonstrates that blood vessels not only provide inductive signals (Lammert et al., 2001) but also cellular components (progenitor cells) other than the mesoangioblasts, a recently characterized class of vessel-associated stem cells (Sampaolesi et al., 2003), for tissue generation and repair. In the testis, the vascular progenitors identified probably contribute to regulation of Leydig cell number during aging and under conditions of Leydig cell hyperplasia/tumorigenesis.

Materials and methods

Materials and tissues

EDS was synthesized in our laboratory from ethylene glycol and methane sulfonylchloride as described by Jackson and Jackson (1984); BrdU and dimethylsulfoxide were purchased from Sigma-Aldrich. Testes and other organs from male Wistar rats of different ontogenetic stages (Charles River Laboratories) were dissected immediately after decapitation of the animals. Human testes, obtained from patients undergoing orchiectomy as the primary treatment of prostatic carcinoma, were chilled and cut 1–2 h after surgery.

EDS treatment and proliferation assays

Adult (3-mo-old, 340–380 g) male Wistar rats were housed three per cage under standard laboratory conditions. To induce Leydig cell depletion, animals received single i.p. injections (75 mg EDS/kg body weight, dissolved in 25% dimethylsulfoxide). The rats were killed 1–48 d after EDS injection. For assessment of mitotic cells, EDS-treated animals received i.p. injections of BrdU (150 mg/kg body weight, dissolved in 0.09 M NaOH) 2 h before killing. Tissues were dissected immediately after decapitation of the animals and were either frozen in liquid nitrogen or fixed in Bouin's fluid. The rats were used according to government principles regarding the care and use of animals with permission (G8151/591-00.33) of the local regulatory authority.

Mouse strains

The generation of nestin-GFP transgenic mice, expressing GFP under the control of the nestin gene promoter and a transcriptional enhancer that resides in the second intron of the gene, has been described previously (Mignone et al., 2004). To address certain questions regarding a potential neural crest origin of Leydig cells, we used mice with targeted mutations in genes of tyrosine kinase receptors of the ErbB family (Britsch et al., 1998). The mutants examined either express ErbB2/β-galactosidase fusion proteins or represent ErbB3 knockouts.

Immunohistochemical and fluorescence analyses

Immunohistochemistry, based on peroxidase activity, and specificity controls on paraffin sections were performed essentially as described previously (Davidoff et al., 1993, 1999, 2002) using commercially available primary antibodies: anti-CytP450 (working dilution 1:300), anti–rat (MAB 353, 1:500), anti–human (AB 5922, 1:300) nestin, rabbit anti-NG2 (NG2 chondroitin sulfate proteoglycan; 1:300), and anti-MOSP (mouse anti-myelin/oligodendrocyte-specific protein 1:2,000) from CHEMICON International; anti-NF-H (1:200), anti-GFAP (1:1,000), anti-SMA (1:400), anti-GAP43 (1:1,000), anti-GDNF (2 μg/ml), and anti-N-CAM (1:200) from Sigma-Aldrich; anti-TrkA (1:100) as well as anti-PDGFR-β (P-20; 1:50) from Santa Cruz Biotechnology, Inc.; rat anti–mouse CD34 (1:100) from BD Biosciences; and mouse anti–human CD34 (1:100) from DakoCytomation.

In case of double staining, the second antigen was detected by alkaline phosphatase activity. We used substrate staining (rather than immunofluorescence) deliberately in these experiments to clearly identify and examine transdifferentiating cells in their normal tissue context (i.e., together with all neighboring cells). For visualization of BrdU, sections were pretreated with 1 M HCl in PBS/0.3% Triton X-100 for 30 min at 37°C followed by 30 min with PBS/2% normal rabbit serum before incubation with anti-BrdU (clone BMC 9318, diluted 1:20 in PBS containing BSA and sodium azide; Roche) for 18–24 h at 4°C.

For detection of GFP fluorescence, adult nestin-GFP transgenic male mice were transcardially perfused with 4% PFA in PBS. After additional immersion fixation for 4 h at RT and impregnation with 30% sucrose-PB, the testes were frozen in liquid nitrogen, and cryostat sections (8, 10, and 20 μm) were mounted onto histological slides. After drying for 30 min at RT, the sections were coverslipped with DakoCytomation fluorescent mounting medium. Cell nuclei of certain sections were additionally stained with DAPI.

Immunoblot analyses

Soluble and particulate fractions of tissue homogenates, prepared as described previously (Middendorff et al., 2002), were size fractionated by SDS-PAGE under reducing conditions in 6% (for detection of nestin and NF-H) or 9% (CytP450, GFAP) acrylamide gels and transferred to nitrocellulose membranes. In the immunoblots shown, equal amounts (80 μg protein) of either soluble (nestin, NF-H) or particulate (CytP450, GFAP) fractions were applied, and rat brain proteins were coanalyzed to serve as positive controls in case of the neural antigens examined. The membranes were probed with antibodies against nestin (MAB 353, diluted 1:1,000; CHEMICON International), CytP450 (AB 1244, 1:1,000; CHEMICON International), NF-H (1:750; Biotrend), or GFAP (1:450; Sigma-Aldrich). After incubations with peroxidase-linked anti–mouse (1:2,000; Pierce Chemical Co., Perbio Science) or anti–rabbit (1:2,000; Sigma-Aldrich) secondary antibodies, signals were detected using ECL (Amersham Biosciences) on x-ray films.

Photographic documentation

Photographic documentation was made using an Axioskop microscope (Carl Zeiss MicroImaging, Inc.) equipped with the analogous 36 × 24-mm microscopic camera (MC-100; Carl Zeiss MicroImaging, Inc.) and a 9 × 12-cm Polaroid camera. The photographic negatives (black and white) and slides (colored) were subsequently scanned and additionally processed using Adobe Photoshop 7.0 and Adobe Illustrator 10 (Adobe Systems, Inc.).

GFP fluorescence was viewed using a fluorescence microscope (Axioskop-2; Carl Zeiss MicroImaging, Inc.) equipped with appropriate filter combinations. Images were taken with a digital camera (AxioCam HRc; Carl Zeiss MicroImaging, Inc.) and stored at a size of 1300 × 1030 pixels. Images were assembled with Adobe Photoshop and Adobe Illustrator programs (see above).

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