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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

The scaffold attachment factor HET/SAFB1 maps to a chromosomal locus that displays extremely high loss of heterozygozity (78%) in estrogen depend of the breast cancers. My mentors lab has shown that HET/SAFB1 functions as a co-repressor of estrogen receptor transcriptional activation. We hypothesize that HET/SAFB1 is a tumor suppressor gene. To test this hypothesis we created a mouse knockout model.

During the first year of my grant we established 2 independent lines of HET/SAFB1 knockout mice.

During the second year of my grand we found that loss of HET/SAFB1 function resulted in multiple phenotypes including prenatal and postnatal lethality, growth retardation, male infertility, female sub-fertility and hyperplasia of the mammary gland.

During the third year we found that low levels of Estradiol and IGF-1 repress early development of mammary gland in knockout females. The hormone stimulation (E2) increased branching and lobulo-alveolar development, increased proliferation (BrdU) and co-localization of ER+ and proliferation.

These studies show that HET/SAFB-1 is involved in the hormonal regulation of development and maturation of the reproductive system and the mammary gland. Tumorogenesis studies are ongoing.

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Introduction

Scaffold Attachment Factor SAFB1 (also known as HET/SAFB1 or HAP) is a member of the nuclear-receptor-interaction protein originally identified as a cofactor of estrogen receptors. Mutation in human SAFB-1 is detected in 78% breast cancers exclusive in estrogen dependent breast cancer suggesting that this gene may play important role in pathology of breast cancer as a co-repressor of estrogen receptor transcriptional activation. We hypothesize that HET/SAFB1 is a tumor suppressor gene. To test this hypothesis we study the function of HET/SAFB1, and created a mouse knockout model.

Full-length mouse HET/SAFB1 cDNA sequence was assembled from the EST database. It shows 85% identity with human SAFB-1 cDNA sequence with scaffolding (SAFB-box) and RNA-binding domains being very well conserved. Human and mouse HET/SAFB1 are expressed in the same tissues including hormone-dependent organs (mammary gland, ovary, testis and uterus).

During first year I found that human and mouse HET/SAFB1 are expressed in the same tissues including hormone-dependent organs (mammary gland, ovary, testis and uterus).

The mouse mammary gland HET/SAFB1 mRNA is present in virgin stage, as well as during pregnancy (11 and 16 days) and involution. To generate HET/SAFB-1 knockout mouse we utilized the mechanism of homologous recombination. A targeting vector was constructed using a mouse genomic clone from a 129 SVJ library where part of HET/SAFB1 gene was replaced by β-galactosidase. At present we have 2 independent lines of HET/SAFB1 knockout mice.

While homozygous HET/SAFB1-/- mice were viable, genotypic distribution indicated that homozygous deficiency resulted in significant pre- and postnatal lethality. Also, the HET/SAFB1-/- mice showed dwarfism, most likely caused by low serum IGF-I levels. Since we had previously shown that HET/SAFB1 interacts with and affects activity of the estrogen receptor, we focused our characterization on hormone-responsive tissues. We found that HET/SAFB1 play a critical role in sex-specific development and physiology. Progressive degeneration of the testicular germinal epithelium associated with low testosterone levels resulted in male sterility. Female HET/SAFB1-/- showed delayed pubertal development, oviductal stenosis, and decreased number of germ cells resulting in female sub-fertility.

Low level of Estradiol and IGF-1 response for delayed mammary gland development HET/SAFB1 -/- females. During unsuccessful multipregnancy or Estradiol treatment was increased branching and lobulo-alveolar development of the mammary gland, increased proliferation (BrdU) and co-localization of ER+ and proliferation in SAFB1-/- gland .

In conclusion, HET/SAFB1 is critical factor during embryonic development, hormonal regulation of growth, development and maturation of the reproductive system and mammary glands.

Body

During first year I planed to study:

1. **Task1: Finish generation of targeting construct. Establish methods for ES cell screening.**

This part of work was finished October – November 2000 year. Figure 1 **(see** Appendices Fig 1, a) demonstrated design of construct and figure 1 (b) illustrated Southern blot analysis of ES clones. We had 5 positive ES clones out of 329 clones screened.

2. **Task2: Apply recombinant ES cell technology:**

This work was done in Dr. Franco De Mayo's transgenic core facility in the Department of Molecular and Cell Biology December 2000 year.

3. **Task3, 4: Identify and breed chimeric animals which have passed the mutation into the germline. Breed germline chimeras to generate heterozygous animals.**

We obtained: 3 chimeric males from clone O1BA3, 1 male from 5F5 clone, 1 male from 3H12 clone. After breeding with C57Black/6 females we found 2 hetereozygous females from 2 chimeris males (from O1BA3 and 5F5). Heterozygous female from O1BA3 line was born 3/13/2001 and female 5F5 line was born 4/18/2001.

4. **Task5: Breed heterozygous animals to generate homozygotes for HET/SAFB.**

Before starting to breed heterozygous/heterozygous mice we breed heterozygous mice with C57Black/6 during period 8/18/2001-1/3/02/2002. We started to breed animal late because the animal facility was closed due to the flood after tropical storm Alison.

1/28/2002 we established two lines of HET/SAFB1 knockout mice from independent clones of embryonic stem cells (**see Appendices Fig 1, b and c**). Analysis of RNA from testis and brain demonstrated that the full-length HET/SAFB1 mRNA is completely absent (**see Appendices Fig 1, d**).

We next performed Western blots on protein extract prepared from mouse brains and embryos E13.5 using an N terminus-specific HET/SAFB1 antibody. Importantly, no full-length HET/SAFB1 or any smaller bands were observed in protein extracts (**see Appendices Fig 1, e**). Thus, we conclude that the targeted allele of HET/SAFB1 is a null mutation at the protein level.

We measured β-galactosidase (LacZ) expression as a reporter of HET/SAFB1 promoter activity in post implantation embryos. At E11.5 β-galactosidase staining was very strong in the developing nervous system, somits, but absent in heart (**see Appendices Fig 1, f**).

We had to increase the number of breeding pairs because only 15% of knockout animals survived.

During second and third year I planed to study:

5. **Task 6: Characterize HET/SAFB1 expression in mammary gland from wild type, heterozygous, and homozygous null mice at 9 key development stages (at postimplantation embryos (15 and 19 days), born, puberty, pregnancy (11.5 and 16.5 days), lactation (1d and 10d) and involution. Furthermore, analyze ductal growth by *in vivo* BrdU labeling.**

We analyzed morphological and histological structures of the mammary gland at juvenile period (4 weeks), puberty 6 and 10 weeks, 4 months, 3.5 day pregnancy and 1 day lactation.

We found that sexual maturation of HET/SAFB1 knockout females was delayed (Table 1) and mammary glands developed 2-3 weeks later than wild type and heterozygous females. 4 and 6 weeks old knockout females had significant smaller mammary gland, 10 weeks older virgin knockout females has similar morphological structure of the mammary glands compared with wild type and heterozygous females **(see Appendices Fig 2, a).** One 10 weeks old knockout female had hyperplasia of the mammary gland.

The distance between the end of terminal endbuds (TEB) and the center of the lymph node of mutant ducts was shorter than controls at 4 and 6 weeks **(see Appendices Fig 2, b)**. The TEB number was significant smaller in mammary gland of knockout females than control mice at 4 weeks of age.

Table 1.

Genotype	Age of First Copulation (in weeks)	P value
+/+	6.1±0.9	
+/-	6.2±0.7	0.999
-/-	8.6±1.6[a]	0.004

[a] p-value from a two sample t-test with Holm's adjustment for multiple comparisons with +/+ genotype (n=13 per group)

HET/SAFB1 knockout females were smaller and had deficiency of IGF-1. We think that in some cases immature mammary gland of HET/SAFB1 knockout females correlated with small size of animal **(see Appendices Fig 3)**.

Analysis of the mammary gland after 17ß –Estradiol treatment. Mammary gland ductul growth in virgin mice depends on the estrogen signaling pathway. We looked ar the level of estradiol in 4 weeks old mice.

Table 2

Genotype females	Number	Estradiol (pg/ml)	P value to +/-	P value to +/+
+/+	2	37.5±17.6		
+/-	4	34.75±8.4		
-/-	3	17±10.8	P=0.028	P=0.09

Because HET/SAFB1 knockout females have low level of estradiol we distinguish between local effect on ER function at mammary gland and systematic hormonal influences. To control serum estrogen level, matched littermates were ovariectomized on 6 weeks old, 3 weeks rest and then treated with either E2 pellets during 7 days (17ß –Estradiol 0.25 mg/pellet) or placebo. Dwarfism of knockout females independent of estradiol level because the weight of E2 treated knockout females did not increase and knockout females were smaller compare with wild type **(see Appendices Fig 4, a)**. After 7 days of treatment, uterine weight was decreased for both the knockout and wild type mice **(see Appendices Fig 4, b)**, but histology analysis demonstrated increase of inflammation effect in knockout uterus (5/6 knockout mice and 1/5 wild type mice) **(see Appendices Fig 4, c)** and one knockout female had abnormally high proliferation of the uterus **(see Appendices Fig 4, d)**.

HET/SAFB1 knockout mammary gland showed intensive ductul growth **(see Appendices Fig 5, a, b and c)**, and increased ratio of BrdU positive cells **(see Appendices Fig 5, d)**.

Quantification of ER positive cells and Progestreone receptor (PgR) positive cells did not demonstrate significant differences between knockout and wild type mammary gland (**see Appendices Fig 6**). For analysis of localization of ER receptor positive cells and proliferative cells we used double immunofluorescence labeling (ER positive (red color) and BrdU positive (green color) and found co-localization of ER and proliferative cells in knockout mammary gland after E2 treatment (**see Appendices Fig 7**).

Only 33% of females (5 animals total) gave birth and only one of them lactated, therefore I could not analyze stage 11.5 and 16.5 days of pregnancy and lactation. Since we found that the knockout females have oviductal stenosis and the development of pregnancy stops on the 4-th day, I studied mammary glands at the 3.5 days of pregnancy. To confirm pregnancy we flushed uterus and oviduct to count embryos.

Analysis of the mammary gland at 3.5 days pregnancy showed hyperproliferation and more branching ducts after second and third pregnancies **(see Appendices Fig 8, a (whole staining) and b (H&E staining)**. The level of progesterone was similar in HET/SAFB1$^{-/-}$, HET/SAFB1$^{+/-}$ and HET/SAFB1$^{+/+}$ pregnant females (**see Appendices Fig 8, c**). The estradiol level in 3.5 day pregnant knockout females was decreased to about 25% (**see Appendices Fig 8, c**). Number of Estrogen Receptor positive cells in end buds and ductuls significantly decreased during 3 unsuccessful pregnancies (**see Appendices Fig 8, d**). Number of PgR positive cells was decreased in the ductuls during third pregnancy. We realize that these experiment are not finished because of the low number of animals (2nd pregnancy: +/+ n=2, -/- n=1; 3nd pregnancy: +/+ n=2, -/- n=3).

Analysis of the mammary gland of the first day of lactation did not show significant difference between wild types and homozygotes, and therefore the reason for the absence of lactation is yet to be determined (n=5).

Since I have limited number of knockout females I utilized alternative methods for analysis of mammary gland: transplant mammary gland to recipient females. I dissected mammary glands from wild type females and knockout females (4 week old) and transplanted fragments mammary gland to recipient females (RAG$^{-/-}$ lines). Finally, recipient female had mammary gland from knockout females at left side and mammary gland from the wild type at their right side. I found however 4 recipient females no significant differences in the development of mammary gland after 3 weeks after transplantation. I have another 5 recipient females 6 months old for analysis mammary gland at the age of 1 year.

3. Task 7:

Characterize morphological and functional changes in HET/SAFB-1 heterozygous and homozygous mice as compared to wild type littermates.

Embryonic lethality.

Analysis of progeny crosses of heterozygous animals showed abnormal mendelian ratio. The number of homo- and heterozygotes was considerably reduced at 83% and 33% compared to the expected from normal mendelian distribution 1:2:1.The ratio was very similar in the two lines derived from independent ES clones (**Table 3**).

To understand when and why embryos die we studied embryos on post implantation stage of development beginning with 10.5 days of pregnancy until newborn stage 1-6 days. Intercrossing of heterozygous mice produced wild-type, heterozygous, and knockout offspring with the ratios shown in **table 4**.

Table 3

Line	Offspring	+/+	+/-	-/-	Ratio +/+: +/-: -/-	p-value[a]	p-value[b]
O1BA3	Total(n=816)	323	431	62	1: 1.3: 0.19	<0.0001	
	Female(n=430)	180	223	27	1: 1.2: 0.15	<0.0001	0.180
	Male(n=386)	143	208	35	1: 1.5: 0.24	<0.0001	
5F5	Total(n=638)	250	347	41	1: 1.4: 0.16	<0.0001	
	Female(n=318)	134	163	21	1: 1.2: 0.16	<0.0001	0.274
	Male(n=320)	116	184	20	1: 1.6: 0.17	<0.0001	

[a]Based on chi-square test for goodness-of-fit to compare the observed distribution of genotypes with the Mendelian 1:2:1 ratio.
[b]Based on likelihood ratio test comparing distribution of genotype between females vs. males from a loglinear model.

Table 4

	Days of Development	+/+	+/-	-/-	Ratio +/+: +/-: -/-	p-value[a]	p-value[b]
Embryos	10.5 (n=48)	14	26	8	1: 1.9: 0.60	0.417	0.973
	11.5-16.5 (n=87)	27	45	15	1: 1.7: 0.60	0.185	
	18.5 (n=34)	9	20	5	1: 2.2: 0.60	0.372	
Newborns	1 (n=114)	35	61	18	1: 1.7: 0.50	0.061	0.048
	4-6 (n=51)	15	34	2	1: 2.3: 0.13	0.002	

[a]Based on exact test for goodness-of-fit to compare the observed distribution of genotypes with the Mendelian 1:2:1 ratio.
[b]Based on likelihood ratio test comparing distribution of genotype across days of development from a loglinear model.

INTERPRETATION OF STATISTICAL RESULTS

Due to smaller number of embryos or newborns in some genotypes, the exact test was used to evaluate goodness of fit with the Mendelian ratio. No statistically significant difference in the Mendelian ratio was detected based on the observed distribution of genotypes for the embryos on any of the days considered. Furthermore, there was no statistically significant difference in this distribution across the 3 time points of evaluation (p=0.973).

In contrast, there was a statistically significant deviation from the expected Mendelian ratio among the newborns. This deviation was more pronounced in newborns evaluated on days 4-6 (p=0.002) than on day 1 (p=0.061). As a consequence, a statistically significant difference in the was observed in the comparison of the distribution of genotypes between day 1 and day 4-6 in the newborns (p=0.048).

The outcome of the breeding experiment is schematically illustrated in **(see Appendices Fig 9, a)**. Genotyping of embryos and newborn pups demonstrated 2 critical points when HET/SAFB1$^{-/-}$ mice die. Approximately 40% of knockout embryos die before E10.5 of

development and the cause of lethality is unknown. Approximately 50% of knockout mice died during 1 and 2 of postnatal development.

The newborn HET/SAFB1$^{-/-}$ mice had difficulties with suckling (no milk in stomach), and had cyanotic skin and small size **(see Appendices Fig9, b)**. All these are indications of anemia and the dysfunction of oxygen exchange because pups have to hold their breath while suckling milk. Histological analysis of E18.5 embryos showed the absence of red blood cells in the liver and incomplete differentiation of epithelium cells of the terminal respiratory alveoli **(see Appendices Fig 9, c).**

The reason for the deficiency of the erythrocytes in the developing liver could be a decreased number of the vascular channels, therefore we analyzed the formation of vascular channels in19 day embryonic livers using CD31 antibody **(see Appendices Fig 9, c).** The number of blood vessels in the liver of HET/SAFB1$^{-/-}$ embryos was reduced by 35% (162 KO/ 458 WT X 100%), and the vessel area was reduced by 30% (2659 KO/ 9517 WT x 100%) compared to the wild type counterparts. However, the adult HET/SAFB1$^{-/-}$ mice and wild type has similar level of the erythrocytes (8.59±0.09 X10^6/μl and 8.7±0.2 X10^6/μl respectively, n=3 per group, 4-6 weeks old, p=0.2) and normal histological structure of the liver. Thus, slow development of hematopoetic system and incomplete maturation of the lung may be the possible reasons for lethality observed in newborns.

Dwarfism.

Knockout mice could be distinguished from their littermates by their smaller size **(see Appendices Fig 10, a).** At 3 weeks of age the difference in weight between knockout mice and their heterozygous and wild type littermates was 45% for males and 24 % for females **(see Appendices Fig 10, b).** After weaning the weight difference decreases but knockout mice never reach the same size as wild type and heterozygous animals. To exclude the influence of an overall change in the body weight, we expressed the weight of the organs as a percentage of body weight and found that adult knockout mice have significant not different in the weight of the liver (4.8% KO/ 5% WT, p=0.5), brain (0.41% KO/ 0.43% WT, p=0.02), heart (0.66% KO/ 0.64% WT, p=0.7) and significant increase weight of the kidney (0.53% KO/ 0.7% WT, p< 0.01).

Retardation of size starts pre-natally at 14 days of embryonic development. Beginning at E15 knockout embryos and newborns 1 and 2 days were 15-20% smaller than littermates **(see Appendices Fig 10, c).** Analysis of the skeleton formation of the newborns demonstrated decreased size of a skeleton with normal formation of bones **(see Appendices Fig 10, d demonstrated highly express of the phenotype, *bottom*)**. There are 7 knockout and wild type newborns from 4 offspring was measurement of the skulls (#1 and 2), length of the spine from first vertebra to vertebra of the pelvis (#3) and width of the thorax (#4). Whole skeleton were 10% smaller them littermates and thorax were 22% smaller and independent from sex **(see Appendices Fig 10, d *bottom).***

The insulin-like growth factor (IGF) system is an important regulator of fetal growth and differentiation. IGF-1 is involved in embryonic processes of growth and potentially differentiation beginning from E13 (Liu et al) and controls growth during the whole life, therefore we measured the level of IGF-1 in the 3-4 month old homozygous, heterozygous and wild type animals. HET/SAFB1$^{-/-}$ knockout mice had the blood level of IGF-1 reduced by 50% compared to HET/SAFB1$^{+/-}$ and HET/SAFB1$^{+/+}$ mice and the difference was independent from the sex of the animals **(see Appendices Fig 10, e).**

Thus, the low level of IGF-1 and anemia may explain the small size and high newborn lethality of HET/SAFB-1$^{-/-}$ knockout mice.

Male reproduction system.

Regular breeding of knockout males with heterozygote and wild type females never resulted in pregnancy. Heterozygous males were fertile and showed similar number of pups with wild types.

Table 5

Breeding Pairs MxF	N Males	n Females	Pregnant Females n (%)	Average number of pups per litter	p-value[a]
+/+ x +/+	2	5	5 (100)	5.6 ± 1.52	
+/- x +/+	6	11	10 (91)	6.1 ± 1.70	0.999
+/+ x +/-	5	11	11 (100)	6.4 ± 1.86	0.999
+/- x +/-	8	17	17 (100)	6.1 ± 1.85	0.620
+/+ x -/-	3	14	5 (36)	3.2 ± 0.45	0.086
-/- x +/+	**3**	**9**	**0**		

[a] p-value from a two sample t-test with Holm's adjustment for multiple comparisons with +/+ x +/+ breeding pair.

Breeding of HET/SAFB-1$^{-/-}$ mice with superovulated C57BL/6 females showed 100% sterility in males **(see Appendices Fig 11, a)** and change lordotic behavior (only one male out of four mated).

Knockout males are hypogonadal, and paired testicular weights are reduced by approximately 80% in comparison with wild type and heterozygous males (wild type, 0.17g ± 0.02; knockout 0.025±0.005 **(see Appendices Fig 11, b and c)**.

Hypogonadism and sterility associated with level of the steroids (testosterone and estradiol) in first. Serum testosterone levels of knockout males were significantly decreased as compared with heterozygous and wild type males of the same age **(see Appendices Fig 11, d).** Level of estradiol was similar in HET/SAFB-1 knockout, heterozygous and wild type mice **(see Appendices Fig 11, e).**

To determine when abnormalities in the testis development start we examined the histology of the testis at various ages. Immature testis (3 weeks old) from HET/SAFB1$^{-/-}$ animals had a similar architecture compared with heterozygous and wild type males but smaller diameter of the seminiferous tubules **(see Appendices Fig 12, a)**. Mature testes (3 months old) demonstrated arrest of spermatogenesis **(see Appendices Fig 12, a)**. Sperm assay confirmed that spermatozoids were absent in epididymis (data not shown). At 3 months, seminiferous tubules exhibited a spectrum of epithelial dysgenesis, degeneration **(see Appendices Fig 12, a)**. We detected the Leydig cell hyperplasia adjacent to several degenerated tubules in the 3 months old males **(see Appendices Fig 12, a)**. In the 9 months old males Leyding cells hyperplasia and tubular degeneration affected most part of the testis **(see Appendices Fig 12, a).**

Testicular tubular degeneration often consists of necrotic spermatogenic cells, perhaps with reduced numbers of germinal epithelial cells. TUNEL staining demonstrated apoptosis of germ cells and spermatocytes of the knockout males **(see Appendices Fig 12, b)**. We confirmed the specific

loss of germ cells during embryonic development, maturation and in mature mice by staining testes of the newborn, 3 and 9 months old males with antibody recognizing the germ cell nuclear antigen (GCNA-1). No differences in architecture of the developing testis, number and position of germ cells was observed in the newborn **(see Appendices Fig 12, c)** and 3 months old testes (data not shown), however testes of the 9 months old knockout males had a smaller number or complete absence of germ cells **(see Appendices Fig 12, c).**

Seminal vesicles of HET/SAFB1$^{-/-}$ males are 2 times smaller compared to wild types and heterozygotes (wild type, 0.44g ± 0.07; heterozygous, 0.56g ± 0.11; homozygous, 0.29g ± 0.075) **(see Appendices Fig 12, c).** In addition, seminal vesicles of knockout males have simple columnar epithelium without branching into secondary and tertiary folds **(see Appendices Fig 12, d).**

In conclusion, HET/SAFB1$^{-/-}$ males are sterile, have reduced size of the reproductive system, progressive testicular tubular degeneration and low level of testosterone.

Female reproduction system.

Only 36% of homozygous females are fertile, as revealed by the test of natural mating with wild type males (Table 5). We monitored 15 virgin HET/SAFB1$^{-/-}$ females mating daily for the presence of vaginal plugs. Copulation plug was detected in 86% (13 from 15) of HET/SAFB1$^{-/-}$ compared with 93% HET/SAFB1$^{+/-}$ and HET/SAFB1$^{+/+}$ (14 from 15) mice. This observation suggests that knockout female's breed and ovulate normally.

Deficient of the IGF-1 and growth retardation may be accompanied by pubertal delay, we compared time first copulation knockout females with HET/SAFB1$^{+/-}$ and HET/SAFB1$^{+/+}$ mice. First copulation of HET/SAFB1$^{-/-}$ females occurs on average 2.4 weeks later than in the heterozygous and wild types mice (Table 1). This result demonstrated that the sexual maturation of knockout females is postponed.

The reason of infertile is the loss of the embryos after fertilization. To establish when embryonic viability is lost, we selected knockout females which did not develop pregnancy after first (n=2) or second copulation (n=3). Embryos were isolated at days 3.5 p.c. and scored for their number and morphological appearance. Normally at this stage the embryos should be in the uterus, however we found all the embryos in the oviducts of knockout females (table 6).

Table 6

Genotype females	Age First Copulation (in weeks)	Were taken embryos	Morphological appearance		Average No. of embryos
			% normal	% survival after 24h. cultivation *in vitro*	
+/+	6.1±0.9	uterus	94	83	5.3±1.9
+/-	6.2±0.7	uterus	96	90	5.6±2.9
-/-	8.6±1.6[a]	oviducts	42	50	2.8±1.1[b]

Data shown in are infertility phenotype SAFB-1 knockout mice. Comparison are shown of preimplantation embryos isolated from SAFB-1$^{-/-}$ (n=5), SAFB-1$^{+/-}$ (n=6), and SAFB-1$^{+/+}$ (n=6) females. Embryos was isolated from oviducts and uterine flushing (day 3.5 pregnancy), counted and scored for morphology. Values are the mean ± SD, ttest; [a]$p<0.01$, [b]$p<0.05$.

Embryos from the knockout females develop normally before morulae. After 24 hr of cultivation *in vitro* 50% of the embryos survived and developed into blastocyts. In contrast, 83% and 90% blastocyts survived from HET/SAFB1$^{+/+}$ and HET/SAFB1$^{+/-}$ females after cultivation (Table 6).

Thus, preimplantation development is markedly normal before 3 p.c., and after it virtually all blastocysts died by day 3.5 and 4 p.c. in HET/SAFB1$^{-/-}$ females. 36% of knockout females had successful pregnancy (16 newborns born from 5 females) and 50% embryos can be cultured to a blastocyst stage indicating that the contribution of oocytes to this effect is small.

Histological structure of the immature (4 weeks old) oviducts demonstrated thinner muscle in the oviductal wall and simple columnar epithelium in HET/SAFB1 knockout females **(see Appendices Fig 13, a)**. The oviducts of the mature knockout mice (4 months old) which had problem with transport of embryos in uterus had oviductal atrophy **(see Appendices Fig 13, a)**. The basic reason for non development of pregnancy is that oviductal stenosis impaired the recovery of blastocysts from the knockout uterus.

To test the endocrine function in HET/SAFB1$^{-/-}$ pregnant females we measured the levels of 3 hormones important during pregnancy: progesterone, estradiol and testosterone. The ovaries at 3.5 days of pregnancy develop large corpora luteas, where progesterone is produced during pregnancy. The level of progesterone was similar in HET./SAFB1$^{-/-}$, HET/SAFB1$^{+/-}$ and HET/SAFB1$^{+/+}$ females **(see Appendices Fig 8, c)**. The estradiol level in 3.5 day pregnant knockout females was decreased to about 25% **(see Appendices Fig 8, c)**. The testosterone level in knockout females at the same stage of pregnancy was about 36% lower than that of the wild types (0.16 ± 0.003 / 0.44 ± 0.05 ng/ml KO/WT, n=5, $p < 0.001$). Low testosterone level is characteristic for the HET/SAFB1$^{-/-}$ a knockout mouse, as it was detected also in 4-weeks-old females (data not shown) as well as in males.

To determine the function of the ovary itself during pregnancy, and involvement of the hypothalamic-pituitary-ovarian axis we performed reciprocal ovarian transfer experiments. 3 out of 6 females had litters (2 pups per litter) after transplantation of the knockout ovary to wild type females. 3 out of 5 control females were pregnant and had 6, 1 and 8 pups, respectively. We suggest that ovaries function normally.

Thus, slow/ incorrect development during maturation of the female reproductive tract is the reason for a later puberty and unsuccessful development of pregnancy in SAFB1 knockout females.

Reduced number of embryos

5 from 14 HET/SAFB1$^{-/-}$ females after crossing with wild type males had 3.2 newborns per litter (Table 5). The HET/SAFB1$^{-/-}$ females with oviductal stenosis at 3.5 days pregnancy also had reduced number of embryos (2.8 embryos vs. 5 embryos in wild type) (table 6). All knockout females (n=5) after first successful pregnancy never became pregnant again. To investigate further the defective ovaries in HET/SAFB1$^{-/-}$ mice we analyzed anatomical and histological structure of the newborn, 4 weeks, 4, 8 and 10 months ovaries.

Gross anatomical examination of newborn, 4-week-old and 6-week-old SAFB1$^{-/-}$ mice revealed normal structure of the reproductive tract. The ovaries of the 6-week-old knockout females had similar weight compared with the wild type (0.0023 ± 0.0005g KO/ 0.0025 ± 0.0008g WT).

Histological examination of the 4-week-old immature ovaries demonstrated a reduced number of primary follicles in the SAFB1$^{-/-}$ ovaries as compared with wild type control mice (wild type, 16.5 ± 1.14; heterozygous, 12.3 ± 0.25; homozygous, 6.7 ± 1.84) (Figs 7d), suggesting that SAFB1 increases apoptosis and/or decreases proliferation in the germ cell population during ovarian development embryonic or postnatal period **(see Appendices Fig 13, b and c)**. We found progressive atrophy of the 4, 8 and 10 months old ovaries of the HET/SAFB1 knockout females **(see Appendices Fig 13, b)** and reduction of the number of primordial/primary and secondary follicles **(see Appendices Fig 13, c)**.

We analyzed the specific loss of primary follicles before or after birth and organization of the organ structure by staining the newborn ovaries with antibody recognizing germ cells (GCNA-1). No

differences in number of germ cells and architecture of the organ were observed in the newborn ovaries (n=4) (Fig 7c). This result suggesting that population of the primary follicles decreased during ovarian development postnatal period.

Thus, reduced number of the primary follicles during oogenesis after birth during maturation is reason of progressive reduced number of the embryos with next ovulation and secondary sterility.

Task 8. Characterize changes in chemically induced carcinogenesis between HET/SAFB homozygous, heterozygous and wild type littermates. Mammary tumors will be induced by transplantation of a syngeneic pituitary isograft under the kidney capsule and then two weeks later gavage with DMBA.

I have limit at number knockout females. In part 3 I suggested to change the background of knockout females from 129/C57/black 6 to Balb/c to increased the effect of tumor development. At present time I backcrossed 7 generation of HET/SAFB1 +/- mice with background Balb/c.

Key research accomplishments

I Overall phenotype HET/SAFB1 knockout mice.

1. Low level of IGF-1, anemia, and underdeveloped lungs explain the small size and high lethality of HET/SAFB-$1^{-/-}$ knockout mice.
2. HET/SAFB1 $^{-/-}$ males show progressive degeneration of the testicular germinal epithelium and hyperplasia of Leyding cells dependent of testosterone production. HET/SAFB1 knockout males have low level of the testosterone.
3. Basic reason for non development pregnancy of the HET/SAFB-$1^{-/-}$ knockout females is that oviductal stenosis impaired recovery of blastocysts from the knockout uterus.
4. Reasons of the secondary sterility are progressive degeneration of the ovaries and uterus.
5. Lack of HET/SAFB-1 causes decrease number germ cell during development.

II Mammary gland analysis of the HET/SAFB1 knockout females.

6. Low level of Estradiol and IGF-1 repress development HET/SAFB1 -/- mammary gland.
7. Increased branching and lobulo-alveolar development during pregnancy and after E2 treatment.
8. Increased proliferation (BrdU) after E2 treatment
9. Co-localization of ER+ and Proliferation cells in HET/SAFB1 -/- mammary gland after E2 treatment.

Reportable Outcomes

Manuscript

1. **Ivanova M**, Michaelis K, Dobrycka K.M, Jiang S, Barski O. A., Divisova Y, Lee A and Oesterreich S. SAFB1 is a Gene Essential for Reproduction. In preparation.

2. I have begun to write the manuscript about mammary gland phenotype.

Abstracts

1. Oesterreich S., Lee A., Deng W., Kang K., **Ivanova M.**, Townson S. The estrogen receptor co-repressor SAFB – mechanism of action and biological significance. 10 SPORE Investigations' Workshop, July 13-16 2002

2. **Ivanova M***., Oesterreich S. Is HET/SAFB a tumor suppressor gene in breast cancer? Generation of a mouse model. Era of Hope, September 25-28 20002.

3. Dobrzycka* K. M., Kang K., **Ivanova M.,** Lee A., Oesterreich S. Scaffold Attachment factor B1 as a breast cancer tumor suppressor gene. Gordon Research Conference , January 5-10, 2003.

4. Dobrzycka KM, **Ivanova M***, Kang K, and Oesterreich S. The Scaffold Attachment Factor B1 As A Potential Tumor Suppressor in Breast Cancer – A Role in the Cell Cycle Checkpoints? 25th Annual Department of Molecular and Cellular Biology Graduate Student Symposium. Baylor College of Medicine. Houston, TX. May, 2003.

5. **Ivanova M*.** and Oesterreich S. The Scaffold Attachment Factor SAFB-1 is an Estrogen Receptor co-repressor and potential tumor suppressor gene in breast cancer: generation of a SAFB-1 knockout mice to validate these function *in vivo.* Mouse Models of Cancer. AACR. February 19-23, 2003

6. Dobrycka K.M., **Ivanova M.**, Kang K., Oesterreich S. The Scaffold Attachment Factor B1 As A potential Suppressor in Breast Cancer – a Role in the Cell Cycle Checkpoints? 25th Annual Dept. of Molecular and Cellular Biology Graduate Student Symposium. Baylor College of Medicine. May 23, 2003.

7. Oesterreich S, Dobrzycka KM, Kang K., and **Ivanova M***. The Scaffold Attachment Factor SAFB1 as Potential Tumor Suppressor Gene in Human Breast Cancer. AACR Breast Cancer Conference, Huntington Beach, CA. October, 2003.

8. Dobrzycka KM, **Ivanova M**, and Oesterreich S. Characterization of Mouse Embryo Fibroblasts (MEFs) from SAFB1 Knockout Mice. Graduate Student Symposium, Baylor College of Medicine, Houston, TX. October, 2003.

9. Dobrzycka KM, **Ivanova M**, and Oesterreich S. The scaffold Attachment Factor B1 and the Cell Cycle Control- Is There A Connection? 1st Annual Cancer Center Retreat and Symposium. Houston, TX. November, 2003.

10. Meyer R*, Adkins B, Dobrzycka K, **Ivanova M**, Kang K, Townson S, and Oesterreich S. SAFB1 and SAFB2 as Estrogen Receptor Corepressors in Breast Cancer. Keystone Symposia: Nuclear Receptors: Steroid Sisters. Keystone, CO. February, 2004

11. Dobrzycka KM, Kang K, **Ivanova M**, Jiang S, and Oesterreich S. Cells lacking the Scaffold Attachment Factor B1 (SAFB1) show immortalization, and genomic instability. 26th Annual Department of Molecular and Cellular Biology Graduate Student Symposium. Baylor College of Medicine. Houston, TX. April, 2004.

12. Dobrzycka KM, Kang K, **Ivanova M**, Jiang S, and Oesterreich S. Loss of SAFB1 leads to cell immortalization and increased susceptibility to oncogenic transformation. 2004 Department of Medicine Research Symposium. Baylor College of Medicine. Houston, TX. April, 2004.

13. **Ivanova M** and Oesterreich S. Pleiotropic Fertility Phenotypes in Mice Lacking SAFB1 Texas Forum Female Reproduction, Houston, April 22, 2004.

14. Oesterreich S, Dobrzyscka K, Lewis M and **Ivanova M.** SAFB1, Tumorigenesis, and Reproduction – Lessons from the Knockout Mice. Gordon Research Conference, May 9-14, 2004.

15. **Ivanova M**, Divisova Y, Lee A and Oesterreich S. SAFB1 a Gene Essential for Reproduction. Gordon Research Conference, June 6-11, 2004.

16. Dobrzycka KM, Kang K, **Ivanova M**, Jiang S, Lee AV, and Oesterreich S. Loss of Scaffold Attachment Factor B1 (SAFB1) leads to mitotic abnormalities, immortalization, increased transformation, and genomic instability in Mouse Embryo Fibroblasts (MEFs). Gordon Research Conference: Cancer Models and Mechanisms. August 2004.

Conclusions:

We have successfully generated HET/SAFB1 knockout mice. This mice display multiple phenotype: embryonic lethality, growth retardation, deficient of IGF-1 and steroids hormones testosterone and estradiol, sterility and hyperplasia of leydig cells in males, subfertility, secondary sterility, delayed maturation and reduction number of primary follicles in HET/SAFB1 knockout females.

Delayed mammary gland development in young virgin HET/SAFB1-/- females and increased branching and lobulo-alveolar development during pregnancy and after E2 treatment.

References:

1. **Oesterreich S,** Allred DC, Mohsin SK, Lee AVL, Osborne CK, O'Connell P. Loss of heterozygozity at the HET/SAF-B locus on chromosome 19p13 in human breast cancer. Br J Cancer; 84(4): 493-498, 2001.
2. **Oesterreich S,** Zhang Q., Hopp T., Fugua SAW., Michaelis M, Zhao HH, Davie JR, Osborne CK, Lee AV. Estrogen receptor bound to the antiestrogen tamoxifen strongly interact with the nuclear matrix protein HET/SAF-B, a novel inhibitor of estrogen-mediated transactivation. Mol. Endo. 14, 369-381, 2000.
3. Couse JF, Korach KS. Estrogen receptor null mice: what have we learned and where will they lead us? Endocrine Reviews 20(3): 358-417, 1999.
4. Parr BA, Mcmahon AP. Sexually dimorphic development of the mammalian reproductive tract requires Wnt-7a. Nature 395, 707-710, 1998.

Abbreviations:

Scaffold Attachment Factor B: HET/SAFB-1; HET/SAF-B; **SAFB1** (new nomenclature).

The training and accomplishments

(1) Baylor Graduate School of Biomedical Sciences

Cancer (audited) and Introduction to Molecular Carcinogenesis (audited)

(2) Breast Center Courses

Scientific writing and research grants course
Biostatistics course
Clinical breast cancer course
Translational research course

(3) Baylor required training courses

Animal research: Transgenic mouse facility and Animal use
Research ethics training program
Protection of human research subjects

(4) Core postdoctoral training

Six monthly Breast Center data review.
Yearly journal club presentation.

Appendices.

Legends

Figure 1. Generation of SAFB-1 knockout mice by homologous recombination.

A. Genomic structure of the mouse SAFB-1 gene, the targeting vector, and the predicted structure of the targeted allele. Exons are indicated by black boxes. This deletion replaced the 8-13 exons of the mouse HET/SAFB-1, including the translation start codon with a *β-galactosidase* gene. An 800 bp 3' external southern probe detects 7 kb wild type EcoRI fragment and 9.7 kb mutant EcoRI fragment. Abbreviation: E, EcoRI.

B. Southern blot analysis of genomic DNA isolated from targeted ES clones (*left*), and mouse tail (*right*) digested with EcoRI.

C. For genotyping animals, genomic DNA was assessed by PCR using HET/SAFB1 specific primers and primers specific to the LacZ.

D. RT-PCR analysis of HET/SAFB1 as a 320 bp amplicon from reverse-transcribed adult testis and brain mRNA prepared from wild-type, heterozygous and homozygous mice. Primers to mouse β-actin amplify a 267 bp fragment used as a control.

E. Western blot with N terminus-specific SAFB1 antibody on protein lysate from MCF7, 3T3 (as control), wild type and knockout brain and embryos E13.5.

F. Whole mount β-galactosidase staining of embryos at 11.5 days: SAFB-$1^{+/+}$, and SAFB-$1^{-/-}$. Expression of SAFB-1 gene is detected in neural tube, somites and no expression is detected in the heart (-/-, magnification 0.8x).

Figure 2. Development of the mammary gland during life of HET/SAFB-1 knockout females.

A. Slow elongation and branching of the mammary ducts during maturation. 4, 6 and 10 weeks old animals.

B. Quantitative representation of growth of distance from the center of the lymph node to the far end of endbuds. 4, 6 and 10 weeks old mice.

C. The number of branching points in 4 and 6 weeks old mammary glands.

Figure 3. Mammary gland of 4 months old HET/SAFB1 knockout females.

A. Comparison of body weight, level of IGF-1 and size of mammary gland of 4 months old knockout females.

B. Linear correlation between IGF-1 concentration and body weight for HET/SAFB1-/- mice (n=5).

Figure 4. Uterine response to estrogen stimulation in HET/SAFB1 knockout females

A. Body weight of knockout and wild type females after ovariectomy (ovex) and E2 treatment.

B. The ratio of uterine weight (milligrams) to body weight (grams) was calculated.

C. Histological analysis of the uterus after ovariectomy (ovex) and E2 treatment. Increased inflammation of the HET/SAFB1 -/- uterus.

D. BrdU staining of representative uterus from a knockout female. The percentage of BrdU positive cells extra induced by estrogen stimulation myometrium of this knockout female.

Figure 5. Mammary gland response to estrogen stimulation.

A. Whole-mount staining of control and knockout mammry glands after ovariectomy (ovex) and E2 treatment.

B. The number of branching points in comparable boxed areas (n=5 mice per group, 5 areas from one mammary gland, **: $p < 0.001$).

C. H&E staining of the control and knockout mammary gland.

D. BrdU staining of the control and knockout mammary gland.

E. Increased BrdU incorporation in the HET/SAFB1 knockout mammary gland (n=5, * $p<0.01$)

Figure 6. Analysis of Estrogen and Progesterone receptors to estrogen stimulation.

A. Immunohistochemistry staining with Estrogen receptor antibodies of control and HET/SAFB1 knockout mammary gland after ovariectomy and E2 treatment.

B. Quantitation of ER positive cells (n=5 per group, counted 5 areas from one mammary gland).

C. Immunohistochemistry staining with Progesterone receptor antibodies of control and HET/SAFB1 knockout mammary gland after ovariectomy and E2 treatment.

D. Quantitation of ER positive cells (n=5 per group, counted 5 areas from one mammary gland).

Figure 7. Co-localization of ER and BrdU positive cells in mammary gland after estrogen stimulation.

Immunofluorescence staining for ER (red) and BrdU (green) was done on control and knockout mammary gland after ovariectomy and E2 treatment. Yellow color indicated co-localization of ER positive and proliferate cells.

Figure 8. Development of the mammary gland in HET/SAFB-1 3.5 day pregnant females.

A. Whole-mount staining of control and HET/SAFB1 knockout mammary gland at a stage of 3.5 day pregnancy during first (n=2), second (n=2) and third (n=3) undevelopment pregnancy.

Multi formation of secondary ducts and branching.

B. H&E staining and iImmunohistochemistry staining with Estrogen and Progesterone receptors antibodies. Control and knockout mammary gland during 2^{nd} and 3^{nd} undeveloped pregnancyes. We used pseudopregnant control females.

C. Serum progesterone, estradiol and testosterone levels of 3.5 days pregnant females (n = 5 mice per group). Statistical analysis was done with two independent sample t test. Two-tailed P values are <0.05 (one asterisks) or <0.001 (double asterisks).

D. Quantitation of ER positive cells.

E. Quantitation of PgR positive cells.

Figure 9. Prenatal and postnatal survival of the SAFB-1$^{-/-}$ mice.

A. Schematic illustration of the SAFB-1 knock-out phenotypes during embryonic development. 50 % of SAFB-$1^{-/-}$ embryos die before E10. At E11-E19, all surviving knock-out embryos are growth retarded. 40% of the SAFB-$1^{-/-}$ individuals die early in the postnatal period.

B. Wild type SAFB-$1^{+/+}$, heterozygous SAFB-$1^{+/-}$ and homozygous SAFB-$1^{-/-}$ newborn pups on the first day at 8 a.m. Dead SAFB-$1^{-/-}$ newborn is marked.

C. Sections of 19 day embryos stained with hemathoxylin and eosin. Liver sections of $SAFB1^{+/+}$ and $SAFB1^{-/-}$ embryos show the absence of erythrocytes in knockout embryos (n=2, same offspring). Sections of liver 19 day embryos staining with CD31 antibody (n=2, same offspring, *up*). Number vessels and vessels areas was calculated with program "metamorph" (n=2, 3 field of the liver, $p<0.05$). Lung sections of $SAFB1^{+/+}$ and $SAFB1^{-/-}$ embryos (n=2). Bars equal 100 μm.

Figure 10. Growth characteristics of the SAFB1$^{-/-}$ mice.

A. HET/$SAFB1^{-/-}$, HET/$SAFB1^{+/-}$ and HET/$SAFB1^{+/+}$ female mice, 6 weeks old. Knockout mouse is marked.

B. Growth defect of SAFB-1 knockout mice between 3 and 8 weeks of age. The graphs illustrates the of weight of $SAFB1^{-/-}$ males compared to $SAFB1^{+/-}$ and $SAFB1^{+/+}$ males (n=14, $p<0.001$, *up*) and $SAFB1^{-/-}$ females compared to $SAFB1^{+/-}$ and $SAFB1^{+/+}$ females (n=12, 3-6 weeks: $p<0.001$, 7and 8 week: $p<0.05$, *bottom).*

C. Growth defect of the SAFB1 knockout embryos (11, 14 and 15 day) and knockout newborn pups 1 and 2 days from heterozygous crossings of SAFB1 mice (*up).* Data are pooled from two separate breeding lines. The graph illustrates the percentage of weight of $SAFB1^{-/-}$ and $SAFB1^{+/-}$ mice relative to wild type: E11 and E14 (n=7); E15 (n=5); 1 day newborn (n=19); 2 day newborn (n=5).

D. Growth defect of the skeleton (*bottom).* Skeletal preparation of newborn after alizarin red and alcian blue staining. Numbers indicated position for measure. The graph illustrates the percentage of length bones of SAFB-$1^{-/-}$ newborns relative to wild type (n=7 per group, analyzed 4 offspring's, $P<0.01$).

E. Serum IGF -I levels of the 3-4-months-old male and female mice (n = 5 mice per group). Statistical analysis was done a two independent sample t test. Two-tailed P values are <0.01 (one asterisks) or <0.001 (double asterisks). Correlation between IGF-1 concentrations and body weight for $SAFB1^{-/-}$ mice *(bottom).* Statistical *t* test indicates a very significant ($p < 0.01$) positive correlation ($r^2 = 0.9138$).

Figure 11. Sterility of the SAFB-1$^{-/-}$ males.

A. Efficiency of fertilization of SAFB-$1^{-/-}$ males. Efficiency of mating and the number of preimplantation embryos or unfertilized oocytes isolated from C75Bl/6 females after breeding with $SAFB1^{-/-}$, $SAFB1^{+/-}$, and $SAFB1^{+/+}$ males (n=4 per group). Embryos were isolated by oviducts flushing (day 1.5), counted and scored for morphology.

B. Male reproduction system of $SAFB1^{+/+}$, $SAFB1^{+/-}$ and $SAFB1^{-/-}$ at 3 moths of age. T–testis, SV–seminal vesicles.

C. Percentage of the organs weight to total body weight of the 3 months old males (n=9 mice per group).

D. Serum testosterone levels of 3-months-old male mice (n=9 mice per group).

E. Serum estradiol levels of 3-months-old male mice (n = 7 mice per group). Statistical analysis was done a two independent sample t test. Two-tailed P values are <0.01 (one asterisks) or <0.001 (double asterisks).

Figure 12. Role of HET/SAB-1 in gonad development and gametogenesis in males.

Histological section of testis from wild type and knockout males. Note the arrest of the spermatogenesis and the progressive loss of the germinal epithelium during testicular maturation.

A Testis from immature HET/SAFB1$^{-/-}$ males demonstrated similar structure with wild type (*upper row*). Testes from mature HET/SAFB1$^{-/-}$ animals demonstrated arrest of spermatogenesis (*second row*). Seminiferous tubules exhibit a spectrum of epithelial dysgenesis and increased effect of degeneration during life (*third row*). Interstitial Leyding cells demonstrate cellular hypertrophy and hyperplasia adjacent to several degenerated tubules (*second and third row*).

B DNA fragmentation was visualized by TUNEL staining (hematoxylin counterstained). Apoptosis in SAFB1$^{-/-}$ testes is confined to particular seminiferous tubules containing dividing intermediate spermatogonia and spermatocytes.

C Staining testis with an antibody recognizing germ cell nuclear antigen (GCNA-1). No differences were observed in the newborn testis (*upper row*). Knockout testis 9-months old mice have a decreased number of germ cells compared with wild type (*second row*).

D Seminal vesicles of the SAFB1$^{-/-}$ males have simple structure compared with wild type ones. Bars equal 100 μm.

Figure 13. Dysfunction of female reproductive system of SAFB1$^{-/-}$ mice.

A. Histology of the oviducts. SAFB1 knockout females develop oviductul stenosis. Sections demonstrated disorganized structure of the muscle in the oviducts wall and simple columnar epithelium in 4-week-old and 4-months-old HET/SAFB1 knockout females.

B. Histology of the ovary from 4-week-old, 4 and 10 months old wild type and knockout females.

C Decrease in the number of primary follicles contrasts the constancy in the number of secondary follicles in the 4-week-old knockout females compared with heterozygote and wild type animals (n=4 per group, 10 step section both ovaries with interval 25 mkr. Two-tailed P values are <0.01 (asterisks) was accepted as being statistically significant). Decreased number of primary and secondary follicles in the 4, 8 and 10 months old knockout females compared with wild type (n=1 per group, 5 step section both ovaries with interval 25 mkr).

D Staining newborn ovaries with an antibody recognizing germ cell nuclear antigen (GCNA-1). No differences were observed in the newborn ovaries. (SAFB1$^{-/-}$ n=4, SAFB1$^{+/+}$ n=3) Bars equal 100 μm.

Figure 1. Generation HET/SAFB1 -/- mice.

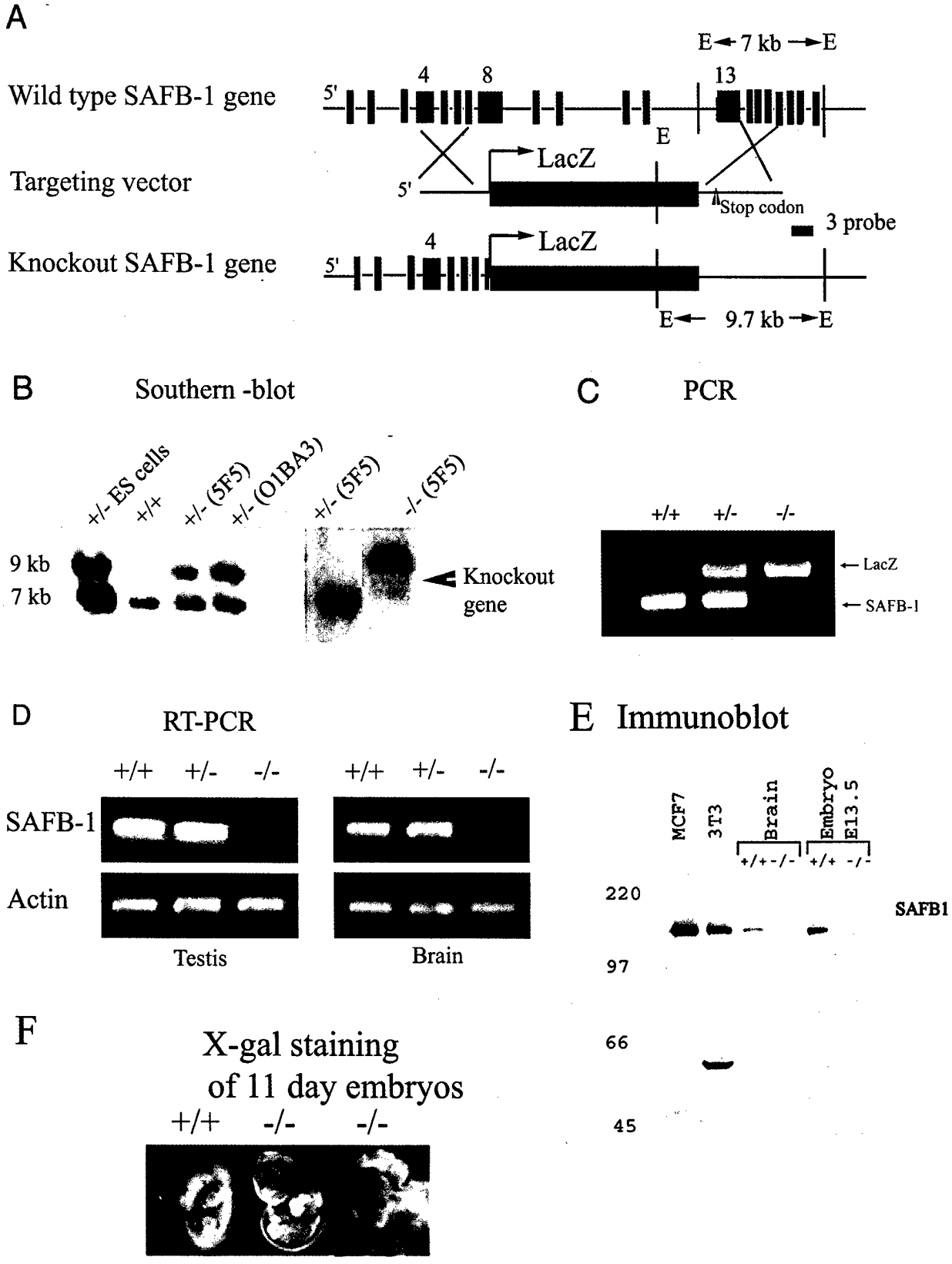
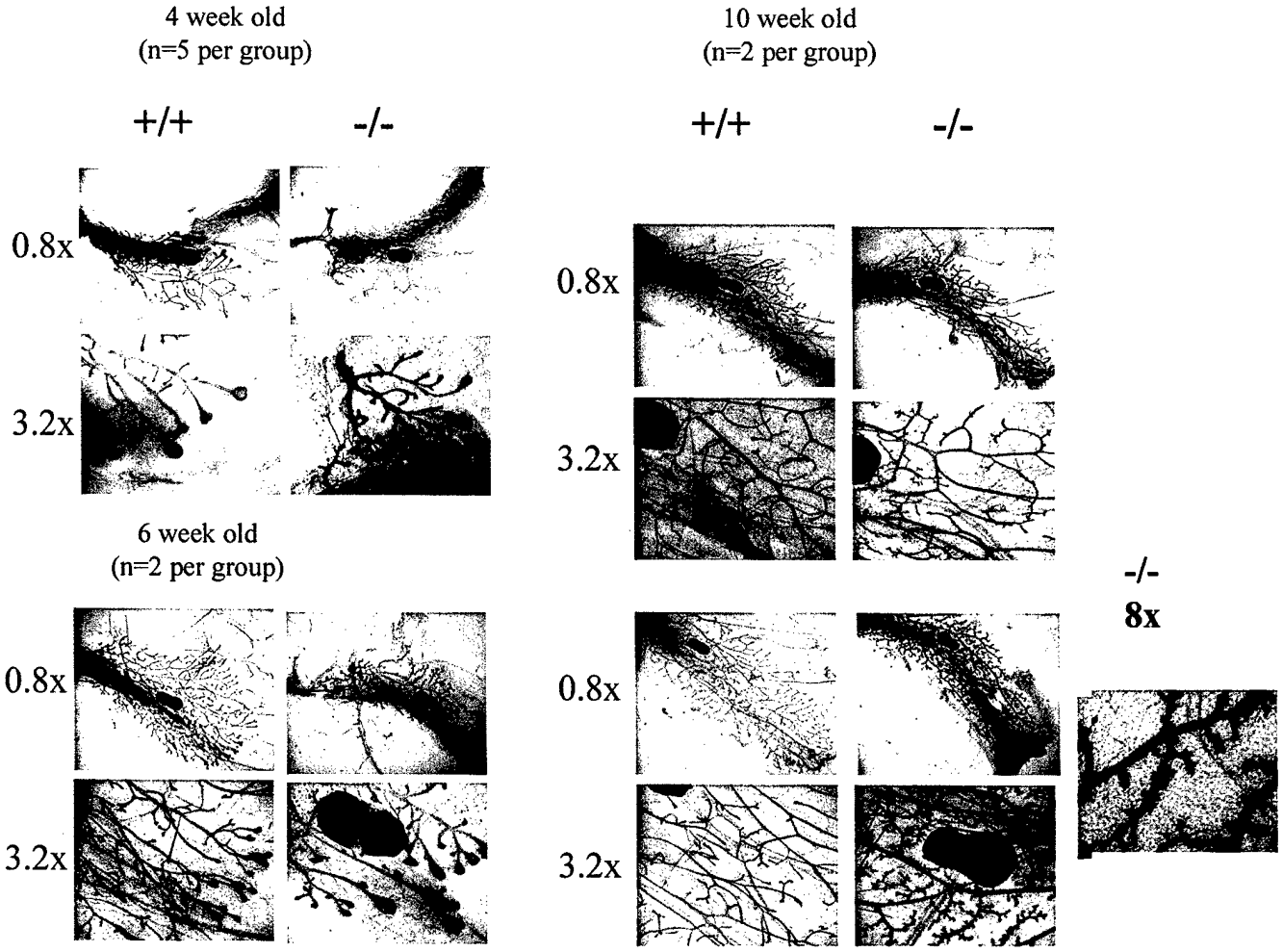
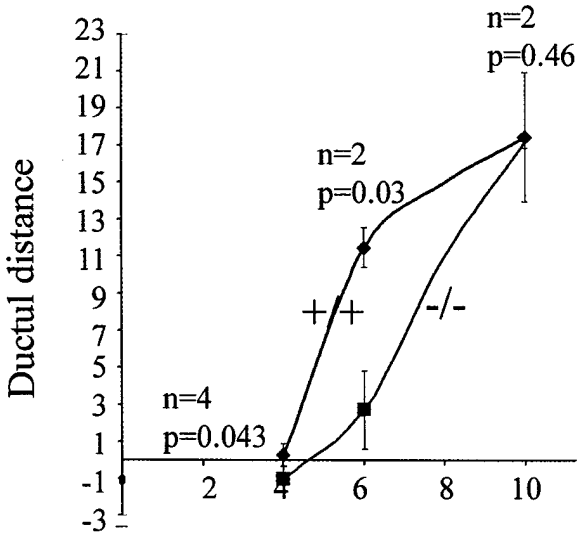


Figure 2. Development of mammary gland during maturation

A. Whole staining of mammary gland.



B. Ductul distance.



C. Terminal EndBuds (TEB) number.

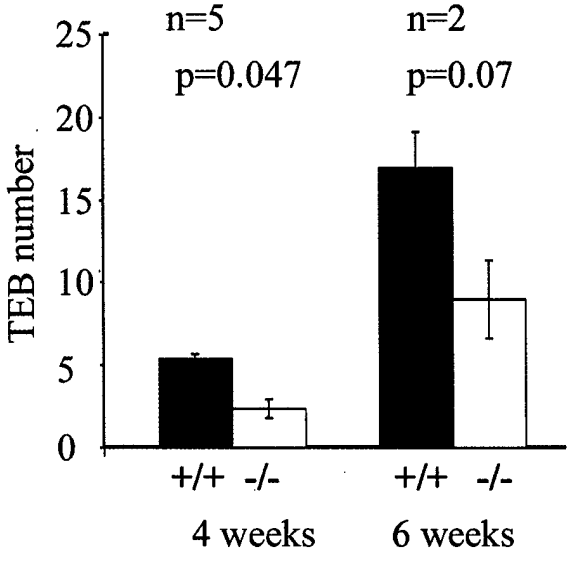
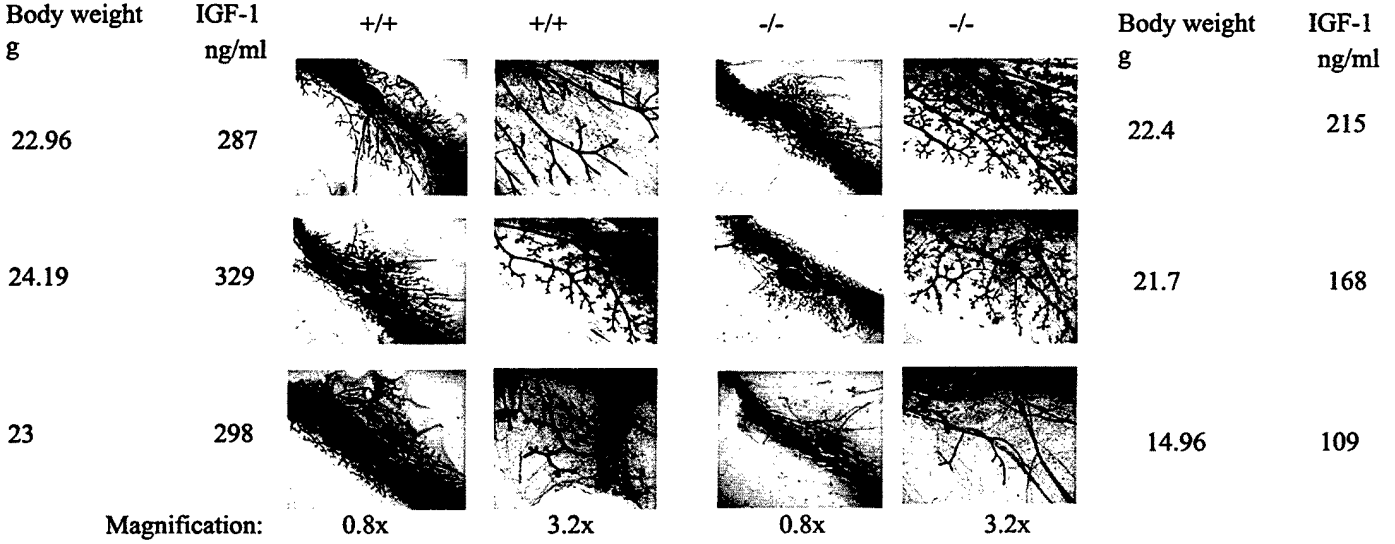


Figure 3.
Mammary gland of 4 months old HET/SAFB-/- females.

A. Whole staining of mammary gland



B. Correlation between IGF-1 concentrations and body weight for HET/SAFB1 -/- mice.

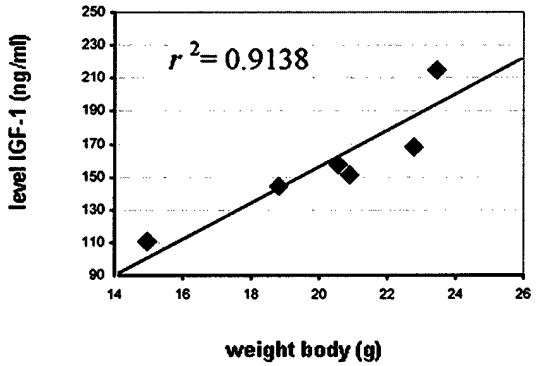


Figure 4. E2 treatment, uterus.

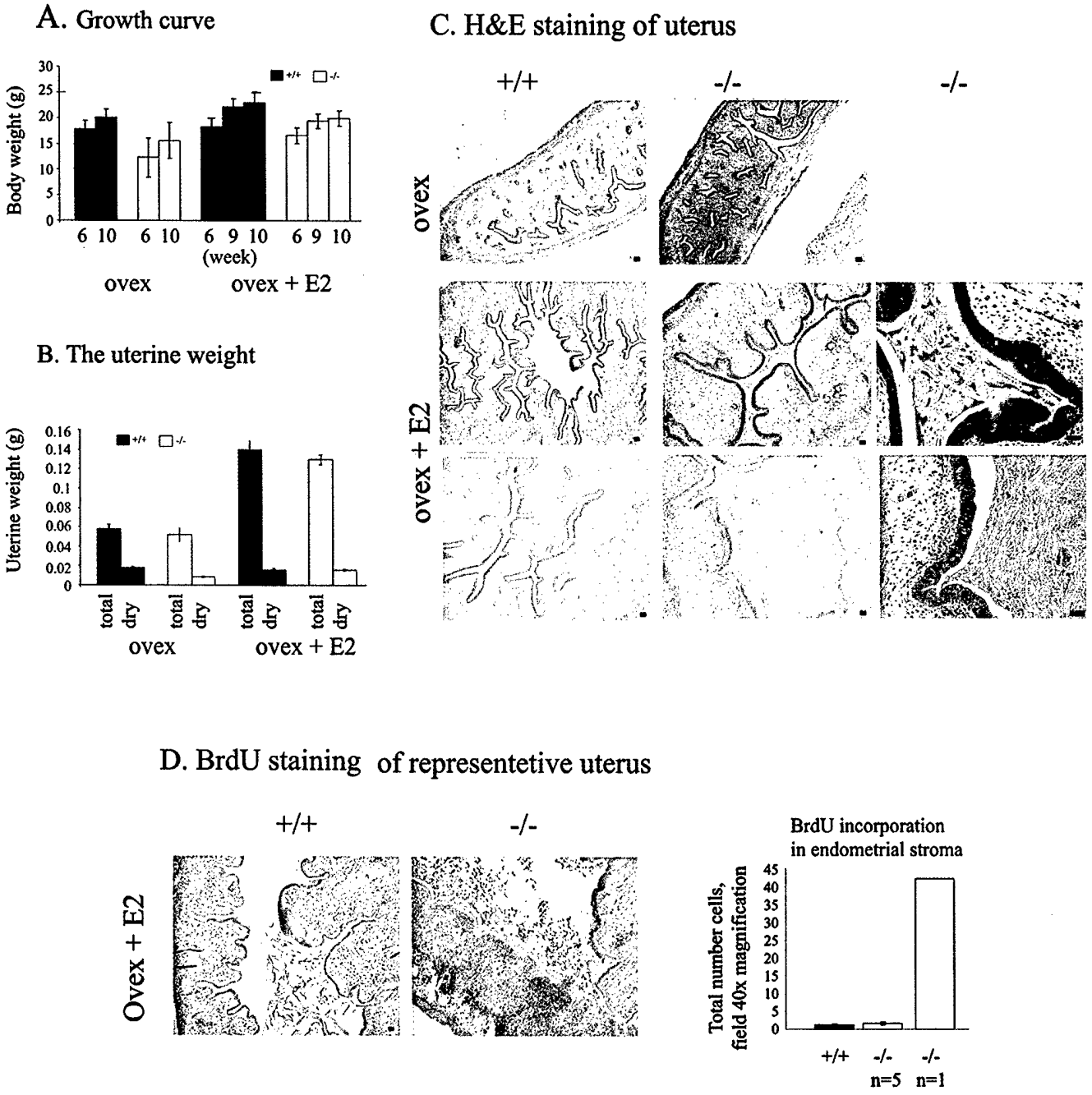
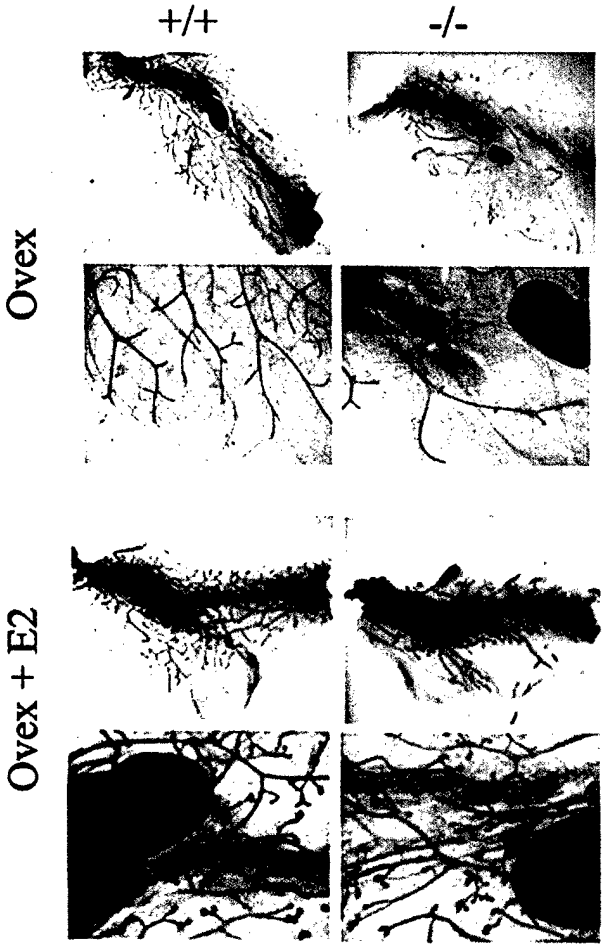
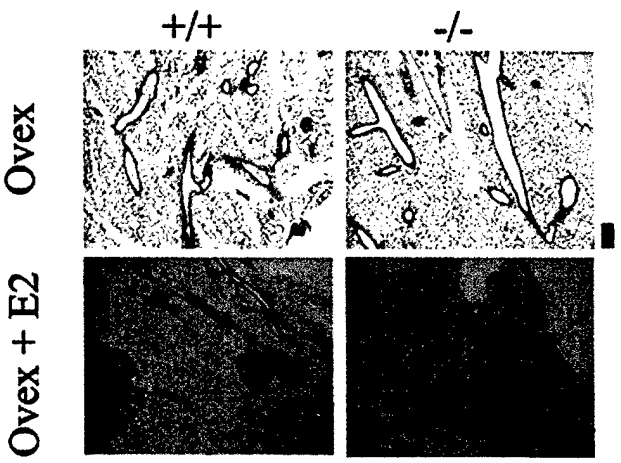


Figure 5. Mammary gland, E2 treatment.

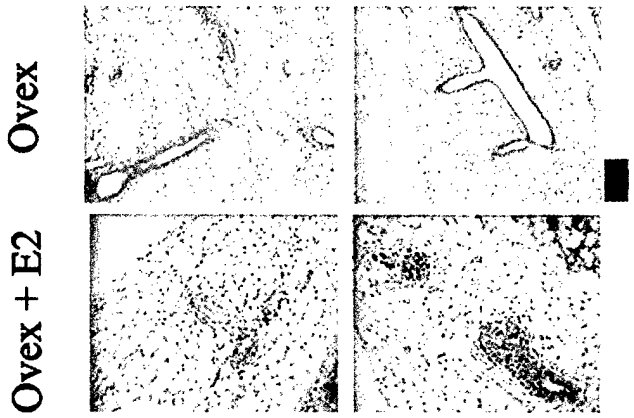
A. Whole staining of mammary gland



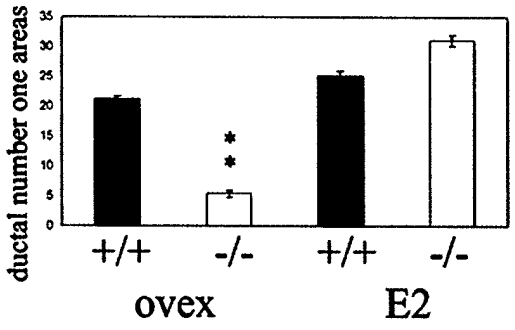
C. H&E staining



D. BrdU staining



B. Ductul number



D. The ratio of BrdU - positive cells

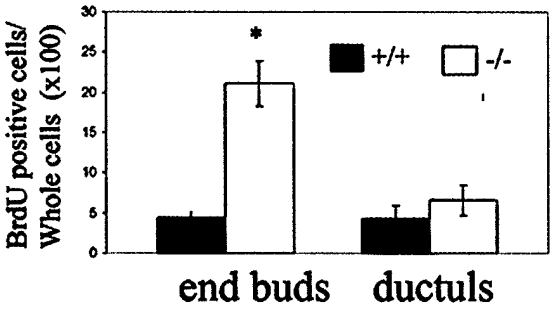
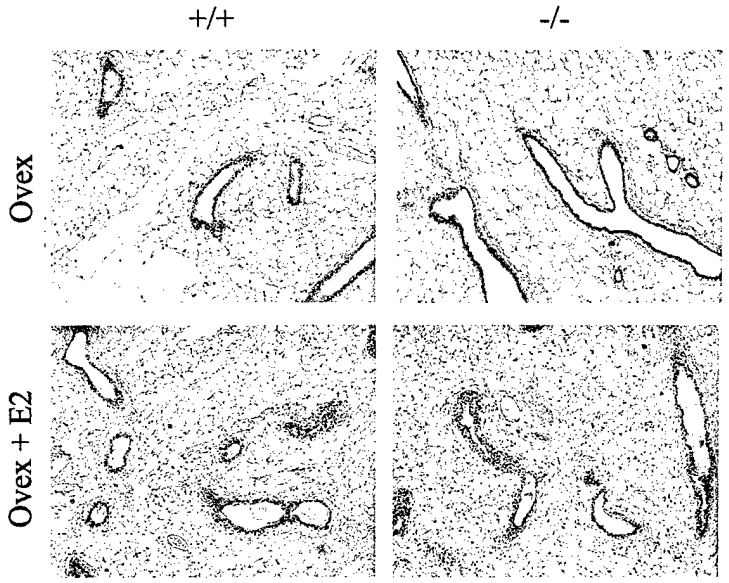
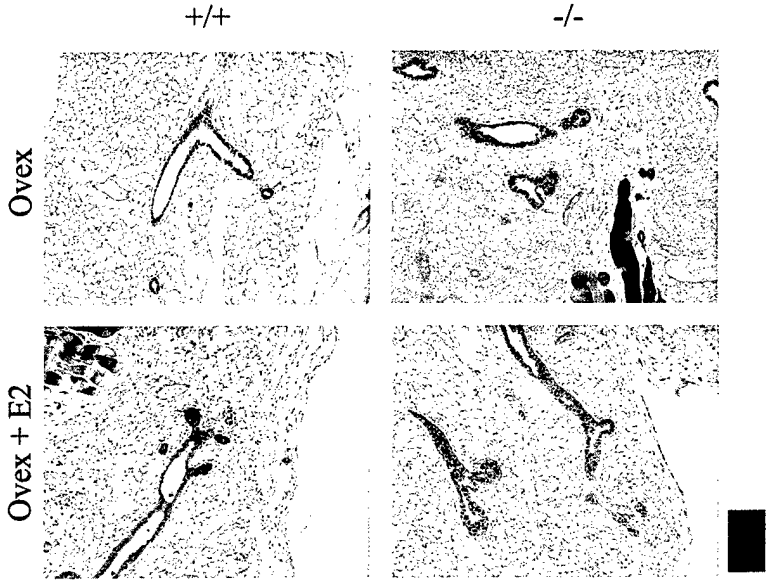


Figure 6. Analysis of Estrogen and Progesterone receptors.

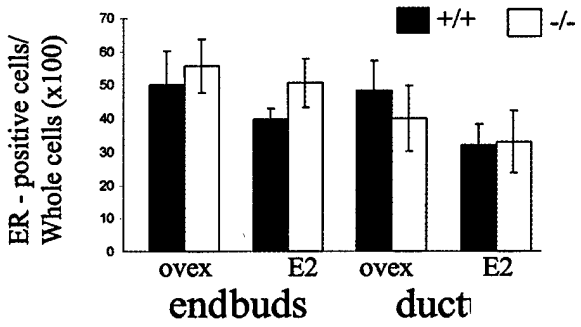
A. Staining with Estrogen receptor antibody



C. Staining with Progesterone antibody



B. Quantitation of ER - positive cells



D. Quantitation of PgR - positive cells

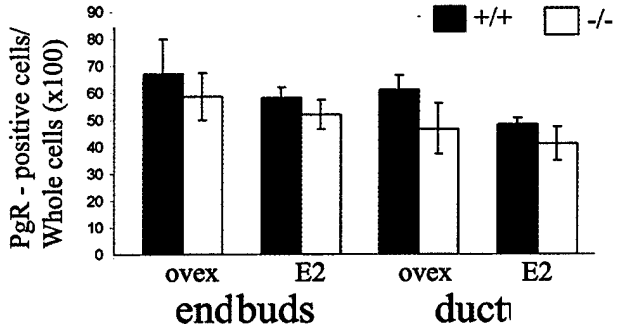
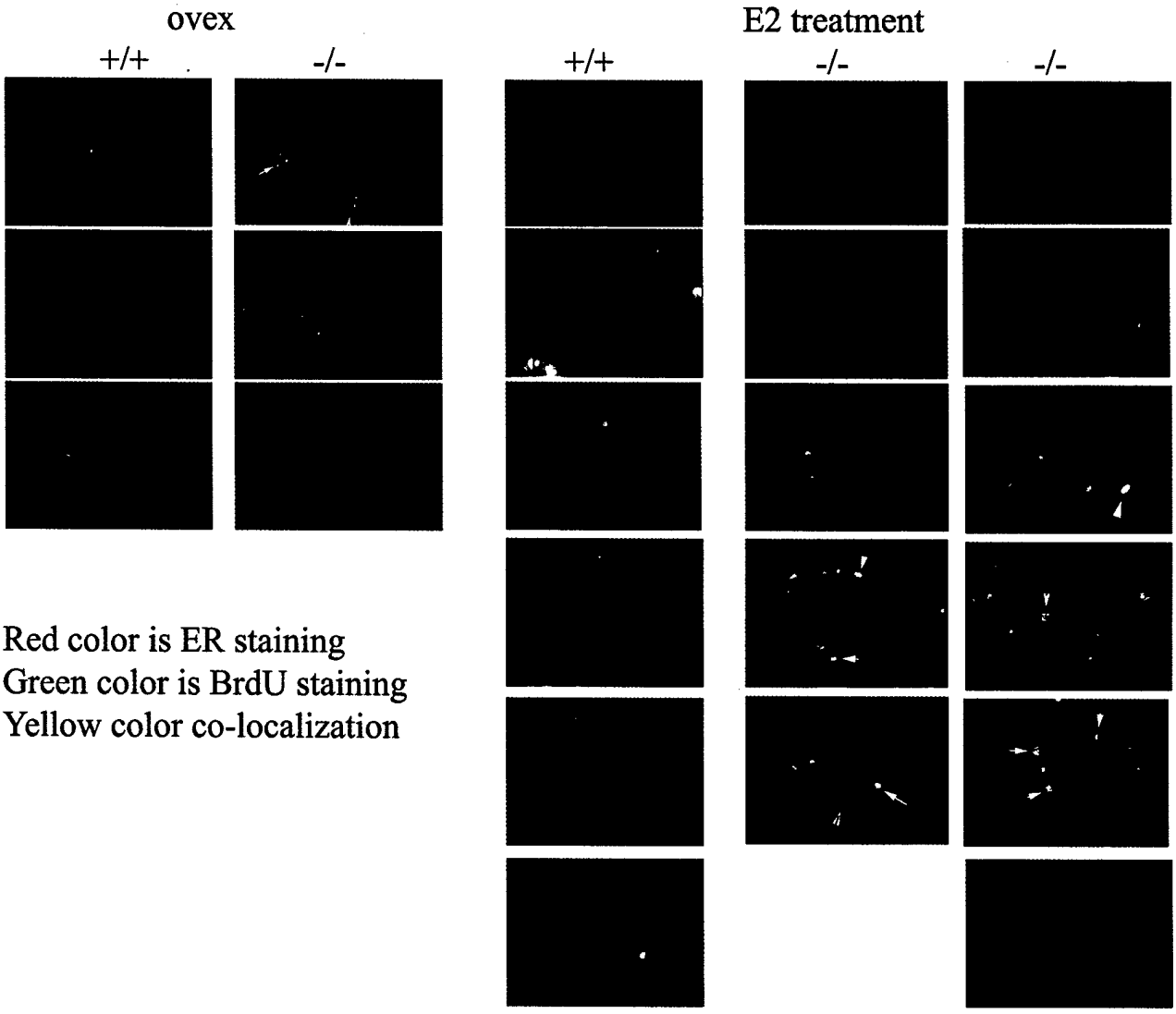


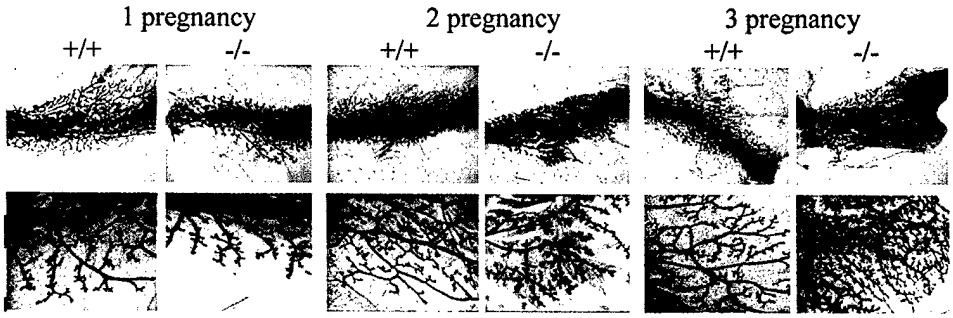
Figure 7.
Co-localization of ER and BrdU positive cells.



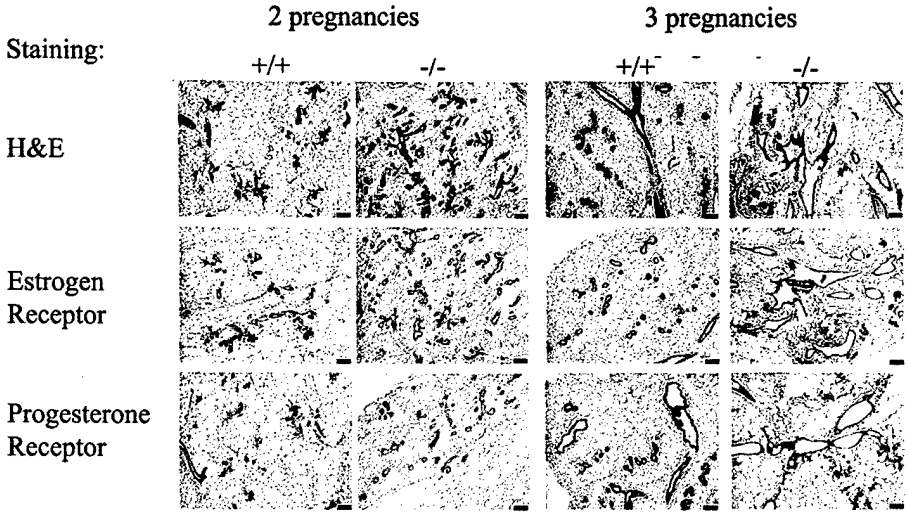
Red color is ER staining
Green color is BrdU staining
Yellow color co-localization

Figure 8. Mammary gland development
3.5 day pregnancy.

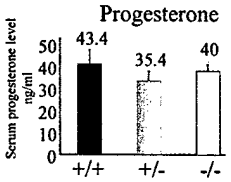
A. Whole staining of mammary gland 3.5 days pregnancy

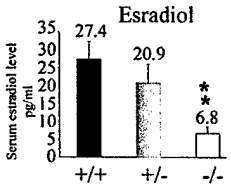


B. Histology of mammry gland 3.5 days pregnancy

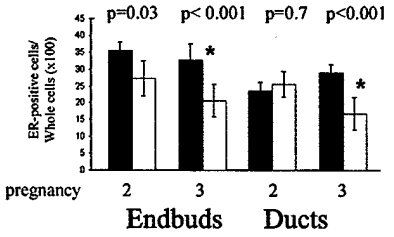


C. Serum level





D. Number of ER positive cells



E. Number of PgR positive cells

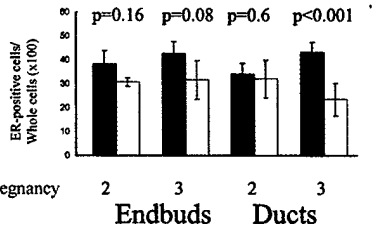
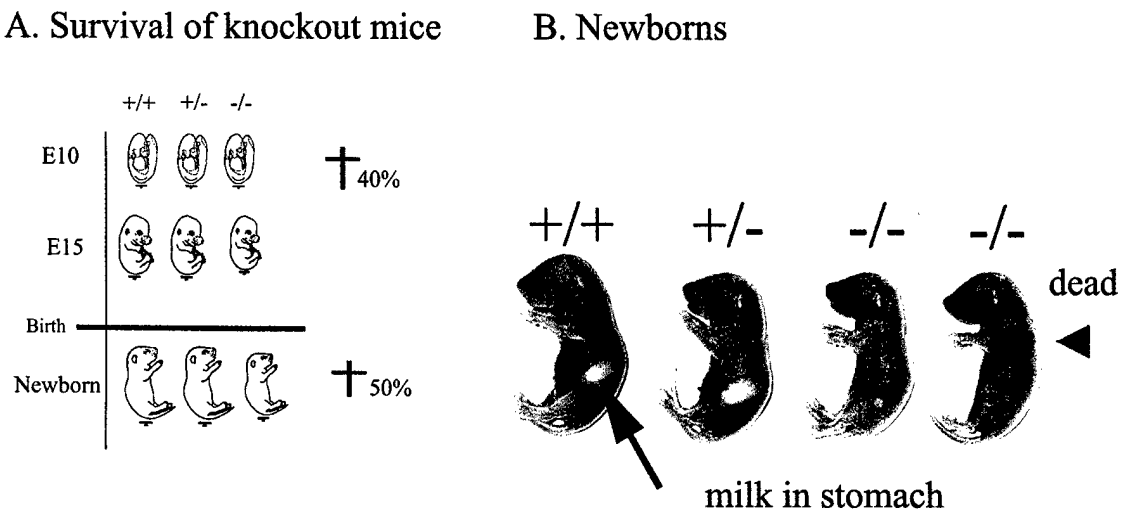


Figure 9. Analysis of embryos and newborns.



C. H&E staining

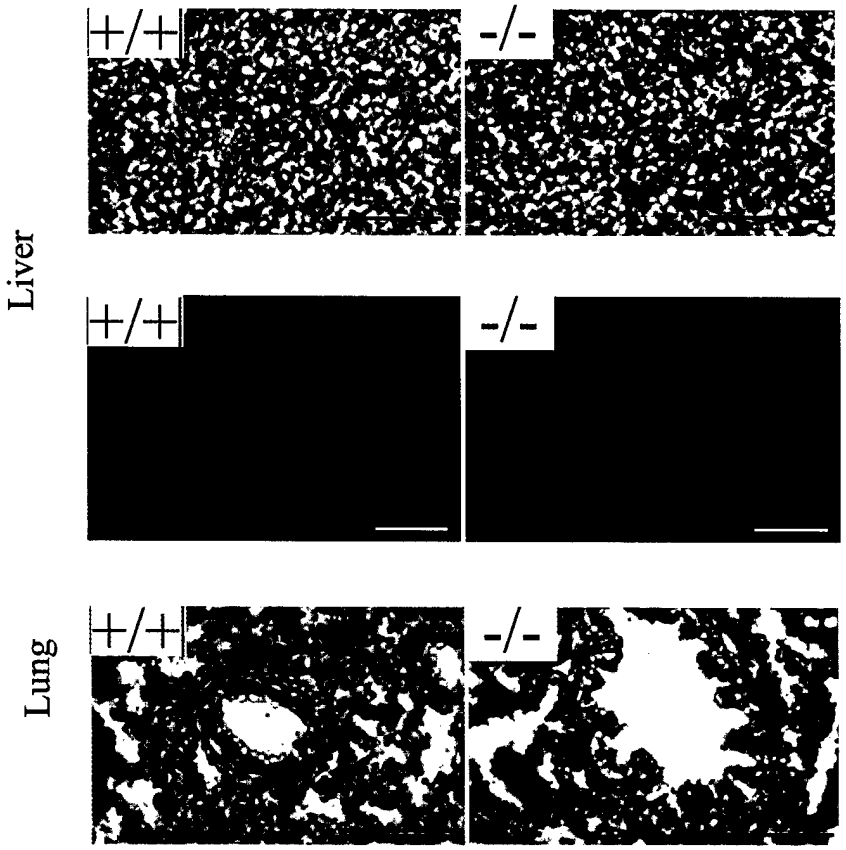
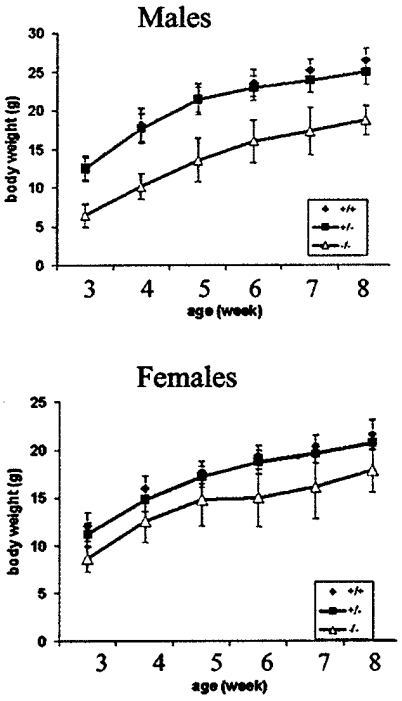


Figure 10. Size of HET/SAFB-/- mice.

A

B



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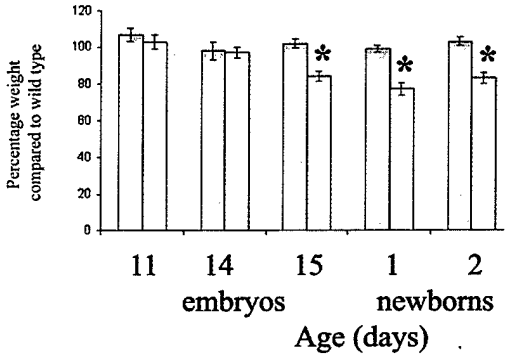
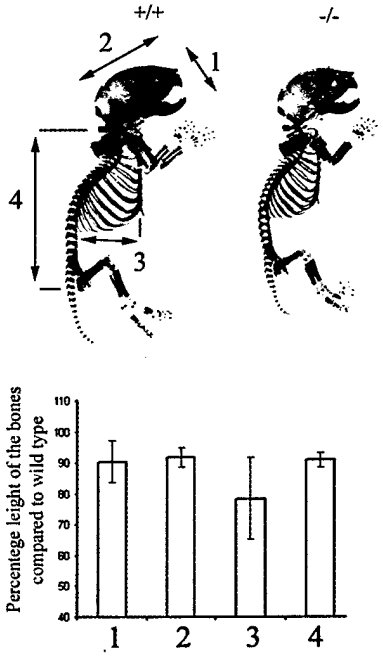


D



E

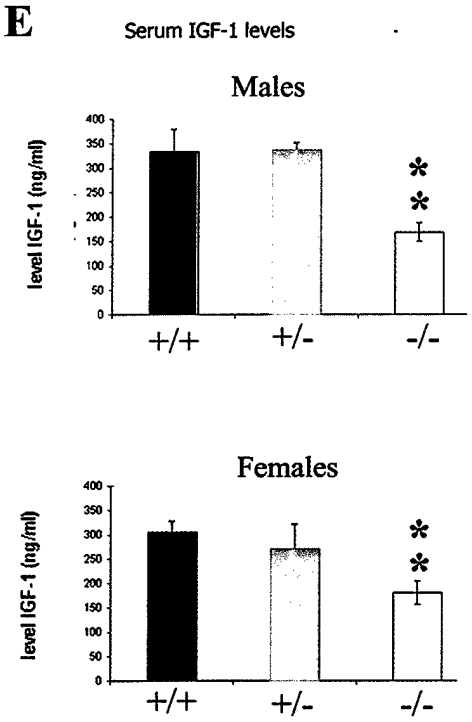
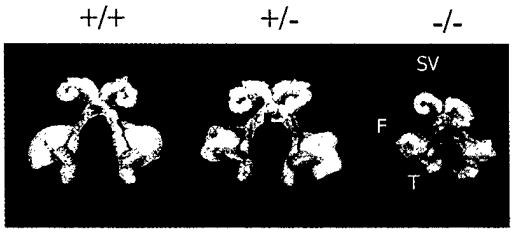


Figure 11. HET/SAFB1-/- males.

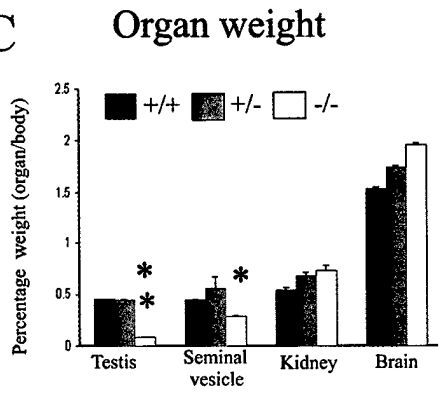
A

Genotype males	No. plug	2-cell embryos	Oocytes
+/+ (n=3)	5	24	15
+/- (n=3)	7	23	15
-/- (n=4)	2	0	53

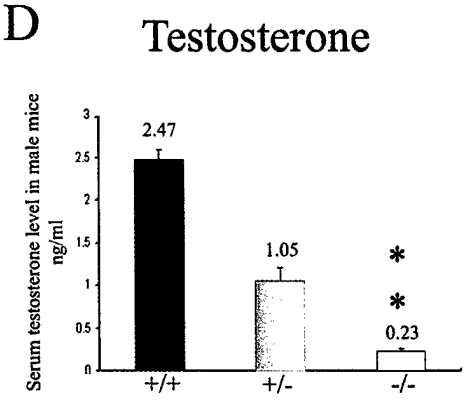
B



C



D



E

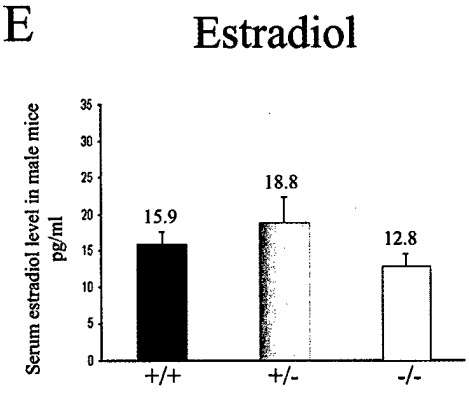


Figure 12. Histology of testis and seminal vesicles of HET/SAFB1 -/- males

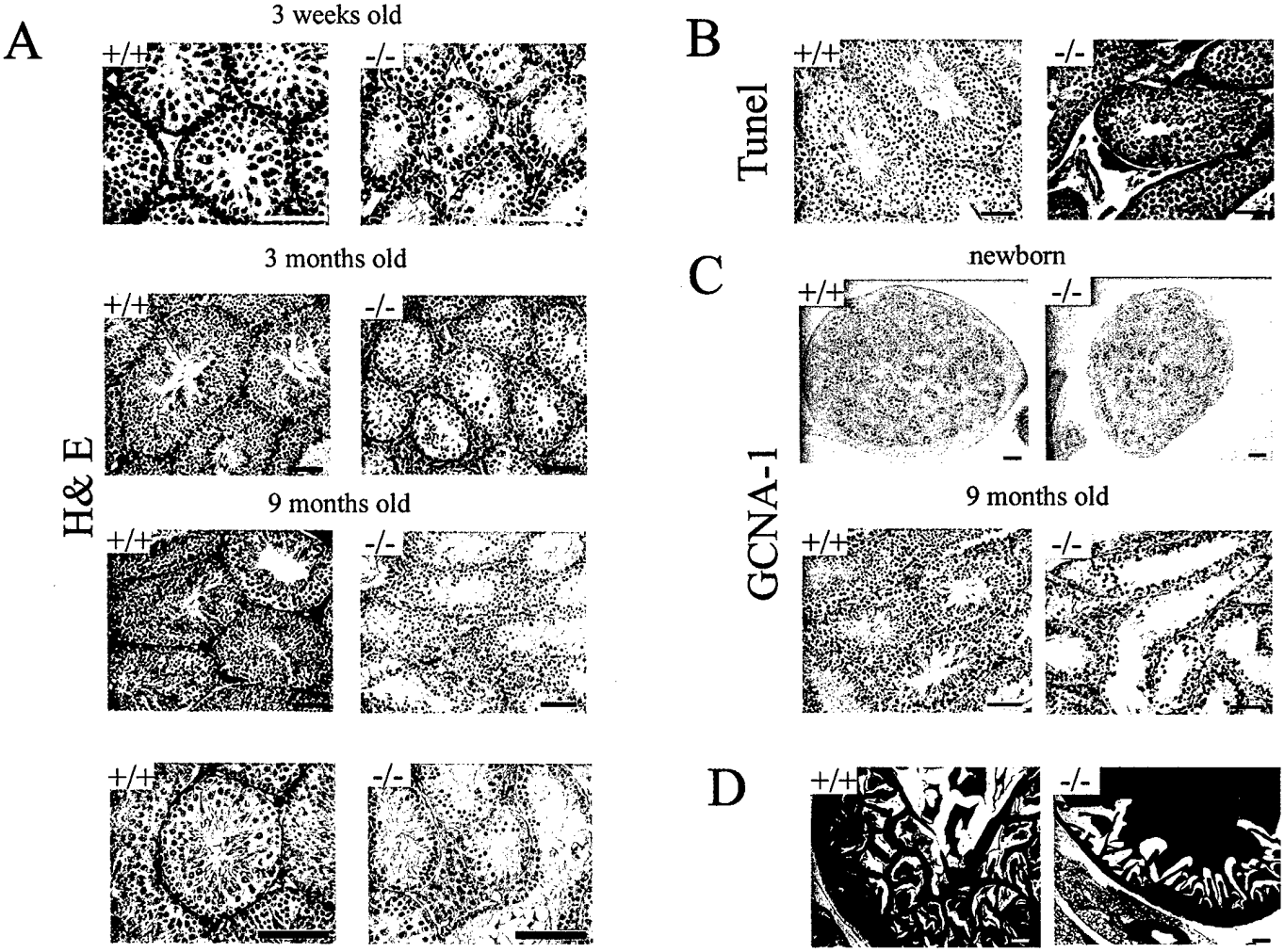


Figure 13. Female reproductive system of HET/SAFB1 -/- mice