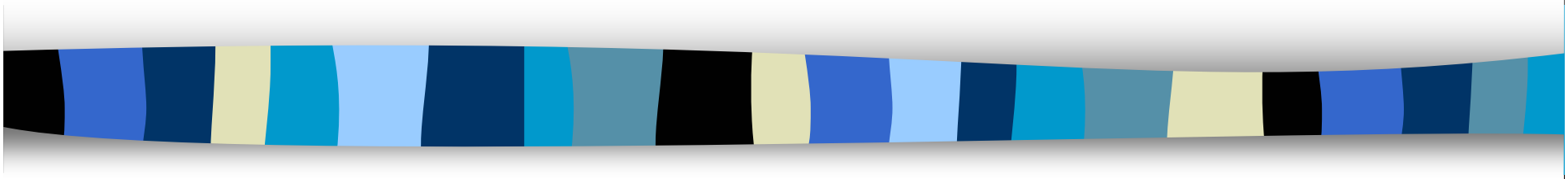
Aplikovaná chemie a biochemie

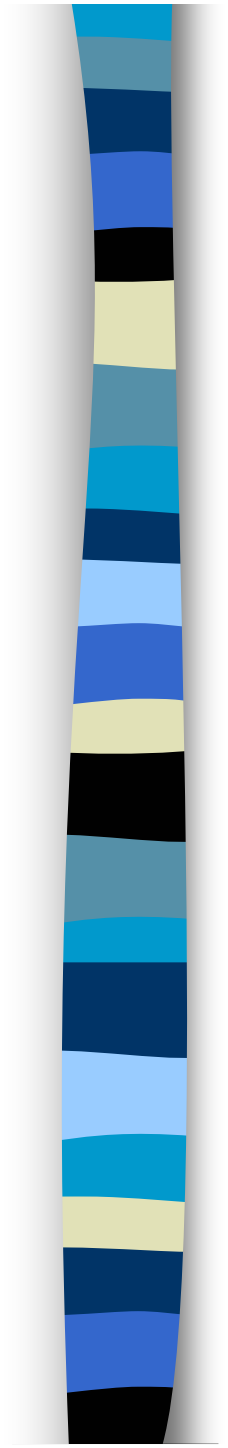


Přednáška č. 6

In vivo modely

Práce s modelovými organismy:

- modely fyziologických stavů; modely onemocnění
- transgenní zvířata – induced expression, knock-out, knock-in; hypomorphic alleles
- tkáňově specifická exprese;



Modelové příklady využití hlodavců:

- genetické modely;
- využití toxických látek;
- virová onemocnění.

Experimentální modely diabetes mellitus:

The best available models for **type I diabetes** mellitus are the **BB rat** and the **NOD mouse.** These demonstrate many of the autoimmune phenomena characteristic of the human disease. They have provided the opportunity to evaluate the effects of manipulating the immune system as well as several environmental factors on the development of diabetes. Thus, neonatal thymectomy, administration of immunosuppressive drugs, and antibodies against various lymphocytes have been effective in preventing diabetes in these models. The BB rat and NOD mouse provide the opportunity to investigate more precisely targeted forms of immune modulation that might then be applicable to patients with type I diabetes mellitus. Although these models are quite useful in studying the etiology of this form of diabetes, the rodents do not develop the long-term complications that are the major clinical problem in patients.

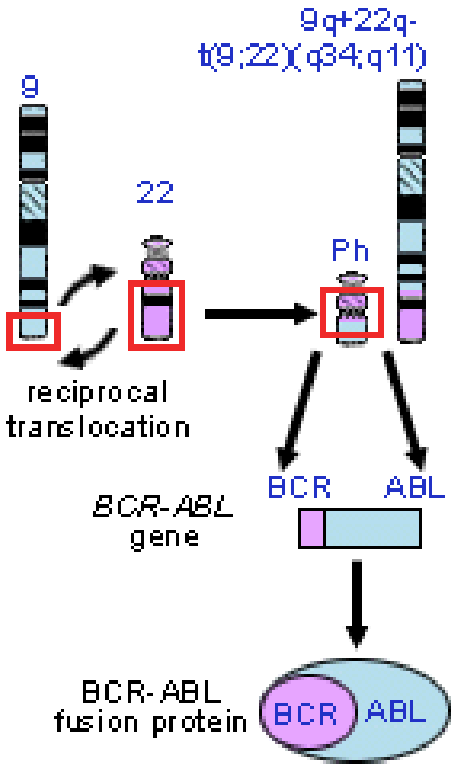
Several genetic models for diabetes exist in different strains of mice. These are relevant to **type II diabetes** because they are also associated with obesity and do not have an absolute insulin deficiency. The **OB/OB and the db/db mice** have been studied because they exhibit tissue resistance to the action of insulin and do not have severe insulin deficiency at the onset of diabetes.

In addition to the genetic forms of diabetes in rodents, the disease can be produced in a wide range of mammals by surgical removal of the pancreas, administration of drugs such as streptozotocin or alloxan, or overfeeding.

Mouse model of chronic myeloid leukemia – viral infection:

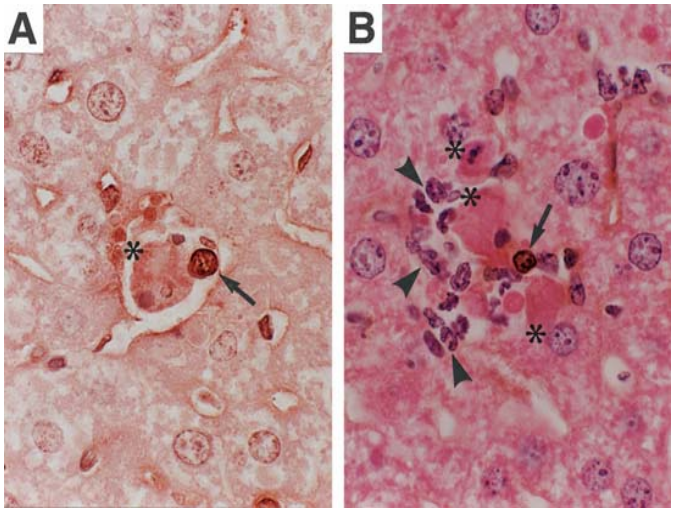
CML is usually diagnosed by finding a specific chromosomal abnormality called the **Philadelphia (Ph) chromosome**. The Ph chromosome is the result of a translocation—or exchange of genetic material—between the long arms of chromosomes 9 and 22 . This exchange brings together two genes: the *BCR* (breakpoint cluster region) gene on chromosome 22 and the proto-oncogene *ABL* (Ableson leukemia virus) on chromosome 9. The resulting hybrid gene *BCR-ABL* codes for a fusion protein with tyrosine kinase activity, which activates signal transduction pathways, leading to uncontrolled cell growth.

A mouse model has been created that develops a CML-like disease when given bone marrow cells infected with a virus containing the *BCR-ABL* gene. In other animal models, the fusion proteins have been shown to transform normal blood precursor cells to malignant cells.



Leukemic white blood cells in CML contain a Philadelphia (Ph) chromosome, the result of a translocation between the long arms of chromosomes 9 and 22. The resulting fusion gene (*BCR-ABL*) produces an altered protein believed to play a key role in the development of CML.

Mouse model of hepatocellular carcinoma – expression of large HBV protein:



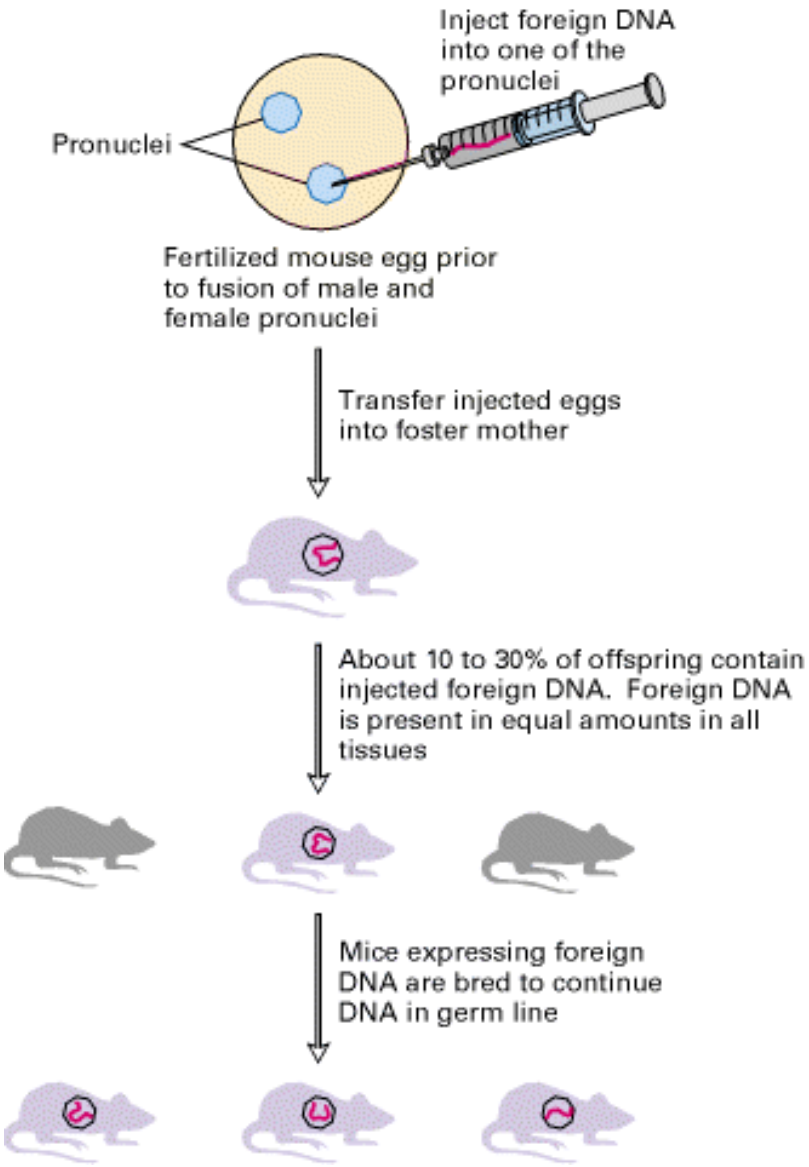
Transgenic mice that overproduce the hepatitis B virus large envelope polypeptide and accumulate toxic quantities of hepatitis B surface antigen (HBsAg) within the hepatocyte develop severe, prolonged hepatocellular injury that initiates a programmed response within the liver, characterized by inflammation, regenerative hyperplasia, transcriptional deregulation, and aneuploidy. This response inexorably progresses to neoplasia. The incidence of hepatocellular carcinoma in this model corresponds to the frequency, severity, and age of onset of liver cell injury, which itself corresponds to the intrahepatic concentration of HBsAg and is influenced by genetic background and sex. Thus, the inappropriate expression of a single structural viral gene is sufficient to cause malignant transformation in this model.

Chisari et al., 1989

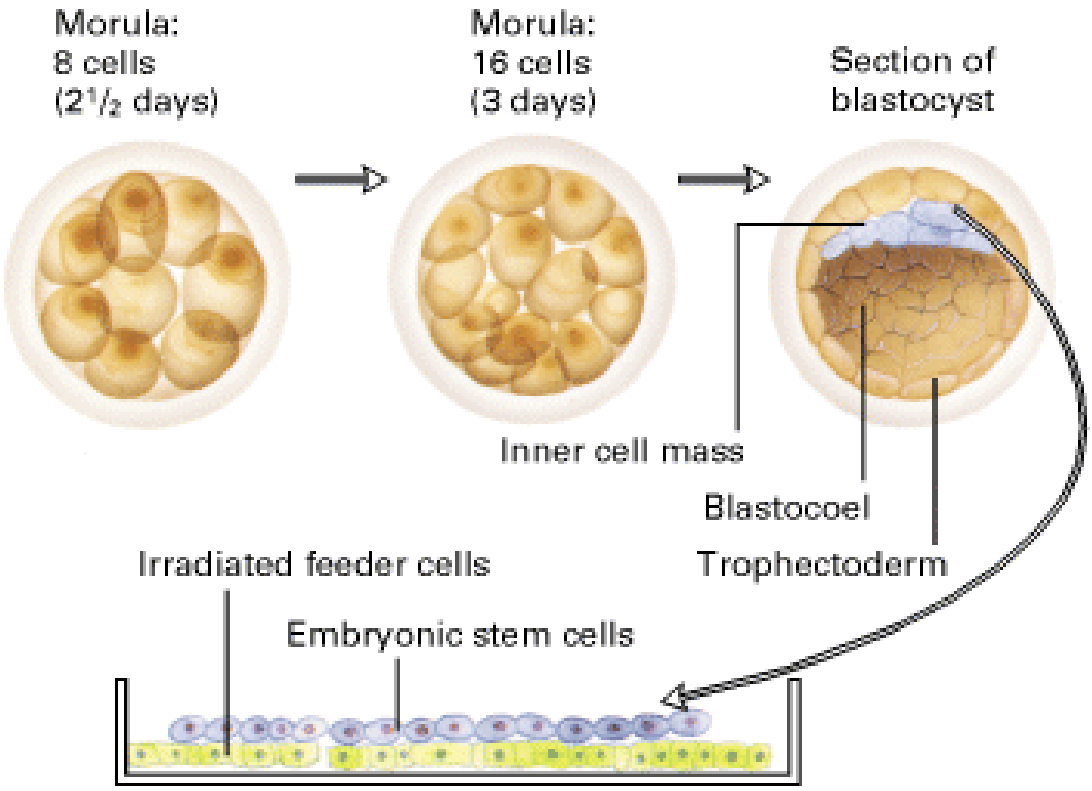
Specifická funkce proteinů:

- transgenní organismy – především myši;

General procedure for producing transgenic mice:



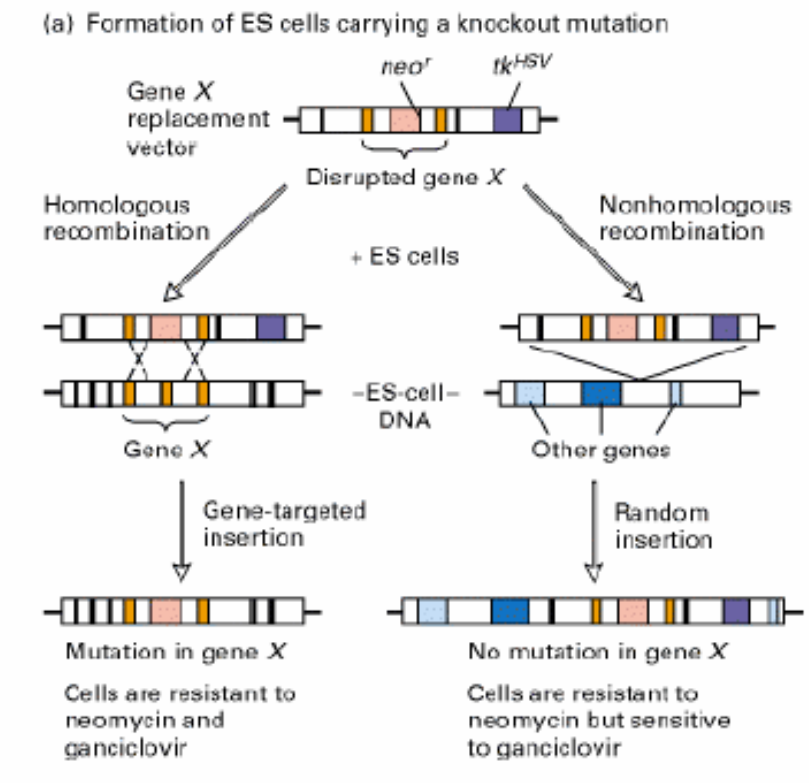
Využití embryonálních kmenových buněk:



Preparation of embryonic stem (ES) cells.

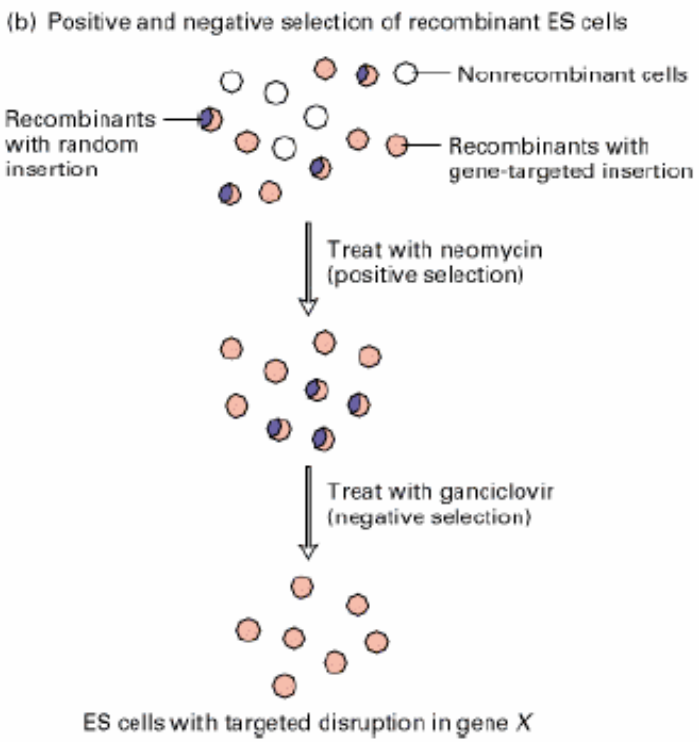
Fertilized mouse eggs divide slowly at first; after 41/2 days, they form the blastocyst, a hollow structure composed of about 100 cells surrounding an inner cavity called the *blastocoel*. Only ES cells, which constitute the inner cell mass, actually form the embryo. Other cells form the trophectoderm, which gives rise to the membranes (amnion and placenta) by which the embryo is attached to the uterine wall. Embryonic stem cells can be removed from the blastocyst and grown on lethally irradiated "feeder cells."

Isolation of mouse ES cells with a gene-targeted disruption by positive and negative selection.

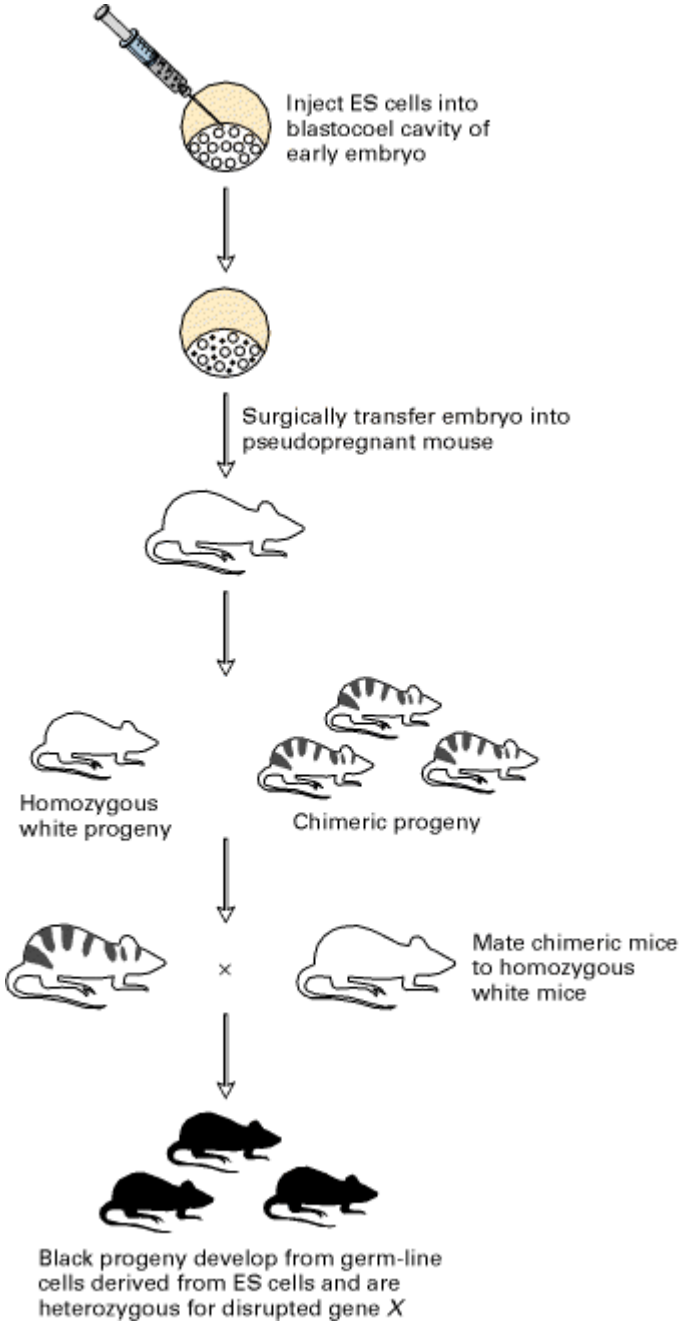


When exogenous DNA is introduced into ES cells, random insertion via nonhomologous recombination occurs much more frequently than gene-targeted insertion via homologous recombination. Recombinant cells in which one copy of the gene *X* (orange) is disrupted can be obtained by using a recombinant vector that carries gene *X* disrupted with *neo*^{r} (light red), a neomycin-resistance gene, and, outside the region of homology, *tk*HSV (purple), the thymidine kinase gene from herpes simplex virus. The viral thymidine kinase, unlike the endogenous mouse enzyme, can convert the nucleotide analog ganciclovir. Thus ganciclovir is cytotoxic for recombinant ES cells carrying the *tk*HSV gene. Nonhomologous insertion includes the *tk*HSV gene, whereas homologous insertion doesn't; therefore, only cells with nonhomologous insertion are sensitive to ganciclovir.

Isolation of mouse ES cells with a gene-targeted disruption by positive and negative selection.



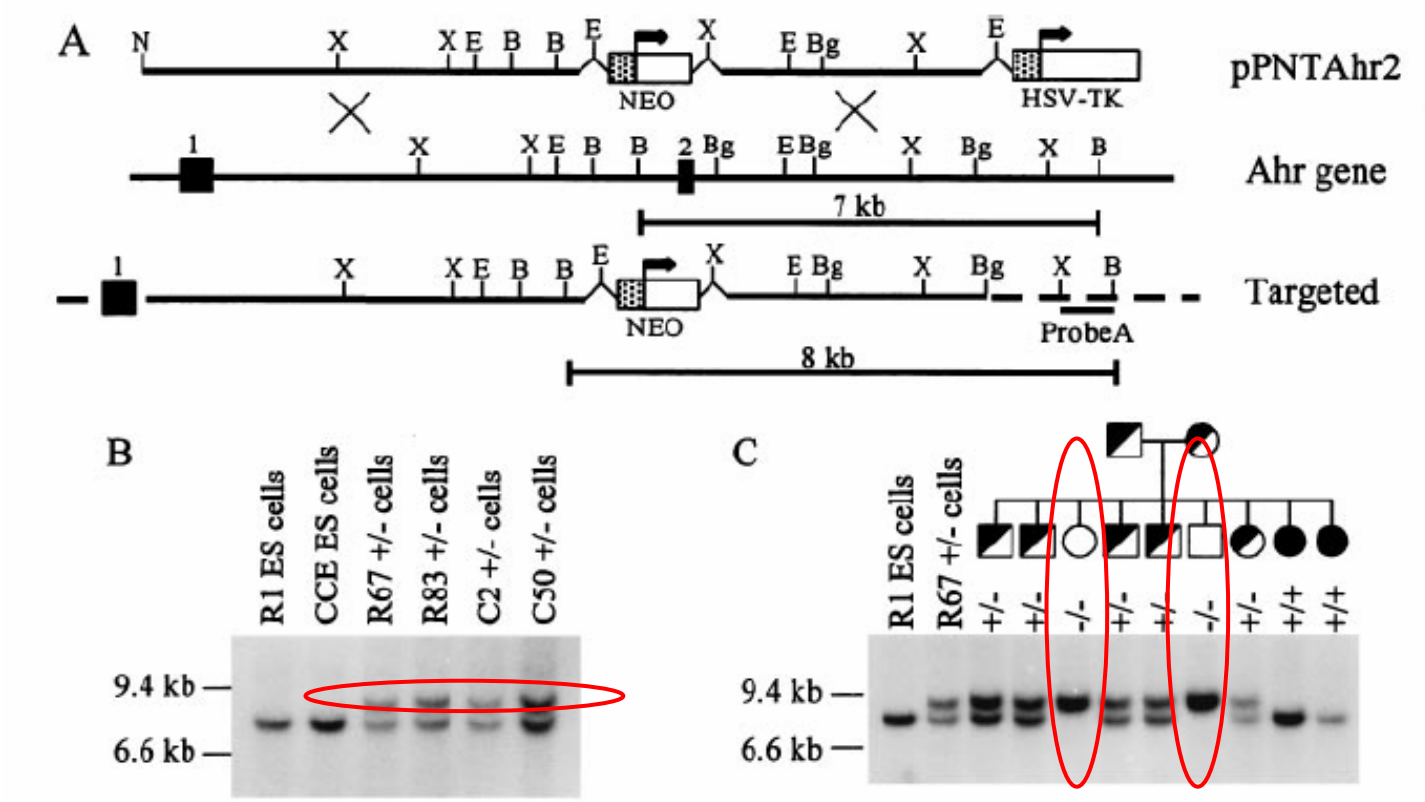
Recombinant cells are selected by treatment with neomycin, since cells that fail to pick up DNA or integrate it into their genome are neomycin-sensitive. The surviving recombinant cells are treated with ganciclovir. Only cells with a targeted disruption in gene *X*, and therefore lacking the *tk*HSV gene, will survive.



General procedure for producing gene-targeted knockout mice.

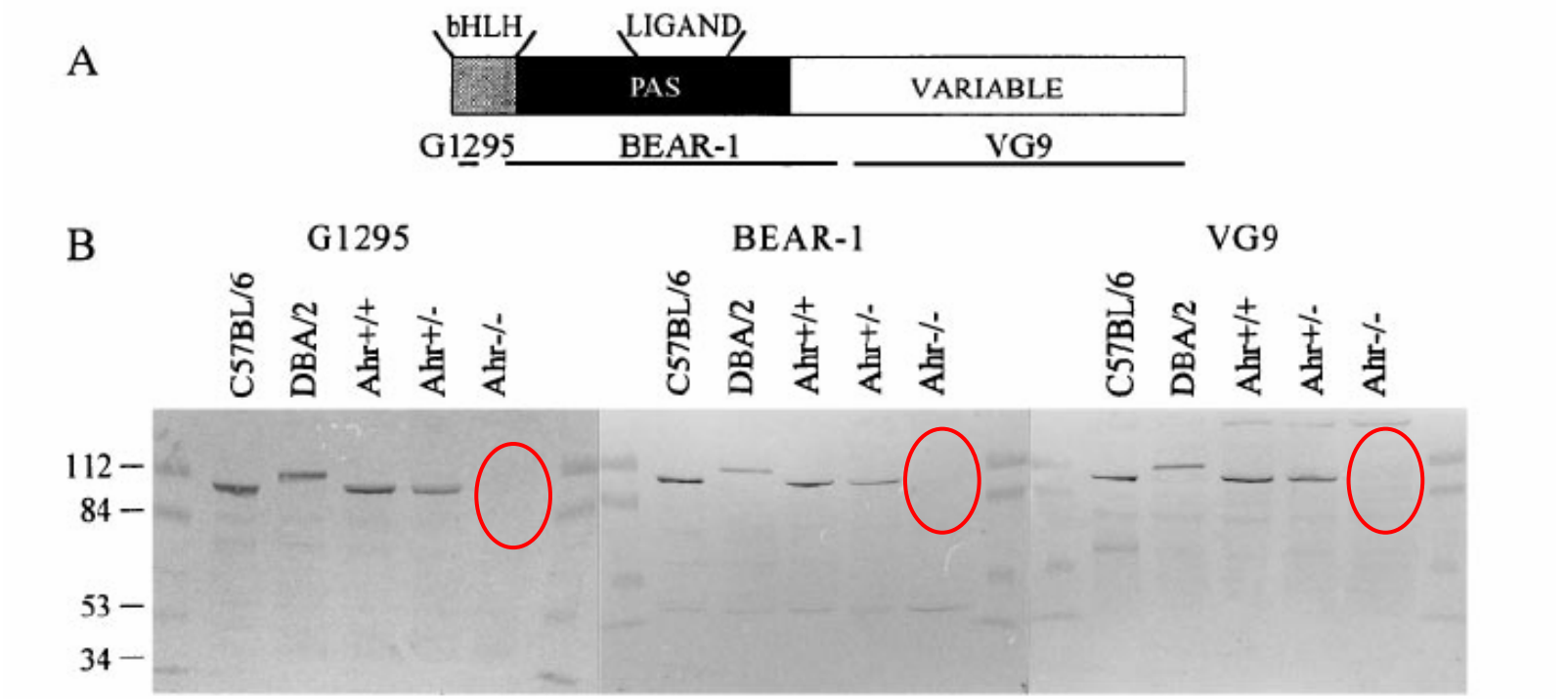
Embryonic stem (ES) cells heterozygous for a knockout mutation in a gene of interest *(X)* and homozygous for a marker gene (here, black coat color) are transplanted into the blastocoel cavity of 4.5-day embryos that are homozygous for an alternate marker (here, white coat color). The early embryos then are implanted into a pseudopregnant female. Some of the resulting progeny are chimeras, indicated by their black and white coats. Chimeric mice then are backcrossed to white mice; black progeny from this mating have ES-derived cells in their germ line. By isolating DNA from a small amount of tail tissue, it is possible to identify black mice heterozygous for the knockout allele. Intercrossing of these black mice produces individuals homozygous for the disrupted allele, that is, knockout mice.

Příprava AhR -/- myši (Schmidt et al., 1996):



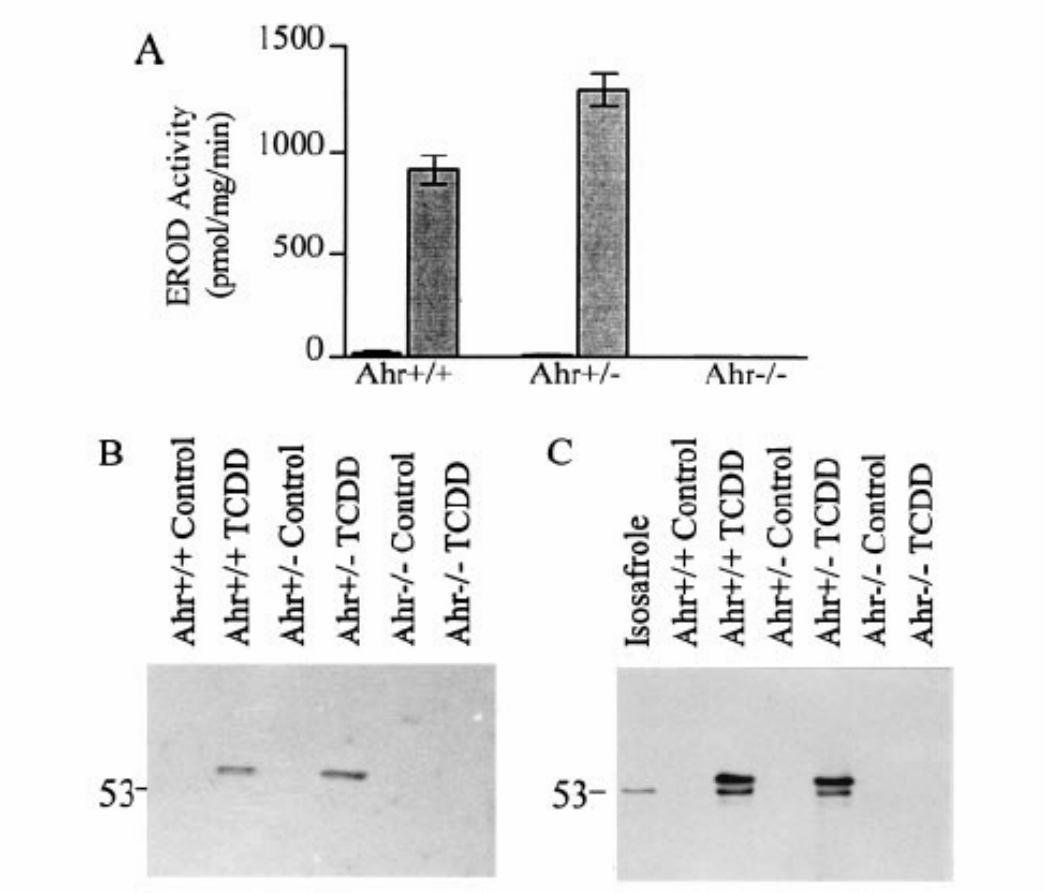
Targeted disruption of the *Ahr* gene. (*A*) Schematic of the *Ahr* targeting construct pPNTAhr2 aligned with the homologous region of the *Ahr* structural gene and the structure of the recombinant allele. Black boxes denote exons, and probe A used to detect the recombinant allele is indicated. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; N, *Not*I; X, *Xba*I. (*B*) Southern blot analysis of parent and targeted ES cell clones.

KO gen nesmí produkovat protein:



The targeted *Ahr* allele produces no AHR protein. (*A*) Schematic of the AHR protein and the regions recognized by the antibodies G1295, BEAR-1, and VG9. (*B*) Western blots of mouse liver cytosol with the three AHR antibodies.

Kontrola ztráty funkce:



Cytochrome P450IA1 and P450IA2 are not induced by TCDD in *Ahr* $^{-/-}$ mice. (*A*) EROD activity of mouse liver microsomal fractions after control or TCDD treatment. Error bars represent the high and low values obtained from two animals. (*B* and *C*) P450 Western blotting with specific antisera. *B* is with a monoclonal antibody specific for P450IA1 and *C* is with a polyclonal antibody recognizing both P450IA1 and P450IA2; the upper band represents P450IA1 and the lower P450IA2.

Studium fenotypu:

- přežívání myší – význam genu pro základní životní funkce a vývoj; embryonic lethal;

- projevy onemocnění;
- vývojové defekty;

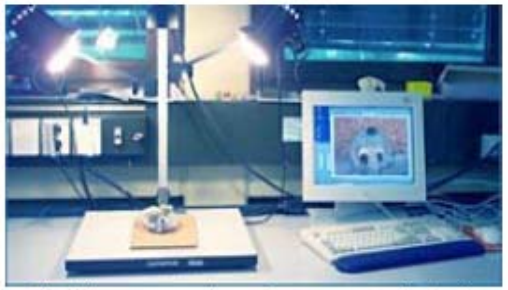
The GMC offers the examination of mouse mutants using a broad standardised phenotypic check-up. The screens in the German Mouse Clinic are designated to the areas of **behaviour, bone and cartilage development, neurology, clinical chemistry, eye development, immunology, allergy, steroid metabolism, energy metabolism, lung function, vision and pain perception, molecular phenotyping, cardiovascular and pathology.**

Př. patologie:

Primary Screen

Mice received in the laboratory of the pathology screen are sacrificed with CO 2. A complete macroscopic examination is performed, including measurement of body weight. S kin, cervical lymph nodes, thymus, spleen, trachea, lung, heart, salivary glands, esophagus, stomach, intestine, liver, pancreatic gland, kidney, urinary bladder, genital organs, brain, cerebellum, adrenal glands, thyroid gland, and parathyroid gland are fixed in 4 % buffered formalin and embedded in paraffin for histological examination, using hematoxylin-eosin stain (H&E).

On request, special stains, immunohistochemistry, X-ray analysis, electron microscopy, FISH, microdissection followed by RT-PCR or PCR analysis, fragmental analysis, Western Blot, and immunoprecipitation could be offered.

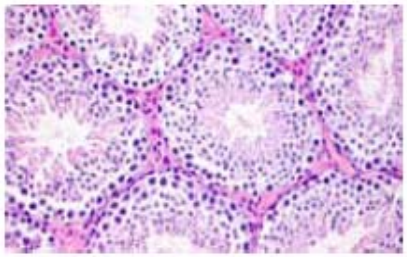


Digital documentation of macroscopic findings

Analysis of mice and rats

Complete dissection

Digital documentation



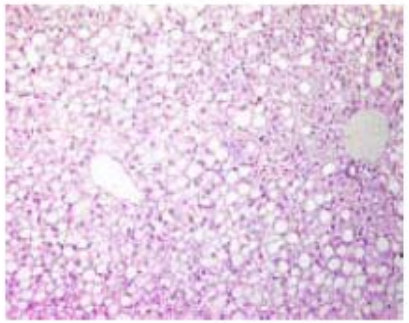
Testis, seminiferous tubules, H&E

Digital documentation of microscopic findings

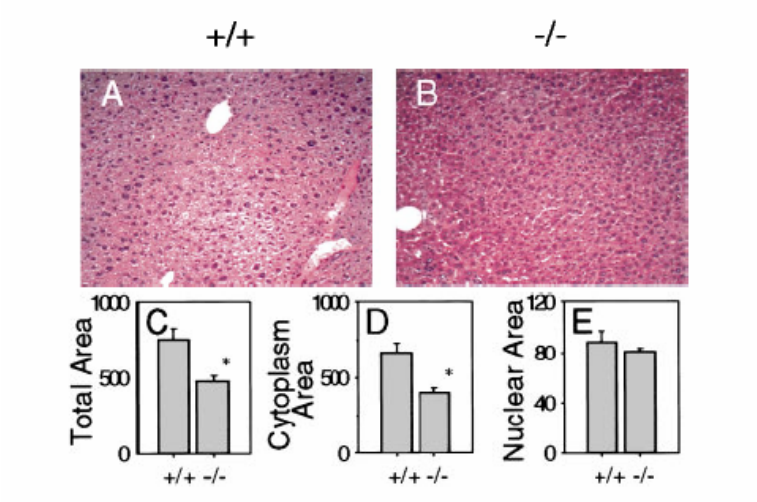
Microscopy:

Complete histological analysis (H&E stain) of all organs, including bone marrow

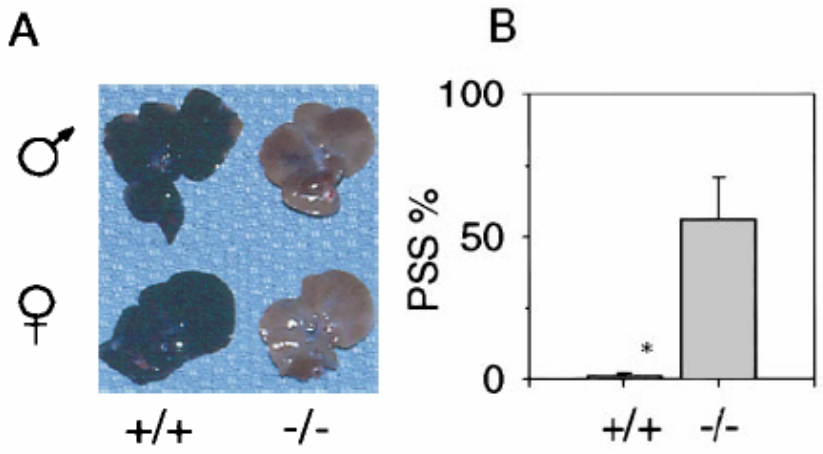
Digital documentation (microscopy)



Steatosis of the liver H&E



Ah-/- mice have smaller hepatocytes than wild-type mice. Livers of 1-year-old mice were fixed in formalin, and 6-mm sections were examined after staining with hematoxylinyeosin.



Livers of *Ah* -/- mice exhibit portosystemic shunting. With perfusion of colloidal carbon, livers of *Ah* +/+ mice become black (*Left*), whereas livers of *Ah* -/- mice remained pink (*Right*). The livers of male miceare shown above the livers of female mice.

Biochemical Pharmacology, Vol. 56, pp. 781–787, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00134-8

COMMENTARY

Ahr Null Alleles: Distinctive or Different?

*Garet P. Lahvis and Christopher A. Bradfield**
McArdle Laboratory for Cancer Research, University of Wisconsin Medical School, Madison, WI 53706-1599, U.S.A.

0090-9556/98/2612-1194–1198$02.00/0
Drug Metabolism and Disposition

Vol. 26, No. 12
Printed in U.S.A.

ANTHONY Y. H. LU COMMEMORATIVE ISSUE

THE ARYL HYDROCARBON RECEPTOR

Studies Using the AHR-Null Mice

FRANK J. GONZALEZ AND PEDRO FERNANDEZ-SALGUERO

Division of Basic Sciences, National Cancer Institute (F.J.G.), and Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Extremadura (P.F.-S.)

ARNT-/- - embrya nepřežívají ED 10.5

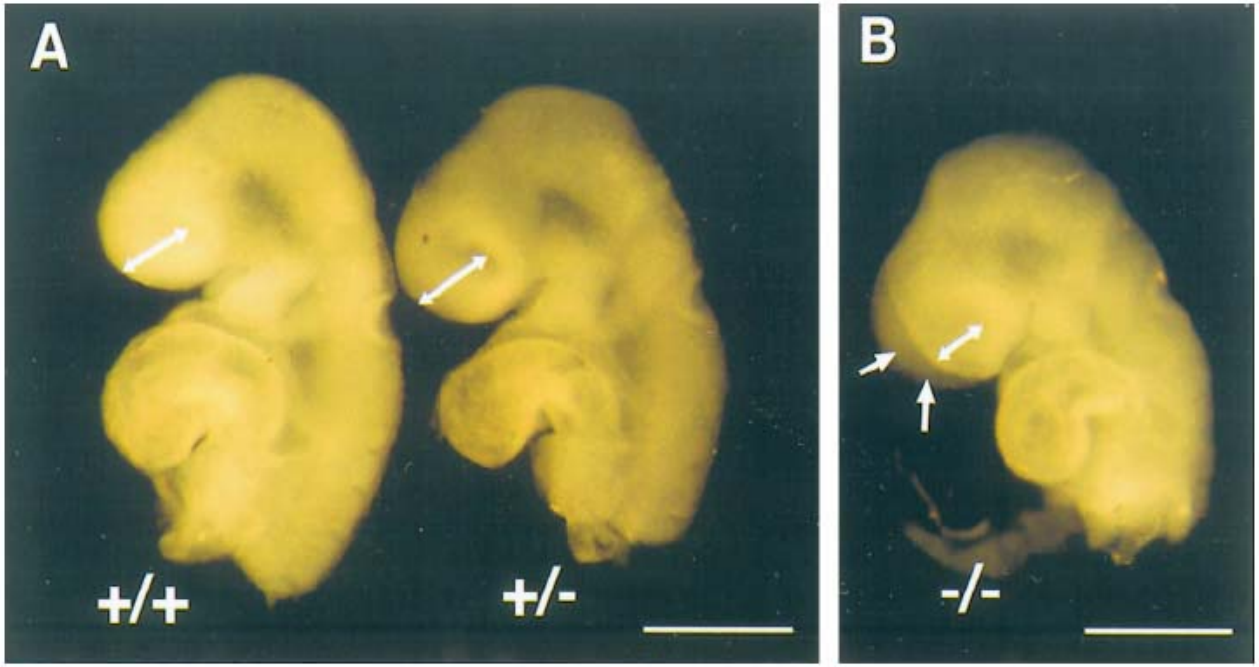
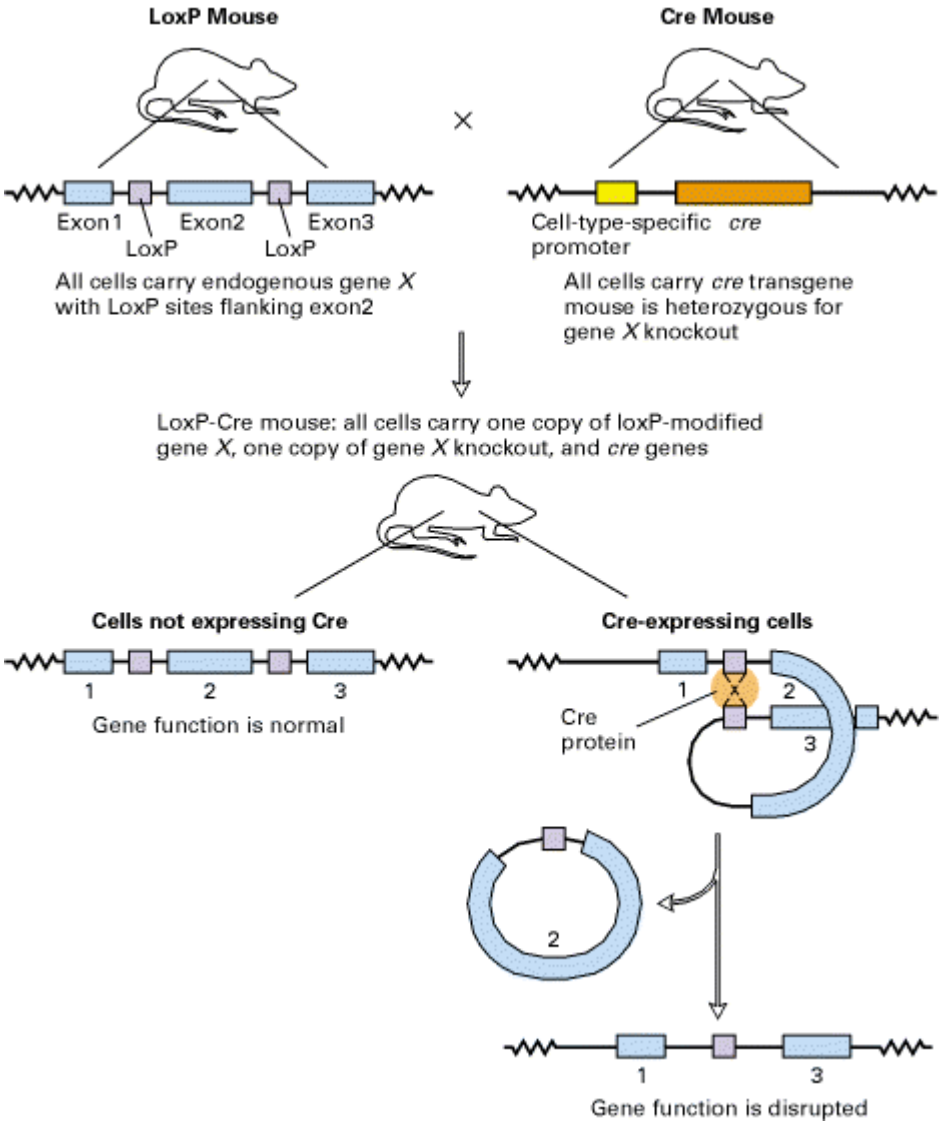
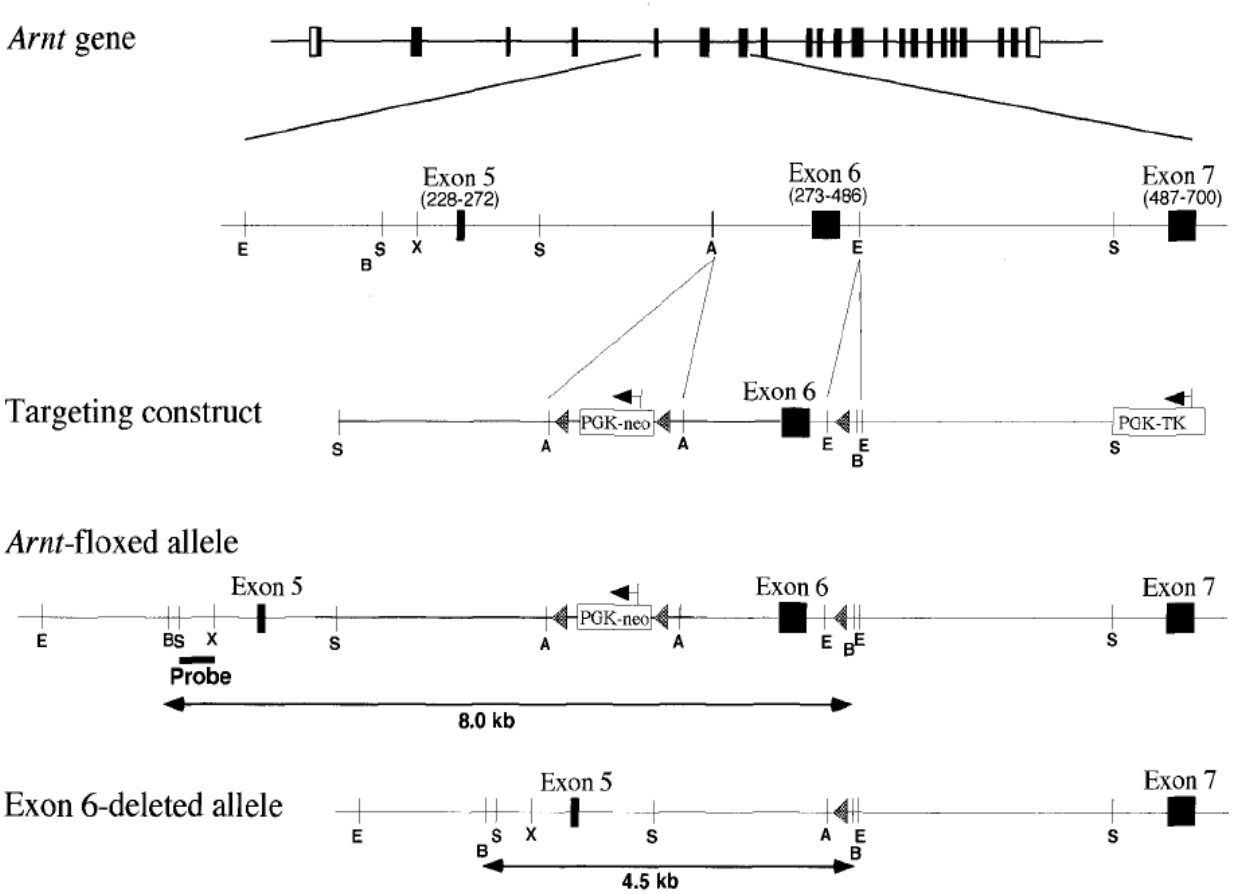


FIG. 4. (A,B) Embryonic phenotypic abnormalities. Embryonic development is similar between GD 9.5 +/+ and +/− embryos (A); however, −/− embryos exhibited neural tube defects ranging from forebrain hypoplasia to unfused head folds, or cleft face (B). The −/− embryo shown in B has unfused head folds (large arrows) and diminished distance from the anterior edge of the forebrain to posterior border of optic placode (double-headed arrows). (Bar, 100 μm).

Cell-type-specific gene knockouts using the loxP-Cre recombination system.



Two loxP sites are inserted on each side of an essential exon (2) of the gene of interest (i.e., gene X) (blue) by homologous recombination. These sites do not disrupt gene function. The loxP-containing mouse is crossed to a transgenic mouse carrying a cell-type-specific promoter controlling expression of the Cre recombinase, which induces recombination between loxP sites. This mouse is heterozygous for a constitutive gene *X* knockout. In the resulting loxP-Cre mouse, Cre protein is produced only in those cells in which the promoter is active, and in those cells recombination therefore occurs between the loxP sites, leading to deletion of exon 2. Since the other allele is a constitutive gene *X* knockout, deletion between the IoxP sites results in complete loss of function in all cells expressing Cre.



Targeted Modification of the *Arnt* Gene. The *Arnt* gene, targeting construct, targeted allele, and schema of Cre-mediated deletion of the *Arnt* gene are shown. *Hatched triangles* represent loxP sites, and the *small arrow* above the PGK-neo cassette shows the direction of transcription that is opposite the direction of transcription of the ARNT gene. The *Bam*HI restriction fragments monitored and detected with the 200-bp probe located in intron 4 are denoted by *double end arrows*. The restriction sites are abbreviated as follows: A, *Avr*II; B,*Bam*HI; E, *Eco*RI; S, *Sac*I; X, *Xho*I. (Tomita et al., 2000).

Conditional liver-specific ARNT knockout:

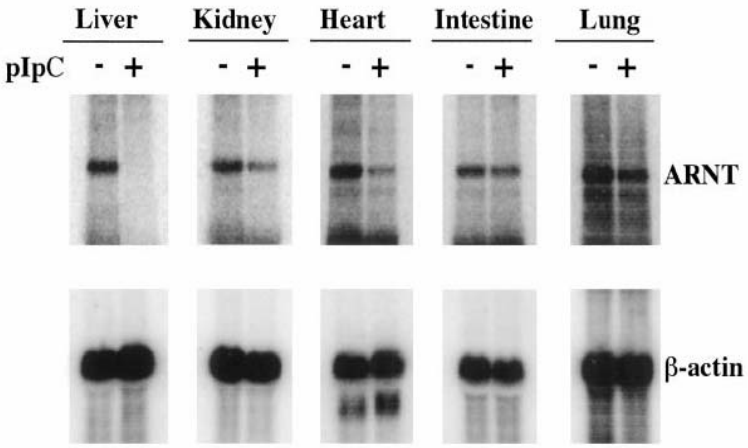


Fig. 4. Expression of ARNT mRNA in Different Tissues of *Arnt-Floxed*/Mx1-Cre Mice

Total RNA (10 μg) was prepared from the indicated tissues of mice treated with pIpC or saline and was subjected to Northern blot analysis. A ^{32}P-labeled cDNA probe was used to detect ARNT mRNA. β-Actin was used as a loading control.

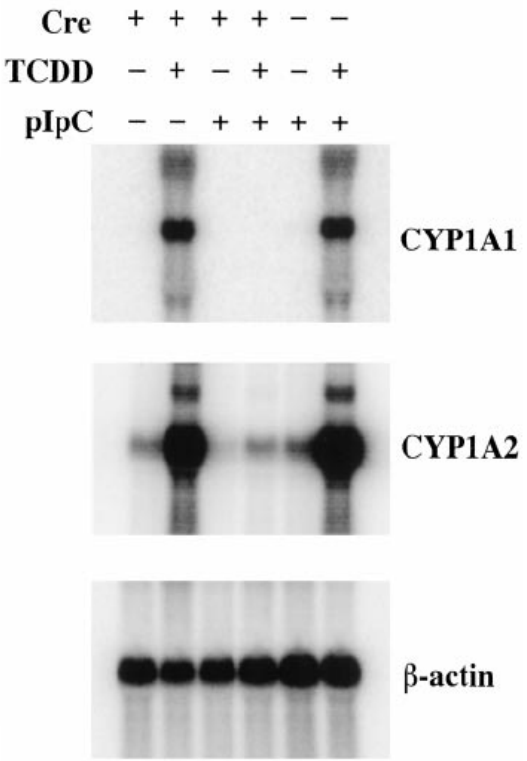


Fig. 5. Expression of Hepatic CYP1A1 and CYP1A2 mRNA in *Arnt-Floxed*/Mx1-Cre Mice

The mice were injected with pIpC or saline followed by administration of TCDD or corn oil. *Arnt-floxed* mice lacking the Cre transgene were used as a control for the effects of pIpC alone on basal and induced expression. Total liver RNA (10 μg) was subjected to Northern blot analysis using ^{32}P-labeled cDNA probes against mouse CYP1A1 and CYP1A2 mRNAs. β-Actin was used as a mRNA loading control.

Konstitutivně aktivní protein – CAAhR:

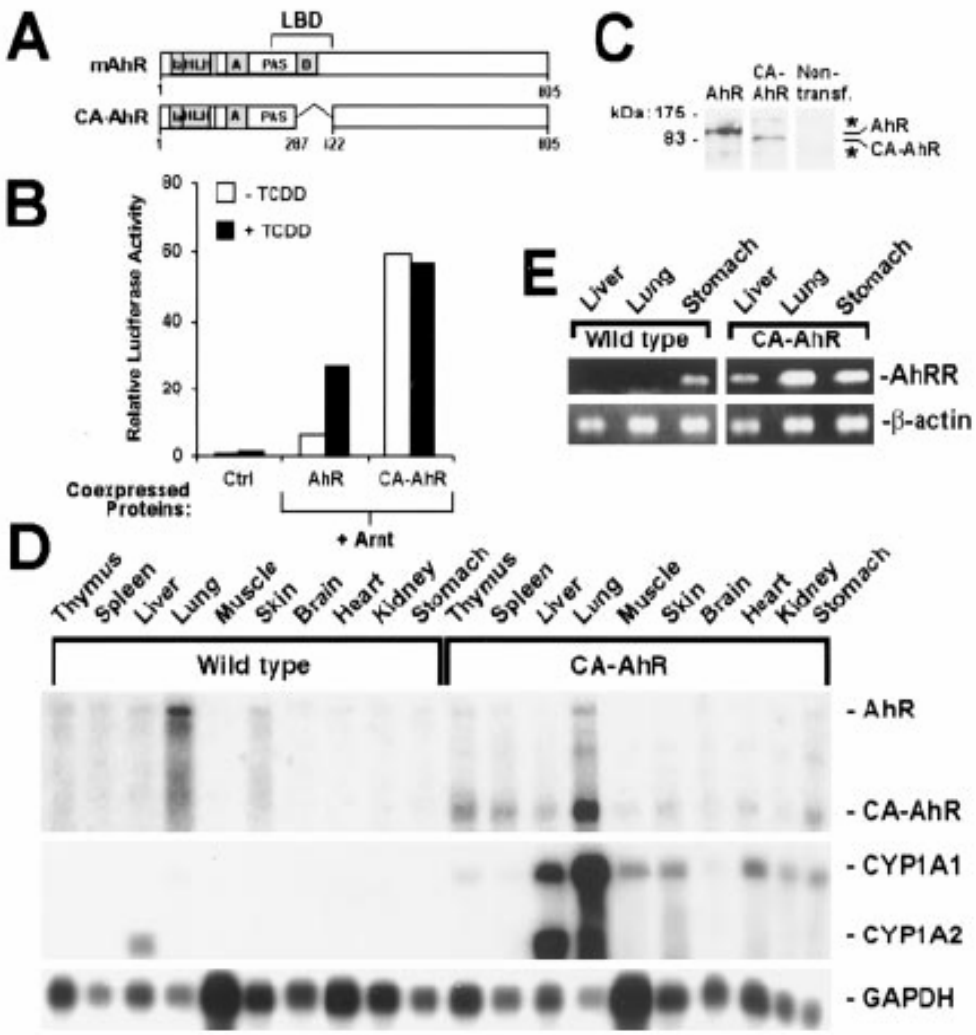


Fig. 1. Constitutive activity of CA-AhR. (*A*) Schematic representation of the wild-type mouse AhR (mAhR) and of CA-AhR. bHLH, basic helix–loop–helix; PAS, Per-Arnt-Sim. (*B*) Functional activity of CA-AhR in Chinese hamster ovary cells. Cells were transiently transfected with an AhR-dependent luciferase reporter gene and expression vectors encoding AhR nuclear translocator (Arnt), wild-type AhR, or CA-AhR. The control lanes (Ctrl) represent activity from the reporter gene alone and empty expression vector. Data are from one experiment performed in duplicate and are representative of at least three independent experiments. (*C*) Detection of the AhR and CA-AhR proteins expressed after transient transfection of Chinese hamster ovary cells. Whole-cell extracts were analyzed by immunoblotting using anti-AhR antibodies. The star indicates nonspecific immunoreactivity. (*D*) Expression and functional activity of CA-AhR in 8-month-old female mice: RNA blot analysis showing expression of the endogenous AhR, CA-AhR, and the target genes *CYP1A1* and *CYP1A2*. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as RNA-loading control of corresponding tissues. (*E*) AhR repressor (AhRR) and β-actin mRNA expression was assessed by reverse transcription–PCR.

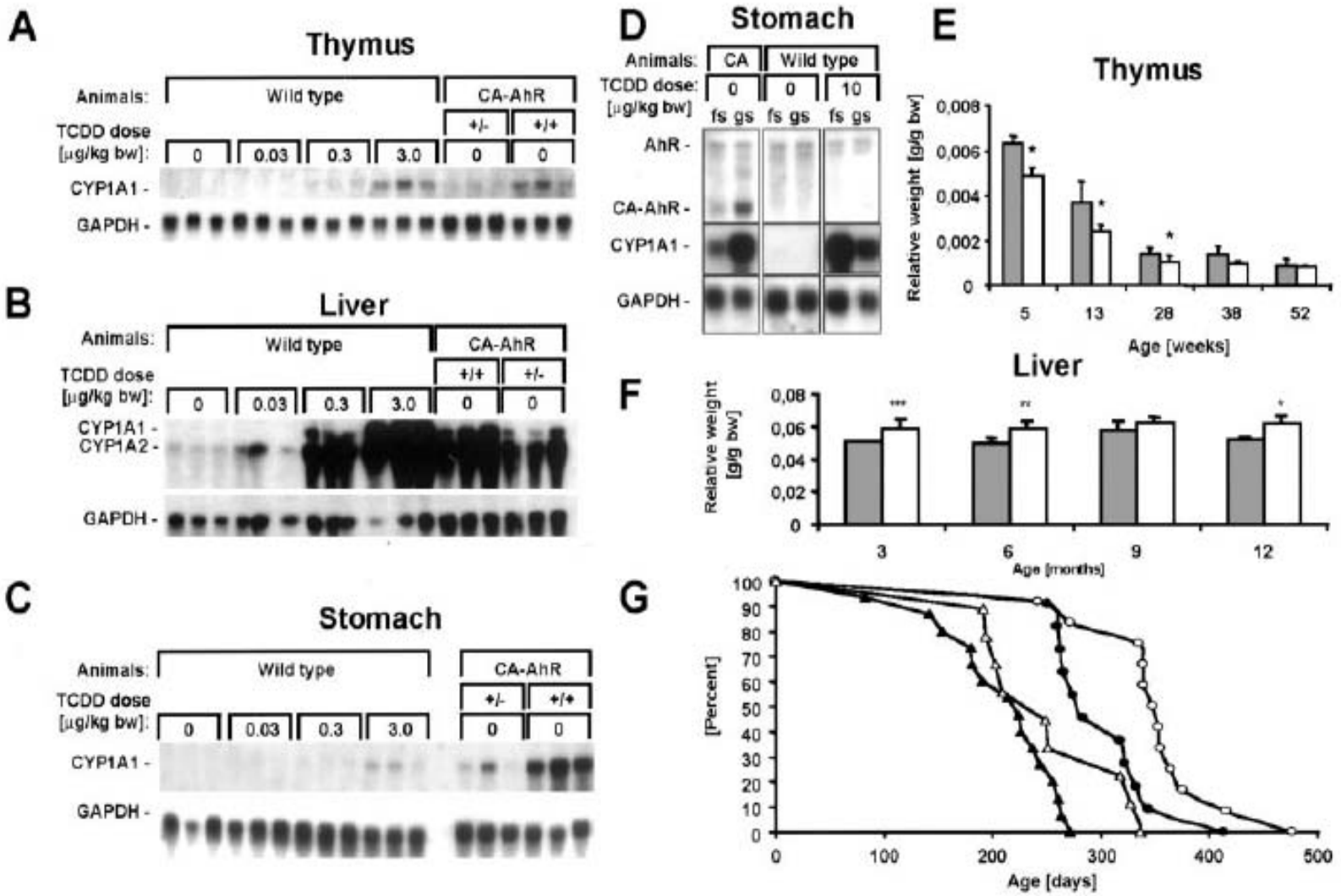
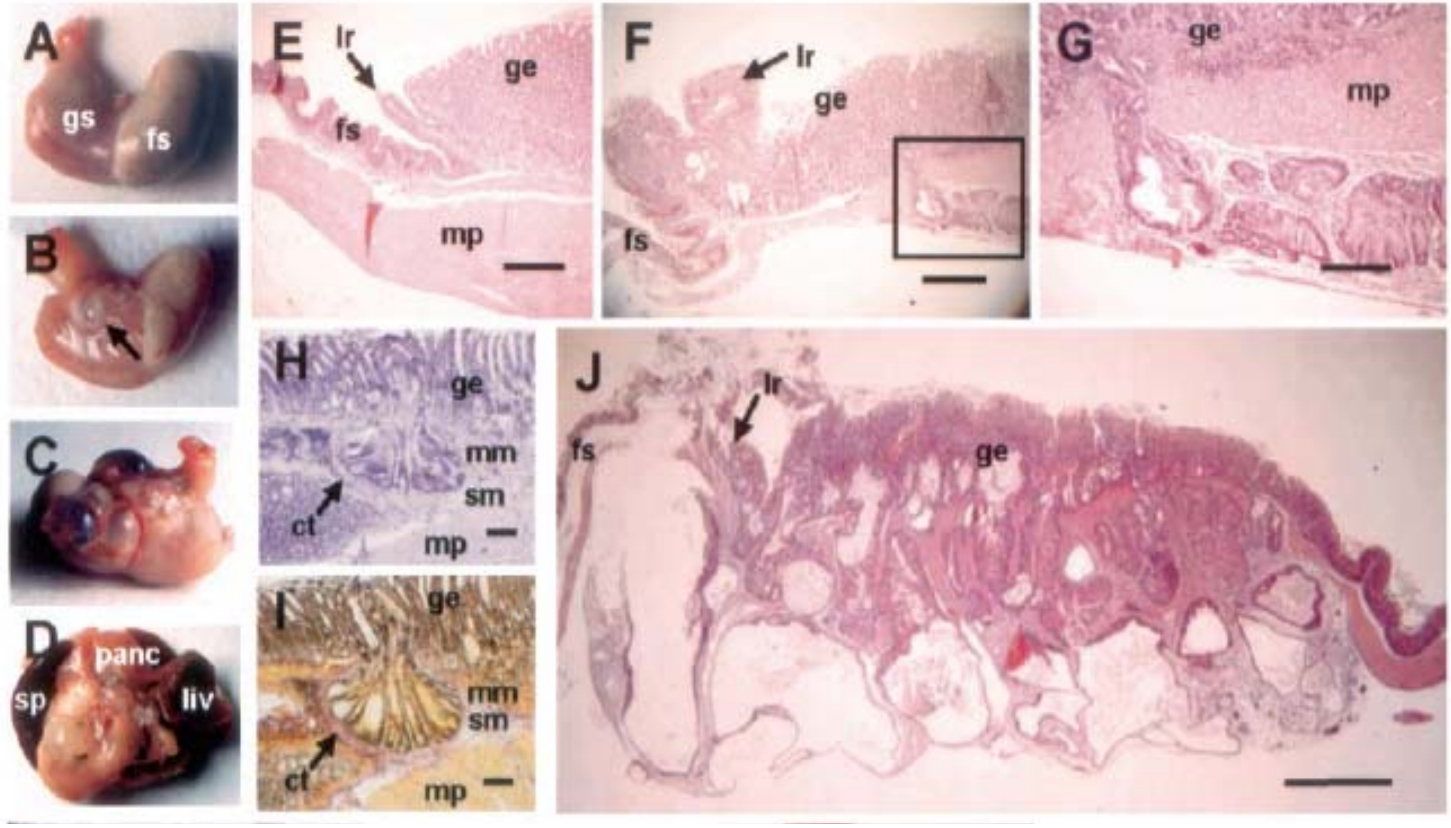


Fig. 2. Functional activity of CA-AhR. (*A–C*) RNA blots showing expression of CYP1A1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in thymus, liver, and stomach of 6-month-old female wild-type, heterozygous, and homozygous CA-AhR mice treated orally 3 days earlier with vehicle or TCDD as indicated. bw, body weight. (*D*) RNA blot showing expression of AhR, CA-AhR, CYP1A1, and glyceraldehyde-3-phosphate dehydrogenase in forestomach (fs) or glandular stomach (gs) from 3-month-old CA-AhR (CA) or wild-type male mice treated orally with vehicle or TCDD 1 day earlier. (*E* and *F*) Alteration of the relative weights of thymus and liver in homozygous CA-AhR animals. The closed bars represent wild-type animals, and open bars represent CA-AhR animals. Thymuses from at least four female animals of each genotype and age and livers from at least five males of each genotype and age were examined. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.005$ as assessed by two-tailed Student's *t* test. (*G*) Ages of the homozygous CA-AhR mice found dead stratified by sex (closed symbols for males, open for females) and independent founder lines (triangles for line A3 and circles for line Y8).

Fenotyp CA-AhR mouse – nádory žaludku:



Neoplastic lesions and intestinal metaplasia in the stomach of CA-AhR mice. (*A*) Normal stomach from a 12-month-old wild-type male. (*B*) At 3–4 months of age single small cysts. (*C*) In older CA-AhR animals (6–12 months old) the number of cystic tumors increased and occupied a larger area of the stomach. (*D*) In the most severe cases (9–12 months of age), the stomach was adherent to adjacent organs such as spleen (sp), pancreas (panc), and liver (liv).

Porovnání různých přístupů:

Huntington's disease is a dominantly inherited disorder characterized by a progressive neurodegeneration of the striatum that also involves other regions, primarily the cerebral cortex. Single gene mutated – huntigtin (*HD*).

Three types of mouse models have been developed:
knockout, transgenic, and knock-in models.

1) Nullizygous animals die during embryonic development, they have demonstrated that huntingtin plays a crucial role in embryogenesis.

2) The neuronal degeneration and behavioral phenotype detected in conditional knockout, in which the huntingtin gene was inactivated in brain and testis at early stages, indicate that huntingtin is required for neuronal function and survival.

3) Transgenic models are those in which the human mutant *HD* gene, or a fragment of it, is inserted randomly into the mouse genome. In this case, the mouse will express a full-length or a fragment of the mutant gene in addition to the two normal copies of the endogenous mouse huntingtin (*Hdh*) gene.

4) Knock-in mouse models have the mutation inserted into the mouse huntingtin gene and can be homozygous or heterozygous for the mutation. Because knock-in mice carry the mutation in its appropriate genomic and protein context, they are the most faithful genetic models of the human condition.