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**Immunolocalization of murine type VI 3 β -hydroxysteroid dehydrogenase
in the adrenal gland, testis, skin, and placenta**

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Abstract

The enzyme 3 β -hydroxysteroid dehydrogenase/isomerase (3 β -HSD) is essential for the biosynthesis of all active steroid hormones, such as those secreted from the adrenal gland, testis, ovary, skin and placenta. The 3 β -HSD enzymes exist in multiple isoforms in humans and rodents. To date, six different isoforms have been identified in the mouse, and these isoforms are speculated to play different roles in different tissues. We previously showed that the murine type VI 3 β -HSD isoform (Hsd3b6) is expressed specifically in the aldosterone-producing zona glomerulosa cells within the adrenal gland and that its overexpression causes abnormally increased aldosterone synthesis, revealing a crucial (or rate-limiting) role of this enzyme in steroidogenesis. However, potential contributions of this enzyme to the steroid hormone synthesis outside the adrenal glands are poorly understood. This paucity of knowledge is partly because of the lack of isoform-specific antibody that can be used for immunohistochemistry. Here, we report the development and characterization of specific antibody to Hsd3b6 and show the results of immunohistochemistry for the adrenal gland, testis, ovary, skin and placenta. As expected, Hsd3b6 immunoreactivities within the adrenal gland were essentially confined to the zona glomerulosa cells, where aldosterone is produced. By contrast, no immunopositive cells were observed in the zona fasciculata, which is where corticosterone is produced. In the gonads, while the ovaries did not show any detectable immunoreactivity to Hsd3b6, the testes displayed intense immunoreactivities within the interstitial Leydig cells, where testosterone is produced. In the skin, positive immunoreactivities to Hsd3b6 were only seen in the sebaceous glands, suggesting a specific role of this enzyme in sebaceous function. Moreover, in the placenta, Hsd3b6 was specifically found in the giant trophoblast cells surrounding the embryonic cavity, which suggests a role for this enzyme in local progesterone production that is required for proper embryonic implantation and/or maintenance of pregnancy. Taken together, our data revealed that Hsd3b6 is localized in multiple specific tissues and cell types, perhaps thereby involved in biosynthesis of a number of tissue-specific steroid hormones with different physiological roles.

Keywords: 3 β -hydroxysteroid dehydrogenase/isomerase, Steroidogenesis

1. Introduction

The enzyme 3 β -hydroxysteroid dehydrogenase/isomerase (3 β -HSD) is essential for the biosynthesis of all classes of steroid hormones, such as those produced in the adrenal gland, testis, ovary, skin and placenta. It is known that steroid hormones are synthesized from either cholesterol or dehydroepiandrosterone sulfate, the pathways of which both require 3 β -HSD enzymatic activity (Payne and Hales, 2004; Simard et al., 2005) (see also Fig. 1): Whatever the end-point steroid products (e.g. mineralocorticoid, glucocorticoid, androgen and estrogen), all active steroid hormones need to be converted from 3 β -hydroxysteroid precursors to hormonally active 3-ketosteroids, a reaction step catalyzed by the 3 β -HSD. Thus, steroid hormone synthesis necessitates this enzyme.

The 3 β -HSD enzymes exist in multiple isoforms in humans and rodents, and to date six different isoforms (designated Hsd3b1, Hsd3b2, Hsd3b3, Hsd3b4, Hsd3b5 and Hsd3b6) have been identified in the mouse. Among these isoforms, type I (i.e. Hsd3b1) and type VI (i.e. Hsd3b6) are the major 3 β -HSDs playing a role in canonical steroid-producing organs, such as the adrenal, gonads and placenta (Abbaszade et al., 1997; Bain et al., 1991). On the other hand, the remaining four isoforms, from type II to V, are expressed in the liver and kidney (Abbaszade et al., 1995; Bain et al., 1991; Clarke et al., 1993). Also, it has been biochemically shown that type IV and V do not function as 3 β -HSDs but function as 3-keto steroid reductases, thereby involved in the inactivation of steroid hormones (Abbaszade et al., 1995; Clarke et al., 1993). Thus, the isoforms from types II to V are not likely to be important for steroidogenesis. Rather, the dominant importance has been assigned so far to the two specific isoforms, Hsd3b1 and Hsd3b6. Interestingly, reinforcing this notion, only the two genes are evolutionally conserved in humans, with the genes named *HSD3B1* and *HSD3B2* being a candidate counterpart of the mouse *Hsd3b6* and *Hsd3b1*, respectively (Luu-The and Labrie, 2010; Simard et al., 2005). Thus, in order to understand the principal roles of 3 β -HSDs, characteristics of the two conserved isoforms need to be well defined.

Importantly, previous reports demonstrate that at the mRNA level, *Hsd3b6* (*HSD3B1*) and *Hsd3b1* (*HSD3B2*) show different tissue-specificity, providing a reason to speculate that the two isozymes may play different roles in different steroidogenic tissues (Payne and Hales, 2004). However, the lack of isoform-specific antibody precludes further analysis of this enzyme family. Because of a high degree of sequence similarity between the two isoforms (93.5% identity at the amino acid level between human *HSD3B1* and *HSD3B2*; 83.9% identity between mouse *Hsd3b1* and *Hsd3b6*; see also Supp. Fig. 1), none of the antibodies so far developed for 3 β -HSD could differentiate between the two isoforms.

We have been interested in the mouse *Hsd3b6*, since our laboratory previously found the expression of *Hsd3b6* in the adrenal zona glomerulosa cells, where aldosterone is produced. Importantly, increased expression of *Hsd3b6* causes abnormal enhancement of aldosterone production and leads to the development of salt-sensitive hypertension in mice (Doi et al., 2010; Ota et al., 2012). Thus, this enzyme appears to have a capacity to determine the rate of steroid biosynthesis. However, although the physiological importance of *Hsd3b6*

has begun to be clarified in aldosterone biology, the relevance of this isoform for the tissues outside the adrenal gland remains largely unknown.

In the present study, as a starting point towards the elucidation of the isoform-specific roles of 3 β -HSDs, we created a specific antibody against Hsd3b6 and determined for the first time the immunolocalization of this isoform within the major steroid hormone-synthesizing organs. The results demonstrate that Hsd3b6 is localized not only in the adrenal zona glomerulosa but also in specific types of cells within the testis, skin, and placenta. In contrast, immunoreactivity to Hsd3b6 was not detected for the ovary or the adrenal zona fasciculata/reticularis, where the alternate isoform *Hsd3b1* was found to be mainly expressed.

2. Materials and Methods

2.1. Animals

C57BL/6 mice aged 8 to 20 weeks were used in this study. For immunohistochemistry, animals were anesthetized and circulationally perfused with ice-cold phosphate buffer containing 4% paraformaldehyde, and the tissues were isolated and immersed in the same fixative at 4°C overnight. For RNA extraction, animals were sacrificed by cervical dislocation and the tissues harvested were immediately lysed into Trizol reagent (Invitrogen). All of the studies were approved by the Animal Experimentation Committee of Kyoto University.

2.2. Quantitative RT-PCR

RNA was extracted using an RNeasy kit (Qiagen) according to the manufacture's protocol. Total RNA was converted to cDNA with random hexamer primers using SuperScript III First-Strand Synthesis SuperMix (Invitrogen), and qPCR was run in duplicate with a specific set of primers shown below using a Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen). As a qPCR device, we used a StepOnePlus real-time PCR monitoring system (Applied Biosystems). Quantification of target cDNAs was done using a standard curve method (Hou et al., 2010). The standard curve was generated by amplifying a dilution series of a standard DNA (ranging from 1 to 10,000 zmol), for which we used linearized plasmid DNA carrying the target amplicon. The data were normalized with *Rplp0*. The primer sets used were following: *Hsd3b1* (NM_008293), Fw: 5'-agc atc cag aca ctc tca tc-3', Rv: 5'-gga gct ggt atg ata tag ggt a-3'; *Hsd3b6* (NM_013821), Fw: 5'-tga tgg gaa gag ggt gga g-3', Rv: 5'-agg tgc tga gag gct tgg a-3'; *Rplp0* (NM_007475), Fw: 5'-ctc act gag att cgg gat atg-3', Rv: 5'-ctc cca cct tgt ctc cag tc-3'.

2.3. Antibody

In order to obtain isoform-specific antibody against Hsd3b6, antiserum was generated by immunizing rabbit with a synthetic peptide comprising the amino acid sequence specific to the type VI enzyme (36 amino acids from 20 to 55 of Hsd3b6). The raised antibodies were

then affinity-purified using the antigen peptide. The purified antibodies were dialyzed into phosphate-buffered saline (PBS) and stored at the concentration of 0.3 mg/ml.

2.4. Immunocytochemistry

For ectopic expression of recombinant Hsd3b1 and Hsd3b6, full-length coding sequences of *Hsd3b1* and *Hsd3b6* were cloned into pEZ vector (GeneCopoeia), and an hemagglutinin (HA) epitope tag sequence was then introduced into their C-terminal ends using In-fusion HD kit (Takara). The resultant constructs (Hsd3b1-HA/pEZ and Hsd3b6- HA/pEZ) were separately introduced into COS-1 cells by lipofection (Lipofectamine 2000, Invitrogen), and these cells were used for immunocytochemistry with anti-Hsd3b6 antibody. In brief, the transfected COS-1 cells, seeded in 8-well chamber slides (Nunc), were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS. Following pretreatment with blocking solution (5% bovine serum albumin; 0.1 % Triton X-100 in PBS) for 30 min at 25°C, the cells were incubated in blocking solution mixture containing anti-Hsd3b6 antibody (1:100 dilution) together with anti HA (rat monoclonal, 3F10, Roche) for 12 h at 4°C. After rinsing with PBS, the cells were then incubated for 1 h at 25°C in blocking solution mixture containing both Alexa594-conjugated goat anti-rabbit IgG and Alexa488-conjugated goat anti-rat IgG (Invitrogen). The cells were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) before microscopic observation.

2.5. Immunoprecipitation and Western blot

For immunoprecipitation assay, COS-1 cells were plated at 1.0×10^5 cells/well in 6-well plate, and transfected with either Hsd3b1-HA/pEZ or Hsd3b6-HA/pEZ expression vector. The cells were lysed in 250 μ L of RIPA buffer (20 mM Tris-HCl [pH 7.6], 150 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing 2mM phenylmethylsulfonyl fluoride, complete protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Nakarai, Japan), and the lysate was incubated with either Hsd3b6 antibody (1 μ g) or anti-HA antibody 3F10 (Roche, rat monoclonal, 1 μ g) for 12 h at 4°C. The immunocomplexes were then precipitated with Protein A-Agarose (for Hsd3b6 antibody) or Protein G-Agarose (for HA) beads (Roche). Following extensive washes with RIPA buffer, immunoprecipitants were eluted from the beads to Laemmli sample buffer. Western blot was performed according to our standard method (Doi et al., 2004). Proteins samples resolved by SDS-PAGE were immunoblotted, and the immunoreactivities to Hsd3b6 and HA were visualized with enhanced chemiluminescence (ECL plus, GE healthcare) using horseradish peroxidaseconjugated anti-immunoglobulin (GE healthcare).

2.6. Immunohistochemistry

The specimens fixed with 4% paraformaldehyde were embedded in paraffin-wax with routine protocol. Five-micrometer-thick sections were cut on a microtome and deparaffinized with xylene and ethanol. For detecting Hsd3b6 immunoreactivity, sections were antigen-retrieved by pressure cooking in Tris-EDTA buffer (pH 9.0) for 5 min, as described elsewhere (Tanaka et al., 2011). Sections were then immersed in PBS containing 0.1% Tween20 and incubated with

anti-Hsd3b6 antibody (1:500 dilution) for 24 h at 4°C. The immunoreactivities were visualized with 3,3-diaminobenzidine (DAB: brown staining) using horseradish peroxidase -labeled anti-rabbit IgG polymers (Dako, EnVision+ System- HRP Labelled Polymer Anti-Rabbit) according to the manufacture's protocol. After color development, sections were dehydrated and mounted, and images were taken under a light-field microscope. Fluorescence immunohistochemistry was also performed with adrenal cryosections (20 µm thick). In this experiment, free-floating sections were incubated with anti-Hsd3b6 antibody (1:100 dilution) for 24 h at 4°C, and the immunoreactivities were visualized with donkey anti-rabbit IgG, conjugated to *red* fluorescent dye Alexafluor-594 (Invitrogen). Absorption experiment was also performed to verify the specificity of antibody binding. To this end, the immunostaining protocol was repeated using absorbed antibody produced by an overnight incubation of the primary antibody with the antigen peptide (10µM) at 4°C.

3. Results

3.1. Tissue distribution profile of *Hsd3b6*

We began this study by analyzing messenger RNA levels of *Hsd3b6* and *Hsd3b1* in different steroidogenic organs, including the adrenal gland, testis, ovary, skin, and placenta (Fig. 2). Quantitative RT-PCR analysis with gene-specific primers demonstrated that *Hsd3b6* and *Hsd3b1* exhibit different tissue-specificity: *Hsd3b6* was highly expressed in the adrenal gland, testis, skin, and placenta, but no appreciable expression was observed for the ovary and other control tissues tested, such as in liver and kidney (Fig. 2). On the other hand, *Hsd3b1* was localized in the adrenal, testis, and ovary, but no expression was observed for the skin, placenta and the other tissues. Thus, *Hsd3b6* and *Hsd3b1* appear to function in a different set of tissues. However, it also needs to be emphasized that the distributions of the two isoforms are *not* mutually exclusive: Rather, they show a substantial overlap of expression: for example, they are both expressed in the adrenal and testis. Thus, discrimination between the two isoforms is essential for further investigation in such tissues.

3.2. Specificity of *Hsd3b6* antibody

In order to examine the specificity of antibody used for *Hsd3b6*, HA-tagged *Hsd3b6* (*Hsd3b6*-HA) and *Hsd3b1* (*Hsd3b1*-HA) were separately expressed in COS-1 cells, and immunoreactivities to anti-*Hsd3b6* antibody were examined immunocytochemically. Double-label staining (Fig. 3A) showed that the cells expressing *Hsd3b6*-HA were indeed immunopositive to both *Hsd3b6* (red) and HA (green) antibodies, whereas *Hsd3b1*-HA transfected cells were only stained with anti-HA (green). These observations demonstrate that the antibody that we made for *Hsd3b6* has no cross-immunoreactivity to *Hsd3b1*.

Immunoprecipitation-Western blot analyses (Fig. 3B) further demonstrate that our antibody was also useful for isoform-specific immunoprecipitation (Fig. 3B). We found, however, that the antibody we made could not be used for Western blot as this antibody could not detect *Hsd3b6* protein that had been denatured (Fig. 3B). This feature of our antibody precludes SDS-PAGE/Western blot analysis of this protein with tissue lysates. Our antibody seems likely to recognize a natively folded structure(s) of *Hsd3b6*.

3.3. Immunohistochemistry of *Hsd3b6* in the adrenal gland

Consistent with the observed isoform specificity, anti-*Hsd3b6* immunoreactivities in the adrenal gland (Fig. 3C) were specifically found in the zona glomerulosa, the outermost cortical layer of the adrenal cortex, where *Hsd3b6* mRNA is known to be expressed (Doi et al., 2010). On the other hand, immunopositive cells were not found in the zona fasciculata, a middle layer of the cortex, which is where *Hsd3b1* transcripts are dominantly expressed. No cross-reaction with the cells expressing *Hsd3b1* provides further evidence that the antibody that we made is indeed *Hsd3b6*-specific. Moreover, we also performed absorption of the antibody with immunizing peptide (Fig. 3C), which led to no positive immunoreactivities within the zona glomerulosa cells, a result further confirming the specificity of the antibody binding observed.

Importantly, paraffin-embedded tissue sections were also applicable for anti-Hsd3b6 immunostaining. Indeed, paraffin sections of the adrenal gland were found to show strong and specific immunoreactivities to Hsd3b6 within the zona glomerulosa cells (Fig. 4 A-D; see immunopositive, round cells that are arranged in globular clusters, lying directly beneath the capsular layer). Moreover, we also observed that the immunolocalization of this enzyme within the cells were mainly cytoplasmic or perinuclear (Fig. 4D), which observation echoes the fact that this enzyme resides in the endoplasmic reticulum (Simard et al., 2005).

3.4. Immunohistochemistry of Hsd3b6 in the testis and ovary

Using the antibody specific to Hsd3b6, we next examined the expression of this enzyme in the gonads (Fig. 5). In the testes, strong immunoreactivities to Hsd3b6 were found only in the interstitial Leydig cells, where testosterone is produced, with essentially no positive immunoreaction in any of parenchymal cells within the testes (Fig. 5, A-C). We found, on the other hand, that the cells in the ovaries were all immunonegative for Hsd3b6, including those comprising the theca layer surrounding the follicles (Fig. 5 D-F; the arrowhead indicates the location of the theca cells responsible for progesterone production in the ovaries). Negative immunoreactivity seen in the ovaries is in parallel to mRNA profiling of this isoform (Fig. 2).

3.5. Immunohistochemistry of Hsd3b6 in the skin

In the skin, positive immunoreactivities for Hsd3b6 were limited to the cells in the sebaceous glands (Fig. 6). Interestingly, the cells outside the sebaceous glands were all immunonegative, including those comprising the dermal papillae and epidermis, which are the sites previously reported to express other steroid hormone-converting enzymes, such as steroid sulfatase and 5- α reductase (Courchay et al., 1996; Hoffmann et al., 2001; Luu-The et al., 1994).

3.6. Immunohistochemistry of Hsd3b6 in the placenta

Finally, immunolocalization of Hsd3b6 was determined in the placenta. For this end, we examined the placental Hsd3b6 expression at embryonic day (E) 8.5, a stage at which the mRNA level of *Hsd3b6* has been demonstrated to be high in the placenta (Arensburg et al., 1999). Interestingly, intensive immunoreactivities to Hsd3b6 were found only in giant trophoblast cells surrounding the embryonic cavity (Fig. 7). These are the cells responsible for production of progesterone in the placenta. No expression of Hsd3b6 was observed in the embryo.

4. Discussion

The 3 β -HSD enzyme family is comprised of multiple, structurally similar isozymes that are encoded by different genes. However, because of the lack of isoform-specific antibody, different physiological roles that they may play in different tissues have remained poorly understood. In the present study, we reported the development and characterization of an isoform-specific antibody toward the murine type VI 3 β -HSD isozyme (Hsd3b6). Using this antibody, we determined for the first time the immunolocalization of Hsd3b6 within the

major steroid hormone-producing organs including the adrenal gland, testis, ovary, skin, and placenta.

The mouse adrenal gland produces two types of steroids with different functional properties: mineralocorticoid (aldosterone) and glucocorticoid (corticosterone), which are the steroids known to regulate fluid mineral balance and glucose metabolism, respectively. We found that in the adrenal gland, positive immunoreactivities to Hsd3b6 were essentially confined to the cells in the zona glomerulosa, which is where aldosterone is produced. In marked contrast, the cells in the zona fasciculata, which is where corticosterone is produced, were found to be all immunonegative for Hsd3b6. Thus, alternative isoform is needed for the corticosterone synthesis. Several investigators, including ourselves, previously showed that *Hsd3b1* is the predominant isoform transcribed in the zona fasciculata cells (Abbaszade et al., 1997; Doi et al., 2010). Although anti-Hsd3b1 specific antibody is not currently available, Hsd3b1 and Hsd3b6 appear to function in different adrenal physiologies that involve corticosterone and aldosterone, respectively.

As for the gonads, the testes showed strong expression of Hsd3b6 particularly within the interstitial Leydig cells. These are the cells responsible for testosterone production. By contrast, no appreciable expression of Hsd3b6 was observed for the ovaries. Thus, our data indicate that Hsd3b6 is involved in male sex steroid hormone production within the testes. On the other hand, estrogen production by the ovaries seems likely to involve the other isozyme, Hsd3b1. Differently from *Hsd3b6*, *Hsd3b1* is expressed in both the testis and ovary. Thus, different gene transcription mechanisms appear to allow the two isoform genes to be expressed in a particular set of tissues and cell types.

In the skin, strong immunoreactivities to Hsd3b6 were observed particularly in the sebaceous gland. Intriguingly, analogous observations have been reported for the human skin. Simpson et al. described that in the human skin, 3 β -HSD enzymatic activities specifically localize in the sebaceous gland (Simpson et al., 1983). Moreover, immunohistochemistry using anti-human 3 β -HSD antibody (which is not isoform-selective) demonstrated that this antibody indeed strongly stained the sebaceous gland (Dumont et al., 1992; Sawaya and Penneys, 1992). Although the identity of isoform(s) expressed was not clear in this experiment, transcriptome analysis independently showed that the human skin has only one expressed 3 β -HSD isoform, which is *HSD3B1* (Rheume et al., 1991). Remarkably, human *HSD3B1* has been thought to be a functional counterpart of mouse *Hsd3b6* (note that numbers assigned to the isoforms do not reflect the similarity of function, since the members of 3 β -HSD family have been chronologically designated according to their order of identification in each species). Such evolutionary conservation and/or a functional convergence between humans and mice strongly suggest that this specific isoform would be indispensable for sebaceous function in both species. It has been proposed that 3 β -HSD activity in the sebaceous glands would provide the ability of glands to control local concentrations of dehydroepiandrosterone and other steroids in the human skin (Labrie et al., 2000; Simard et al., 2005). It will be of interest to explore the role of this enzyme in more

detail using genetically modified mice lacking specifically Hsd3b6.

In the placenta, strong immunoreactivities to Hsd3b6 were specifically found in the giant trophoblast cells surrounding the embryonic cavity. Importantly, the giant trophoblast cells isolated from the placenta are able to produce progesterone *in vitro* under culture conditions (Peng et al., 2002) Thus, the immunolocalization of Hsd3b6 clearly illustrate that this isoform is involved in the local progesterone production within the placenta. Intriguingly, as seen in mice, human placenta also expresses *HSD3B1*, the human counterpart of the mouse *Hsd3b6* (Lorence et al., 1990; Rheume et al., 1991). Moreover, previous studies show that syncytiotrophoblast cells of the human placenta are immunolabeled with pan (non-selective) 3 β -HSD antibody (Mason et al., 1993; Riley et al., 1992). Thus, in humans and mice, this isoform may play an evolutionally conserved role in the placenta. It has been proposed that local progesterone production within the placenta is required for proper embryonic implantation and/or maintenance of early pregnancy in mice and further speculated that a genetic mutation in human *HSD3B1* could be a cause of many unexplained recurrent abortions early in the first trimester in humans (Peng et al., 2002). In order to experimentally address these long-standing questions, a knockout mouse model lacking Hsd3b6 would be worth development for placental analysis.

In conclusion, we reported herein the development and characterization of isoform specific antibody to Hsd3b6. Our work revealed that this enzyme is involved not only in aldosterone production by the adrenal gland but also in the biosynthesis of multitude, functionally distinct steroid hormones that are secreted from specific cell types in specific tissues. Obviously, the exact role of this enzyme in each specific tissue and cell type should be clarified in future in more detail by loss-of-function experiment for this isoform. The antibody that we made should prove valuable for further investigation of isoform-specific roles of 3 β -HSD in normal steroid hormone homeostasis as well as its disorder.

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References

- Abbaszade, I.G., Arensburg, J., Park, C.H., Kasa-Vubu, J.Z., Orly, J. and Payne, A.H., 1997. Isolation of a new mouse 3beta-hydroxysteroid dehydrogenase isoform, 3beta-HSD VI, expressed during early pregnancy, *Endocrinology* 138, 1392-9.
- Abbaszade, I.G., Clarke, T.R., Park, C.H. and Payne, A.H., 1995. The mouse 3 beta-hydroxysteroid dehydrogenase multigene family includes two functionally distinct groups of proteins, *Mol. Endocrinol.* 9, 1214-22.
- Arensburg, J., Payne, A.H. and Orly, J., 1999. Expression of steroidogenic genes in maternal and extraembryonic cells during early pregnancy in mice, *Endocrinology* 140, 5220-32.
- Bain, P.A., Yoo, M., Clarke, T., Hammond, S.H. and Payne, A.H., 1991. Multiple forms of mouse 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase and differential expression in gonads, adrenal glands, liver, and kidneys of both sexes, *Proc Natl Acad Sci U S A* 88, 8870-4.
- Clarke, T.R., Bain, P.A., Greco, T.L. and Payne, A.H., 1993. A novel mouse kidney 3 beta-hydroxysteroid dehydrogenase complementary DNA encodes a 3-ketosteroid reductase instead of a 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4-isomerase, *Mol. Endocrinol.* 7, 1569-78.
- Courchay, G., Boyera, N., Bernard, B.A. and Mahe, Y., 1996. Messenger RNA expression of steroidogenesis enzyme subtypes in the human pilosebaceous unit, *Skin Pharmacol* 9, 169-76.
- Doi, M., Okano, T., Yujnovsky, I., Sassone-Corsi, P. and Fukada, Y., 2004. Negative control of circadian clock regulator E4BP4 by casein kinase Iepsilon-mediated phosphorylation, *Curr Biol* 14, 975-80.
- Doi, M., Takahashi, Y., Komatsu, R., Yamazaki, F., Yamada, H., Haraguchi, S., Emoto, N., Okuno, Y., Tsujimoto, G., Kanematsu, A., Ogawa, O., Todo, T., Tsutsui, K., van der Horst, G.T.J. and Okamura, H., 2010. Salt-sensitive hypertension in circadian clock-deficient *Cry*-null mice involves dysregulated adrenal *Hsd3b6*, *Nat Med* 16, 67-74.
- Dumont, M., Luu-The, V., Dupont, E., Pelletier, G. and Labrie, F., 1992. Characterization, expression, and immunohistochemical localization of 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase in human skin, *J Invest Dermatol* 99, 415-21.
- Hoffmann, R., Rot, A., Niiyama, S. and Billich, A., 2001. Steroid sulfatase in the human hair follicle concentrates in the dermal papilla, *J Invest Dermatol* 117, 1342-8.
- Hou, Y., Zhang, H., Miranda, L. and Lin, S., 2010. Serious overestimation in quantitative PCR by circular (supercoiled) plasmid standard: microalgal *pcna* as the model gene, *PLoS One* 5, e9545.
- Labrie, F., Luu-The, V., Labrie, C., Pelletier, G. and El-Alfy, M., 2000. Intracrinology and the

- skin, *Hormone Res.* 54, 218-29.
- Lorence, M.C., Murry, B.A., Trant, J.M. and Mason, J.I., 1990. Human 3 beta-hydroxysteroid dehydrogenase/delta 5----4isomerase from placenta: expression in nonsteroidogenic cells of a protein that catalyzes the dehydrogenation/isomerization of C21 and C19 steroids, *Endocrinology* 126, 2493-8.
- Luu-The, V. and Labrie, F., 2010. The intracrine sex steroid biosynthesis pathways, *Prog. Brain Res.* 181, 177-92.
- Luu-The, V., Sugimoto, Y., Puy, L., Labrie, Y., Lopez Solache, I., Singh, M. and Labrie, F., 1994. Characterization, expression, and immunohistochemical localization of 5 alpha-reductase in human skin, *J Invest Dermatol* 102, 221-6.
- Mason, J.I., Ushijima, K., Doody, K.M., Nagai, K., Naville, D., Head, J.R., Milewich, L., Rainey, W.E. and Ralph, M.M., 1993. Regulation of expression of the 3 beta-hydroxysteroid dehydrogenases of human placenta and fetal adrenal, *J Steroid Biochem Mol Biol* 47, 151-9.
- Ota, T., Fustin, J.M., Yamada, H., Doi, M. and Okamura, H., 2012. Circadian clock signals in the adrenal cortex, *Mol. Cell. Endocrinol.* 349, 30-7.
- Payne, A.H. and Hales, D.B., 2004. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones, *Endocr Rev* 25, 947-70.
- Peng, L., Arensburg, J., Orly, J. and Payne, A.H., 2002. The murine 3beta-hydroxysteroid dehydrogenase (3beta-HSD) gene family: a postulated role for 3beta-HSD VI during early pregnancy, *Mol Cell Endocrinol* 187, 213-21.
- Rheaume, E., Lachance, Y., Zhao, H.F., Breton, N., Dumont, M., de Launoit, Y., Trudel, C., Luu-The, V., Simard, J. and Labrie, F., 1991. Structure and expression of a new complementary DNA encoding the almost exclusive 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4-isomerase in human adrenals and gonads, *Mol Endocrinol* 5, 1147-57.
- Riley, S.C., Dupont, E., Walton, J.C., Luu-The, V., Labrie, F., Pelletier, G. and Challis, J.R., 1992. Immunohistochemical localization of 3 beta-hydroxy-5-ene-steroid dehydrogenase/delta 5----delta 4 isomerase in human placenta and fetal membranes throughout gestation, *J Clin Endocrinol Metab* 75, 956-61.
- Sawaya, M.E. and Penneys, N.S., 1992. Immunohistochemical distribution of aromatase and 3B-hydroxysteroid dehydrogenase in human hair follicle and sebaceous gland, *J Cutan Pathol* 19, 309-14.
- Simard, J., Ricketts, M.L., Gingras, S., Soucy, P., Feltus, F.A. and Melner, M.H., 2005. Molecular biology of the 3beta-hydroxysteroid dehydrogenase/delta5-delta4 isomerase gene family, *Endocr Rev* 26, 525-82.
- Simpton, N.B., Cunliffe, W.J. and Hodgins, M.B., 1983. The relationship between the vitro activity of 3 beta-hydroxysteroid dehydrogenase delta 4-5-isomerase in human

sebaceous glands and their secretory activity in vivo, *J Invest Dermatol* 81, 139-44.

Tanaka, R., Tainaka, M., Ota, T., Mizuguchi, N., Kato, H., Urabe, S., Chen, Y., Fustin, J.M., Yamaguchi, Y., Doi, M., Hamada, S. and Okamura, H., 2011. Accurate determination of S-phase fraction in proliferative cells by dual fluorescence and peroxidase immunohistochemistry with 5-bromo-2'-deoxyuridine (BrdU) and Ki67 antibodies, *J Histochem Cytochem* 59, 791-8.

Figure Legends

Fig. 1. Schematic representation of the major mammalian steroidogenic pathways. The enzyme 3 β -HSD is required for biosynthesis of all classes of hormonally active steroids.

Fig. 2. Tissue distribution profiles of *Hsd3b6* and *Hsd3b1*. The mRNA levels of *Hsd3b6* and *Hsd3b1* were determined by quantitative RT-PCR using isoform-specific primers. Values are means \pm s.e.m. (n=3, for each tissue) normalized to *Rplp0* expression. N.D., not detectable.

Fig. 3. Specificity of Hsd3b6 antibody. (A) Double-label immunofluorescence of cells expressing Hsd3b1-HA or Hsd3b6-HA. Transfected COS-1 cells were fixed and stained with anti-Hsd3b6 (red) and anti-HA (green) antibodies. Merge shows combined images for Hsd3b6 (red), HA (green), and DAPI staining (blue). (B) Immunoprecipitation (IP)-Western blot analysis. COS-1 cells expressing either Hsd3b1-HA or Hsd3b6-HA were subjected to IP with anti-Hsd3b6 or anti-HA. Then, the resultant immunoprecipitants were immunoblotted with the antibody indicated. Note that anti-Hsd3b6 antibody could selectively immunoprecipitate Hsd3b6-HA but failed to detect denatured Hsd3b6-HA protein in Western blot. Asterisks indicate bands resulting from detection of antibodies eluted from the beads. IB, immunoblot. (C) Immunofluorescence of anti-Hsd3b6 antibody in the adrenal cortex. Immunoreactivities seen in the zona glomerulosa (left) were completely abolished by absorption with immunizing peptide (right).

Fig. 4. Immunohistochemistry of Hsd3b6 in the adrenal gland. (A) Image of the whole adrenal section. (B) and (C) show enlarged views of (A). The vertical lines on the right of (B) indicate the positions of the capsule (C), zona glomerulosa (ZG), and zona fasciculata (ZF). Note that immunolabeling is limited to the ZG, the outermost layer of the adrenal cortex. (C) and (D) are images of the same section before and after hematoxylin counterstaining. Note that label is limited to the cytosol and excluded from the nuclei. Scale bars are 200 μ m in (A), 100 μ m in (B), and 25 μ m in (C) and (D).

Fig. 5. Immunohistochemistry of Hsd3b6 in the testis and ovary. (A) Image of the whole testicular section. (B) and (C) show enlarged views of (A). Note that positive immunostaining is limited to the interstitial cells. (D)(E)(F) Images of the ovary. (E)(F) Higher magnification of the boxed area in (D) with or without hematoxylin staining. The arrowhead indicates the location of theca cells. Scale bars are 200 μ m in (A) and (D), and 50 μ m in the other panels.

Fig. 6. Immunohistochemistry of Hsd3b6 in the skin. Shown are images of the dorsal skin section with or without hematoxylin staining. (B) and (D) show enlarged views of (A) and (C), respectively. Scale bars are 200 μ m in (A) and (C), and 50 μ m in (B) and (D). Note that only the cells in the sebaceous glands are Hsd3b6-immunopositive.

Fig. 7. Immunohistochemistry of Hsd3b6 in the placenta. (A) Image of the whole placenta section at E8.5. (B) Higher magnification of the boxed area in (A). Scale bars are 200 μm in (A), and 50 μm in (B). Note that positive immunoreactivities were essentially confined to the giant trophoblast cells that surround the embryonic cavity.

Figure 2

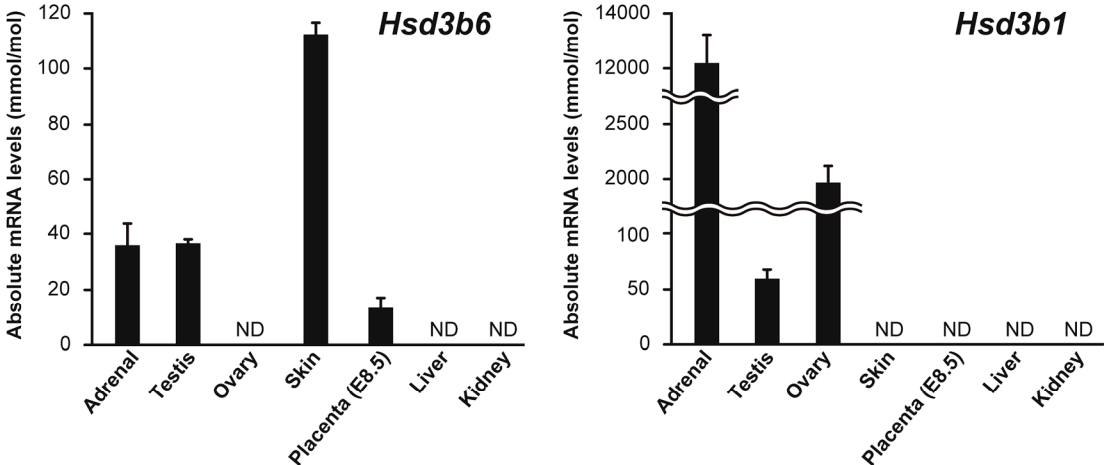


Figure 3

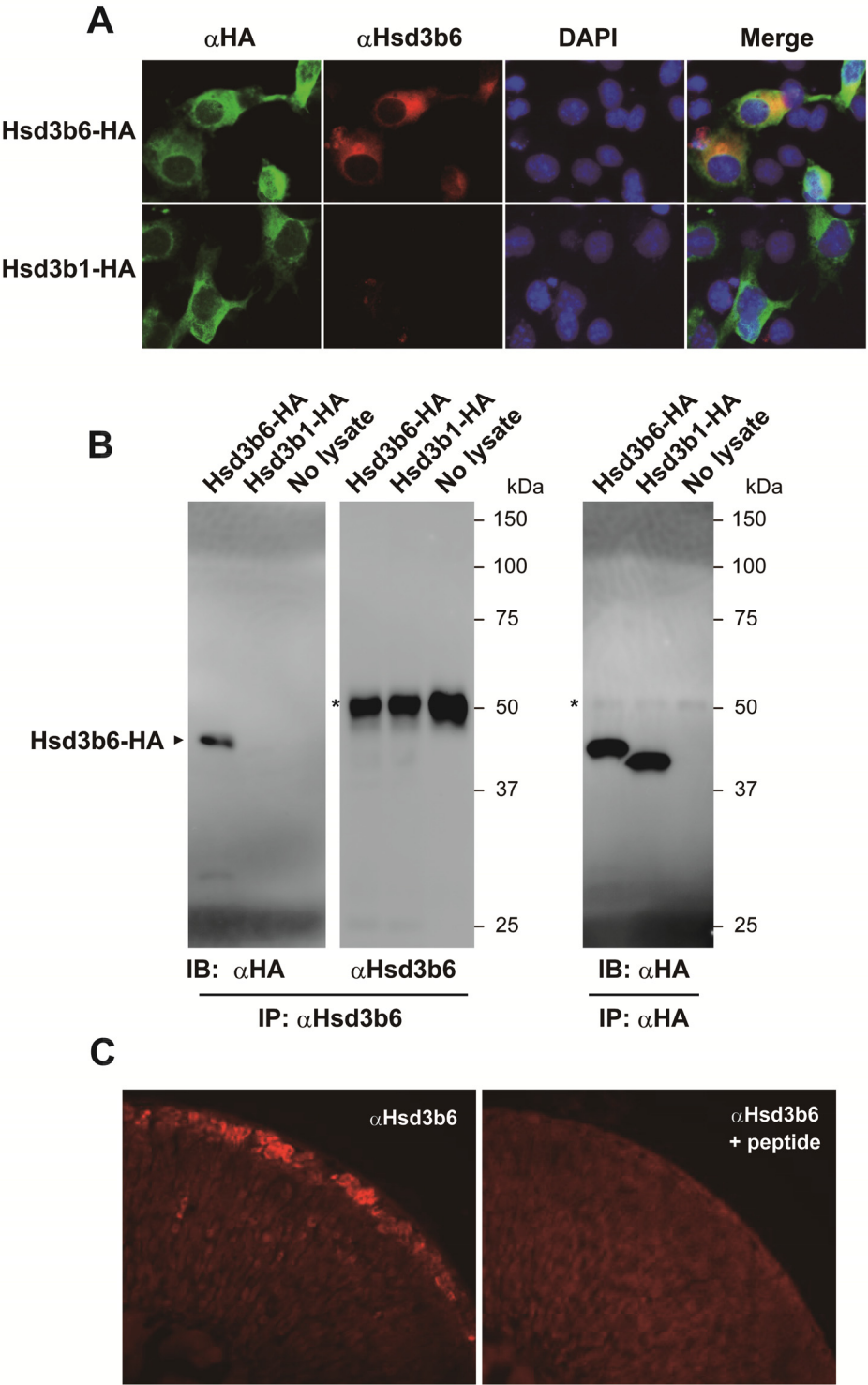


Figure 4

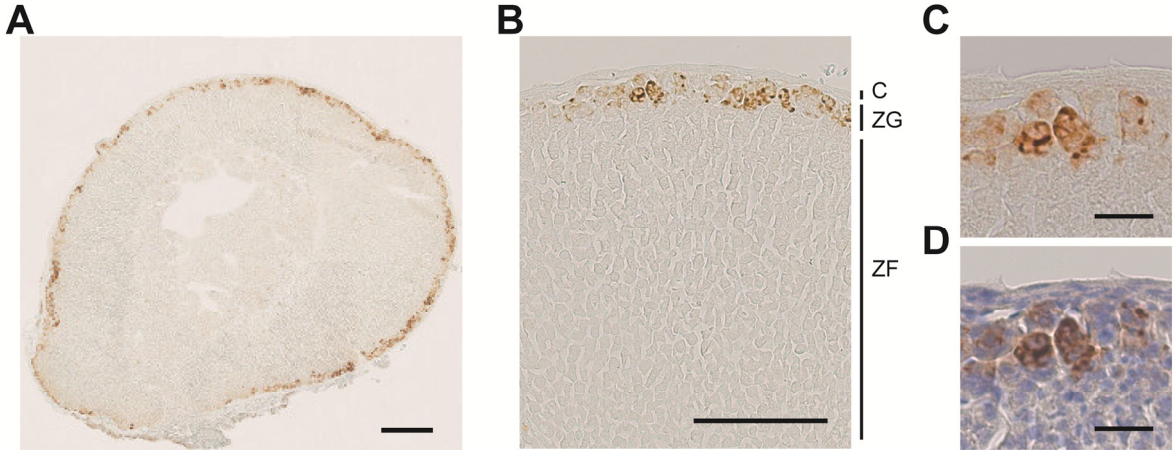


Figure 5

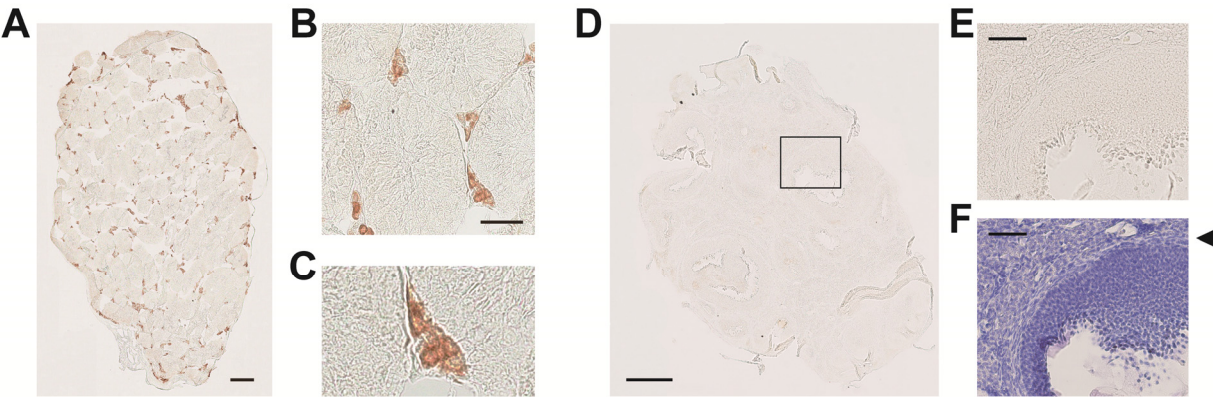


Figure 6

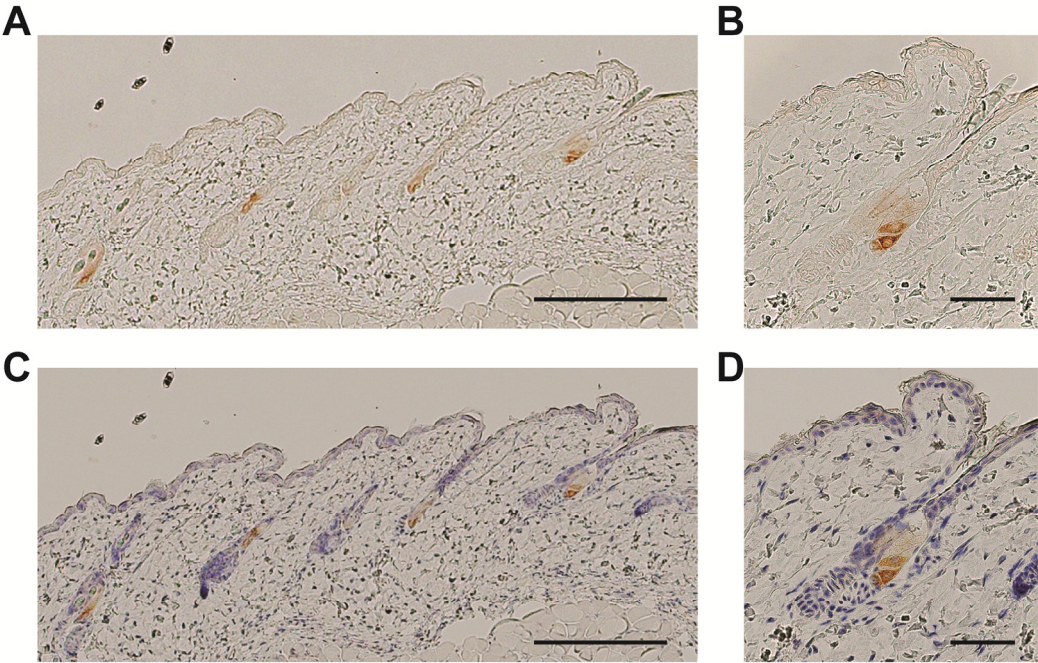
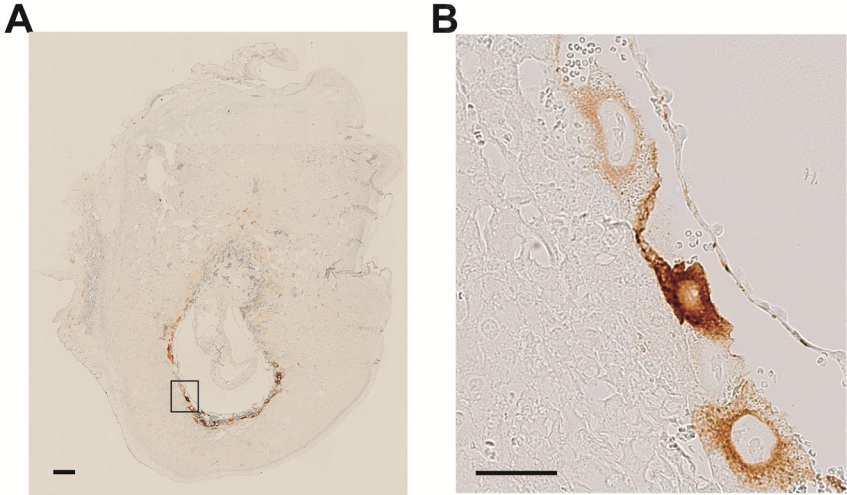
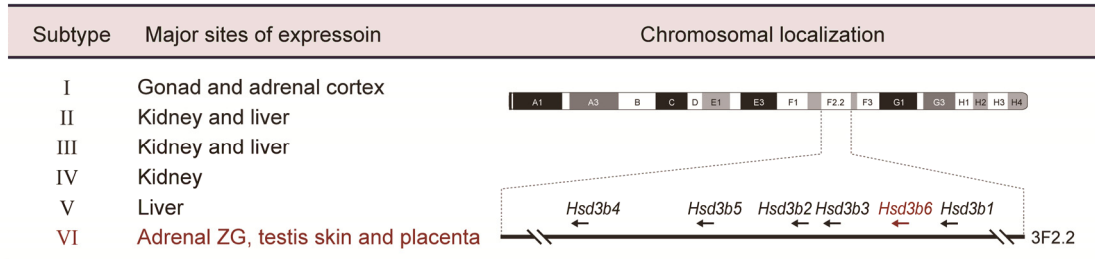


Figure 7

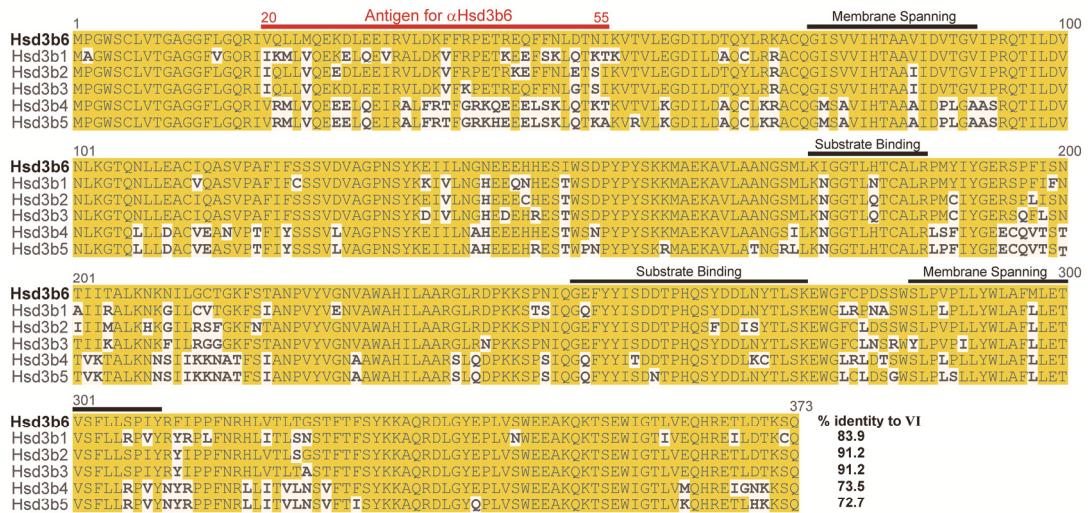


Supplemental Figure 1

A



B



Suppl. Fig. 1. (A) Main expression sites of the mouse β -HSD isoforms and schematic drawing of their genetic loci on the chromosome. The murine β -HSD enzyme family consists of 6 isoforms from type I to type VI, each having a different distribution at the mRNA level (see Fig. 2). Of interest, all the genes (from type I to type VI) form a syntenic cluster at chromosomal position 3F2.2. (B) Comparison of amino acid sequences between the mouse β -HSD isoforms. Amino acid sequences identical to Hsd3b6 are highlighted in yellow. The red line indicates the location of the amino acid sequence of Hsd3b6 (from 20 to 55), a region used as the antigen peptide for the generation of anti-Hsd3b6 antibody.