

# Coxsackievirus-adenovirus receptor (CAR) is essential for early embryonic cardiac development

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## Summary

The coxsackievirus-adenovirus receptor (CAR) is a cell contact protein on various cell types with unknown physiological function. It belongs to a subfamily of the immunoglobulin-superfamily of which some members are junctional adhesion molecules on epithelial and/or endothelial cells. CAR is dominantly expressed in the hearts and brains of mice until the newborn phase after which it becomes mainly restricted to various epithelial cells. To understand more about the physiological function of CAR, we have generated CAR-deficient mice by gene targeting. We found that these mice die between E11.5 and E13.5 of embryonal development. Ultrastructural analysis of cardiomyocytes revealed that the density of myofibrils was reduced and that their orientation and bundling was disorganized. In addition, mitochondria were enlarged and glycogen storage strongly enriched. In line with these

defects, we observed pericardial edema formation as a clear sign of insufficient heart function. Developmental abnormalities likely to be secondary effects of gene ablation were the persistent singular cardiac atrio-ventricular canal and dilatations of larger blood vessels such as the cardinal veins. The secondary nature of these defects was supported by the fact that CAR was not expressed on vascular cells or on cells of the vascular wall. No obvious signs for alterations of the histological organization of the placenta were observed. We conclude that CAR is required for embryonal heart development, most likely due to its function during the organization of myofibrils in cardiomyocytes.

Key words: Cardiomyocytes, Junctions, Heart development, Junctional adhesion molecules, Cell adhesion

## Introduction

The coxsackievirus-adenovirus-receptor (CAR) was originally found as a cell surface protein enabling both viruses to interact with cells (Bergelson et al., 1997; Tomko et al., 1997). Besides this pathological role as a virus receptor, the physiological function of CAR is largely unknown. CAR is a type I transmembrane protein with two extracellular Ig-domains that can support homotypic cell adhesion of transfected cells (Cohen et al., 2001; Honda et al., 2000). It belongs to the CTX-subfamily of the Ig-superfamily, consisting of proteins containing one V and one C2 Ig-domain (Du Pasquier et al., 1999). Some of the members of this subfamily are found at endothelial and/or epithelial tight junctions, such as the junctional adhesion molecules (JAM-A, -B and -C) (Aurrand-Lions et al., 2000; Ebnet et al., 2004; Martin-Padura et al., 1998) and endothelial-selective cell adhesion molecule (ESAM) (Hirata et al., 2001; Nasdala et al., 2002). The latter is selectively expressed on endothelium and platelets, and is structurally most closely related to CAR.

The tissue distribution of CAR is complex and developmentally regulated (Philipson and Pettersson, 2004). Most studies analysing its tissue distribution are based on

mRNA expression data and only a few have directly analyzed the expression of the protein. Strong expression of the CAR protein was found in the central and peripheral nervous system of the mouse embryo as well as in various epithelia and in the heart (Tomko et al., 2000). In adult rodents, CAR expression in the brain was low or absent, and restricted to the ependymal cell layer lining the ventricular system. CAR was abundantly expressed in the epithelia of trachea, bronchi, kidney, liver and intestine. The subcellular localization of CAR on tracheal epithelial cells was confined to epithelial junctions. Immunogold labeling showed co-localization with ZO-1, suggesting its association with tight junctions (Cohen et al., 2001). Others suggested that CAR is located at the lateral surface of epithelia beneath tight junctions and that laterally released adenovirus binds to CAR thereby facilitating dissociation of epithelial cell layers and release of the virus toward the apical surface of the epithelial cell sheath (Walters et al., 2002). Expression of CAR on endothelial cells was described in some reports (Carson et al., 1999; Nasuno et al., 2004; Vincent et al., 2004), yet others found no expression in endothelia (Fechner et al., 2003; Noutsias et al., 2001). These discrepancies suggest that the expression of CAR on these cells

is regulated by inflammatory mediators. Indeed, Vincent et al. showed recently that TNF- $\alpha$  can downregulate CAR expression in endothelial cell cultures (Vincent et al., 2004).

Regulation of CAR expression has also been described for cardiomyocytes. Whereas CAR is very strongly expressed in cardiomyocytes of the embryonal and newborn heart, it is strongly reduced in the adult heart of rats (Kashimura et al., 2004) as well as humans (Fechner et al., 2003). Interestingly, CAR is strongly induced in cardiomyocytes of the adult rat heart by experimental autoimmune myocarditis (Ito et al., 2000). In addition, myocardial infarction in the rat led to a strong upregulation of CAR expression in cardiomyocytes of the infarct zone (Fechner et al., 2003). In human hearts, expression of CAR on cardiomyocytes was clearly found to be upregulated in several cases of dilated cardiomyopathy, whereas no expression was found in healthy tissue (Noutsias et al., 2001). The weak or absent expression of CAR on cardiomyocytes of the adult and healthy heart in contrast to the strong expression of CAR on cardiomyocytes of the developing and diseased heart may suggest a role of CAR during the formation of functional myocardium.

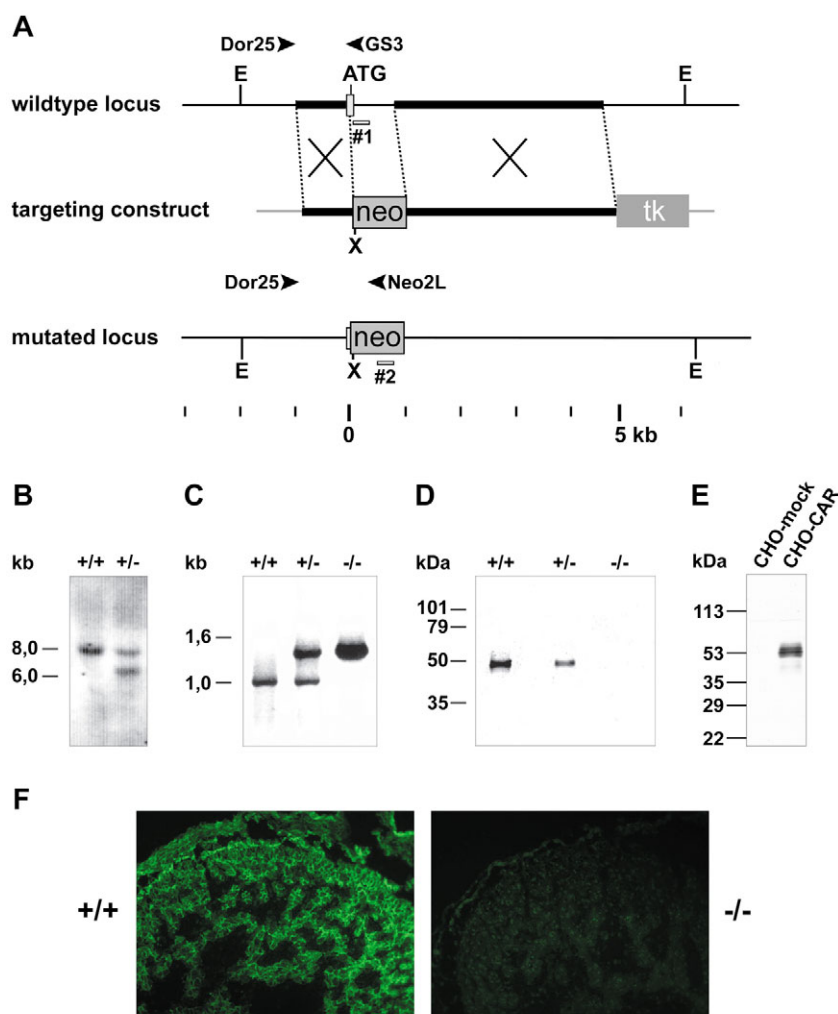
To understand the physiological role of CAR, we have generated and analyzed mice carrying an inactivating deletion in the *CAR* gene. We found that these mice start to die at E11.5 in utero. Whereas no obvious placental phenotype was observed, cardiac development was clearly abnormal and delayed. The ultrastructural analysis of cardiomyocytes indicated that the normal formation of densely and regularly packed myofibrils was disturbed. Our results suggest an important role of CAR in the development of the heart.

## Materials and Methods

### Generation of CAR-deficient mice

A genomic library (BAC-4925) derived from a mouse 129/SvJ II ES cell line was PCR-screened with the primers 5'-GGTTTGAGCATCAC-TACACCG-3' and 5'-TTCAATGTCCAGTGGT-CCCTGG-3' for the presence of exon 2 of the murine *CAR* gene. The screen was performed in collaboration with Incyte Genomics. To construct the targeting vector, a 3.7 kb *NheI* fragment located 3' of the first exon (3' arm) was cloned into the targeting vector pTV0 (kindly provided by Carmen Birchmeier, Max-Delbrück-Center for Molecular Medicine, Berlin, Germany). The 5' arm consisting of a 0.8 kb *PstI/Eco52I* fragment located 5' of the start codon was subcloned into pBluescript II KS+ (Stratagene) and then combined with the first construct to give the final targeting construct (Fig. 1A).

Electroporation, selection and blastocyst injection of E14.1 ES cells (Kuhn et al., 1991) were performed in collaboration with Transgenics/Atugen (Berlin, Germany) essentially as described



**Fig. 1.** Targeted disruption of the *CAR* gene in mice. (A) Targeting strategy. A map of the relevant genomic region containing the first ATG-containing exon of *CAR* (top), the targeting vector (middle) and the mutated locus after recombination (bottom) are shown. Dor25, GS3 and Neo2L represent oligonucleotides used for PCR screening. The positions of probes used for Southern screening are depicted as bars #1 and #2. E=EcoRV, X=XhoI. (B) Detection of wild-type and targeted alleles by Southern blot analysis. Genomic DNA of mice of the F1-generation was digested with *EcoRV* and *XhoI* and hybridized with the combination of probes #1 and #2. DNA of wild type (+/+) mice gave an 8 kb signal (*EcoRV* fragment), +/- heterozygotes gave an additional signal for the 6.4 kb *EcoRV/XhoI* fragment. (C) PCR analysis of genomic DNA with the oligonucleotides Dor25, GS3 and Neo2L described in A. A 1kb product is generated from the wild-type allele and a 1.5 kb product from the targeted allele. (D) Western blot of SDS-PAGE extracts of mouse embryos (E11.5) of the three different genotypes with antibodies against mouse CAR. Note that heterozygotes expressed less CAR protein than homozygous wild-type embryos. (E) Activity and specificity of affinity purified rabbit anti-mouse CAR antibodies illustrated by western blot analysis of mock transfected CHO cells and CAR-transfected CHO cells (as indicated). Molecular mass markers are indicated on the left. (F) Specificity control for affinity purified anti-CAR antibodies (VE15) used for indirect fluorescence staining of the heart region of E11.5 *CAR*<sup>+/+</sup> and *CAR*<sup>-/-</sup> embryos (as indicated).

(Hogan et al., 1994; Joyner, 1993). For Southern blotting, the genomic DNA was *EcoRV/XhoI* restricted and hybridized with two probes specific for the wild type and the targeted allele, respectively (Fig. 1A). Chimeric progeny were identified by coat color, and chimeric males were bred to C57BL/6 females. DNA isolated from yolk sacs of embryos and tail biopsies were used for genotyping animals.

Transmission of the targeted *CAR* locus was confirmed by Southern blotting. Subsequent genotyping was done by genomic PCR using the primers Neo2L (5'-GGCATCAGAGCAGCCGATTG-3', targeted allele), Dor25 (5'-CACTTCTAAATAACTTGCCCAAGACC-3', targeted and wild-type allele), and GS3 (5'-ATCCCGCACAGAGCACGAAG-3', wild-type allele). All analyzed mice had been backcrossed for six generations into the C57Bl/6 background. Embryonal lethality was always exclusively associated with the homozygous disruption of the *CAR* gene.

#### Expression vectors and transfections

For the generation of a mCAR eukaryotic expression vector full-length mouse *CAR* (Gen Bank<sup>TM</sup> accession number: Y10320) was amplified with the Kozak sequence and *Hind*III site-containing sense oligonucleotide 5'-GCGAAGCTTCCGCCATGGCGCGCCTACTGTGCTTC-3' and the *Eco*RI site-containing antisense oligonucleotide 5'-GCGGAATTCCTATACTATAGACCCGTCCTTGCT-3' using Platinum Pfx Polymerase (Life Technologies) and a template cDNA originating from total RNA of mouse heart. The PCR product was ligated into the pcDNA3 vector after digestion with *Hind*III and *Eco*RI (pcDNA3-CAR).

For construction of a mCAR-Fc fusion protein (for transfection of CHO cells), a cDNA fragment coding for the extracellular part of mouse *CAR* covering amino acid residues 1-235 (bp 1-705) was amplified from pcDNA3-CAR using the Kozak sequence and *Hind*III site-containing sense oligonucleotide 5'-GCGAAGCTTCCGCCATGGCGCGCTCTGTGCTTC-3', the *Eco*RI-site containing antisense oligonucleotide 5'-GGAATTCACCTACCTCGGTTGGAGGGTGGGCAAC-3' and Platinum Pfx Polymerase (Life Technologies). After restriction digestion with *Hind*III and *Eco*RI, the PCR product was inserted in *Hind*III/*Eco*RI cut pcDNA3 in frame and upstream of a fragment of human IgG<sub>1</sub> (Gen Bank<sup>TM</sup> accession number: J00228) covering bases 553-1803 (hinge, C<sub>H</sub>2, C<sub>H</sub>3) yielding pcDNA3-CAR-Fc. The cloned constructs were sequenced to confirm integrity. CHO dhfr<sup>-</sup> cells were stably transfected with pcDNA3-CAR plasmid or pcDNA3-CAR-Fc as described (Nasdala et al., 2002). In another approach, a bacterially expressed GST-mCAR fusion protein was generated using the oligonucleotide primers Q1: 5'-CACCGGATCCTTGAGCATCACTACACCCG-3' and GEX1. 5'-GGCTGCGCCGCGGGTGGGACAACGTC-3' and the GST expression vector pGEX-6P-1.

#### Culture of dispersed myocytes from single embryonic hearts

Embryos were collected on embryonic day (E)11.5, and the yolk sac of each embryo was harvested for genotyping by PCR analysis. Embryonic hearts were isolated and the common ventricular chamber was used for the isolation of primary embryonic cardiomyocytes, which was done essentially as described (Kubalak et al., 1995). Cells from the enzymatic digestion were placed in Dulbecco's modified Eagle's medium (with high glucose) supplemented with 15% fetal calf serum, 2 mM L-glutamine, and penicillin/streptomycin, plated on fibronectin-coated LabTec chamber slides (Nalge-Nunc, Wiesbaden, Germany) and cultured in a 10% CO<sub>2</sub> incubator at 37°C. Isolated myocytes were vigorously beating after 1-2 days in culture, and were fixed and stained after 3 days in culture as described below.

#### Antibodies

A first rabbit antiserum (no. 32) was raised against a bacterially expressed GST-mCAR fusion protein. This serum was only used to demonstrate lack of the *CAR* protein in *CAR*-deficient mice by immunoblotting. For all subsequent experiments, affinity purified rabbit antibodies (VE15) were used that had been raised against a *CAR*-Fc fusion protein, produced in transfected CHO cells. For antibody purification, non-IgG proteins were removed from the serum

by caprylic acid (Sigma) precipitation. Antigen-specific antibodies of the serum were affinity-purified on *CAR*-Fc immobilized on CNBr-Sepharose (Amersham Biosciences), and antibodies against the IgG-Fc part were removed by incubation with immobilized human IgG. Rat monoclonal antibodies against ESAM [V1G8.2 (Nasdala et al., 2002)], PECAM-1 [MEC13.3 (Vecchi et al., 1994)] Endomucin [V.7C7 (Morgan et al., 1999)], and  $\beta_1$ -integrin (Lenter et al., 1993) have been described previously. The polyclonal rabbit anti-ZO-1 antibody (Z-R1, Zymed), the mouse monoclonal anti-sarcomeric  $\alpha$ -actinin antibody (EA-53, Sigma), the mouse monoclonal anti- $\beta$ -catenin antibody (clone 14, Becton Dickinson) and mouse monoclonal anti  $\alpha$ -smooth muscle actin antibody (ASM-1, Chemicon) were commercially obtained. Monoclonal rat anti-N-cadherin antibodies were generated in the Vestweber laboratory and will be described elsewhere.

#### Embryological techniques and immunostaining

Only embryos that were alive, defined as those with a beating heart, were used for experiments. For whole mount staining with anti-PECAM-1 antibodies, embryos were fixed in 4% (w/v) freshly depolymerised PFA in PBS (4% PFA/PBS) over night at 4°C and stained essentially as described (Ma et al., 1998). For histological examination of paraffin sections, embryos and placentas were fixed in 4% PFA/PBS over night at 4°C, dehydrated, embedded in paraffin, and sectioned at 4  $\mu$ m, according to standard procedures. Sections were dewaxed, rehydrated, and counter-stained with hematoxylin and eosin. Immunostaining on PFA-fixed specimens was performed as described previously (Kuhn et al., 2002) using the Vectastain<sup>®</sup> Elite ABC kit (Vector Laboratories) to detect the primary antibodies. For Cryostat sections, freshly prepared embryos and placentas were rinsed in PBS, embedded in Tissue Tek<sup>®</sup> O.C.T. (Sakura Finetek), frozen on dry ice, and stored at -80°C before cryosectioning. Cryostat sections (8-10  $\mu$ m thick) were cut using a cryotome (model CM3000, Leica), mounted on poly-L-lysine-coated glass slides (Menzel-Gläser, Nußloch, Germany), and dried. Sections were fixed for 10 minutes at -20°C in methanol and washed in PBS.

Cultured cardiomyocytes were rinsed briefly in PBS, fixed in 4% PFA/PBS for 15 minutes at room temperature, and permeabilized with 0.3% Triton X-100 and 1% BSA in PBS at room temperature. In general, immunostaining of sections and of cultured cells was performed as described previously (Wegmann et al., 2004).

#### Electron microscopy and periodic acid-Schiff staining

For electron microscopy, embryonic hearts of wild type and *CAR* null mice (E10.5-E11.5) were fixed for 2 hours in 2.5% glutaraldehyde (Paesel & Lorei, Hanau, Germany) in Hank's modified salt solution (HMSS) at 4°C. Specimens were transferred to 0.1 M cacodylate buffer, pH 7.4, postfixed for 1 hour in 1% osmiumtetroxide (Paesel & Lorei) in cacodylate buffer, washed again and dehydrated in a graded ethanol series. The 70% ethanol step was saturated with uranylacetate for contrast enhancement and carried out at 4°C over night. Dehydration was completed with propyleneoxide. Specimens were embedded in Araldite (Serva) and polymerized for 48 hours at 60°C. Ultrathin sections were cut using an Ultracut R (Leica), stained with lead citrate and observed in a Zeiss EM 10 transmission electron microscope. The periodic acid-Schiff (PAS) reaction was performed at semithin sections after removal of Araldite with concentrated NaOH in 100% ethanol for 1 hour. The sections were washed several times in 100% ethanol, air-dried, and incubated with 1% periodic acid (Merck) for 10 minutes, washed in distilled water for 5 minutes, incubated in Schiff's reagent (Sigma) for 15 minutes, washed in tap water for 15 minutes and counterstained with Mayer's Hemalaun (Merck) for 5 minutes to turn blue with tap water. Then, the sections were dehydrated in an ethanol-xytol series and mounted in Pertex (Medite, Burgdorf, Germany). For control, paraffin sections were



incubated with amylase (*Aspergillus niger*: 100 mg per 10 ml; Roche) for 1 hour at 37°C.

#### BrdU incorporation

Timed pregnant mice at E11.5 were injected intraperitoneally with 50 mg kg<sup>-1</sup> body weight of 5-bromo-2'-deoxyuridine (BrdU, Sigma) in PBS 2 hours before sacrificing. Embryos were dissected from the uterus, embedded in Tissue Tek® O.C.T. (Sakura Finetek), and sectioned in the transverse orientation. BrdU labeling and visualization of cardiomyocytes using sarcomeric  $\alpha$ -actinin antibodies was performed as described previously (Fedrowitz et al., 2002).

#### TUNEL staining

TUNEL assays were performed using the In Situ Cell Death Detection Kit (Roche) according to the manufacturer's recommendations with minor modifications to co-stain for sarcomeric  $\alpha$ -actinin. Prior to incubation in the TdT enzyme reaction mixture, sections were exposed to sarcomeric  $\alpha$ -actinin antibodies, washed, and incubated with Cy3-conjugated secondary anti-mouse antibodies.

### Results

#### Targeted disruption of the *CAR* gene causes embryonal lethality

The targeting construct used to disrupt the *CAR* gene was generated such that most of the first exon was replaced by a neomycin expression cassette leading to the deletion of the start codon of *CAR* (Fig. 1A). Mice heterozygous for the intended gene disruption showed the expected 6.4 kb hybridization signal for the disrupted locus in addition to the 8 kb signal for the wild-type locus in Southern blot analysis using internal probes (Fig. 1B). Hybridization with the neomycin probe failed to detect any additional sites of integration. PCR analysis of DNA of wild type, heterozygous and homozygous mutant embryos revealed a 1.5 kb product for the mutated allele and a 1 kb product for the wild-type allele (Fig. 1C). Western blots of SDS-PAGE sample buffer extracts of wild type, heterozygote and homozygote embryos revealed that the protein level of CAR was reduced in heterozygous animals whereas the protein was undetectable in homozygous mutant embryos (Fig. 1D).

Heterozygous mice were viable and apparently normal. Genotyping the offspring of mated heterozygous mice revealed that homozygous mice were embryonal lethal. The embryos died between E11.5 and E13.5 post coitum (p.c.) with viable *CAR*<sup>-/-</sup> embryos still present at Mendelian ratios at E10.5, and reduced ratios of 17% at day 11.5, and only 7% and 0% at E12.5 and E13.5, respectively (Table 1).

To analyze the expression of *CAR* in the embryo by

immunohistochemistry we generated rabbit antibodies by immunizing with a recombinant CAR-Fc fusion protein. Purified CAR-Fc was used for immunization as well as for affinity purification of the antibodies. Antibodies recognizing the Fc-part of the fusion protein were removed with IgG affinity columns. The specificity of the affinity purified antibodies was demonstrated in immunoblots on CAR-transfected CHO cells, as shown in Fig. 1E and by fluorescence staining of the heart region of E11.5 *CAR*<sup>+/+</sup> and *CAR*<sup>-/-</sup> embryos as shown in Fig. 1F.

#### Overall vascularization of *CAR*-deficient embryos is normal except for the dilatation of large vessels

To analyze potential effects of the *CAR* deficiency on overall vascularization, blood vessels of *CAR*<sup>+/+</sup> and *CAR*<sup>-/-</sup> embryos at stages E10.5 and E11.5 were visualized by whole mount staining with antibodies against PECAM-1. Only embryos of the same litter were compared and five embryo pairs were analyzed in total. As shown in Fig. 2, *CAR*<sup>-/-</sup> embryos showed a well-developed peripheral vascular system at E10.5 as well as E11.5 with no obvious differences to the wild-type embryos. To examine larger blood vessels in deeper layers of embryonal tissue, paraffin sections of E11.5 embryos were analyzed. Sections were taken from corresponding regions at the position of the posterior limb buds. We detected strong dilatations of the cardinal veins and the aortae in *CAR*<sup>-/-</sup> embryos (Fig. 2).

#### *CAR* deficiency does not affect the embryonal-maternal interphase of the placenta

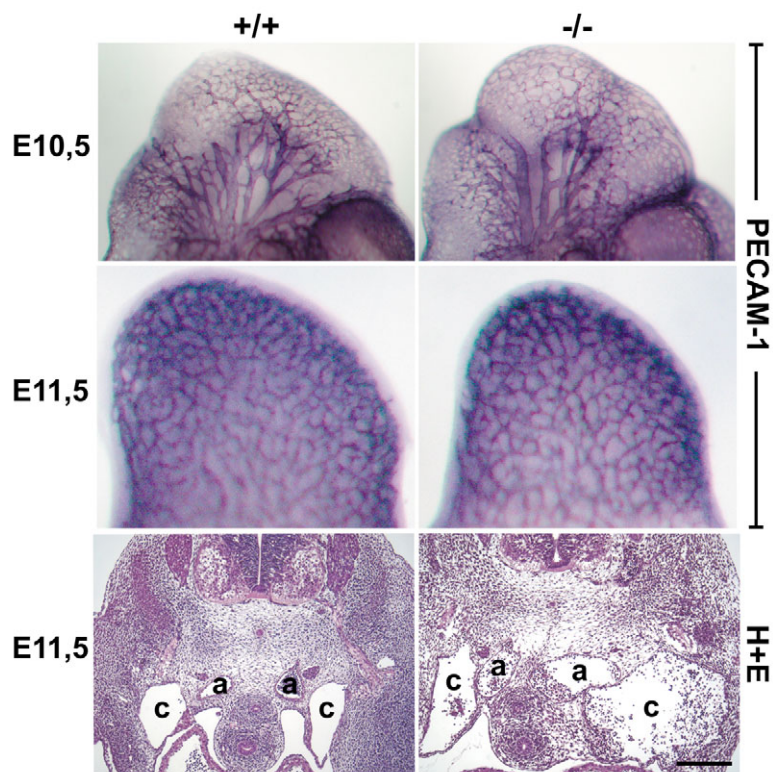
Developmental defects that lead to embryonal lethality during mid-gestation are usually either due to defects in the development of the placenta or of the cardiovascular system. The labyrinth layer of the placenta is the region where the material exchange between the maternal and fetal blood system occurs. This layer is often underdeveloped in mutants with defective placental function. The fetal blood vessels are outlined by endothelial cells, whereas the maternal blood supply passes through arterial sinuses where the endothelial cells have been replaced by trophoblast cells. We stained sections of paraffin-embedded placental tissue with a mAb against the endothelial marker endomucin. Due to the lack of endothelial cells in maternal sinuses, staining of endothelium selectively marked embryonal blood vessels in the labyrinth layer. As shown in Fig. 3A, the labyrinth layer of *CAR*<sup>-/-</sup> placental tissue was not underdeveloped suggesting that the overall organization of this layer was unaffected in mutant embryos. The only morphological changes that we detected were restricted to modest dilatations of fetal blood vessels within the labyrinth zone (Fig. 3A). Analysis of the expression pattern of *CAR* within the labyrinth layer of the placenta revealed that *CAR* was expressed on trophoblast cells, whereas endothelial cells that were identified by staining for PECAM-1 were clearly negative for *CAR* (Fig. 3B). We conclude that the overall organization of the maternal fetal interphase in the placenta is not affected, except for some modest dilatations of embryonal blood vessels.

**CAR is not expressed on embryonal endothelial cells**  
Since we observed dilatations in blood vessels of *CAR*<sup>-/-</sup>

**Table 1. Analysis of progeny from heterozygote (*CAR*<sup>+/-</sup>) intercrosses**

| Day p.c. | +/+      | +/-       | -/-      |
|----------|----------|-----------|----------|
| E9.5     | 28% (11) | 43% (17)  | 30% (12) |
| E10.5    | 28% (18) | 43% (28)  | 29% (19) |
| E11.5    | 25% (54) | 58% (123) | 17% (35) |
| E12.5    | 45% (13) | 48% (14)  | 7% (2)   |
| E13.5    | 55% (18) | 45% (15)  | 0% (0)   |

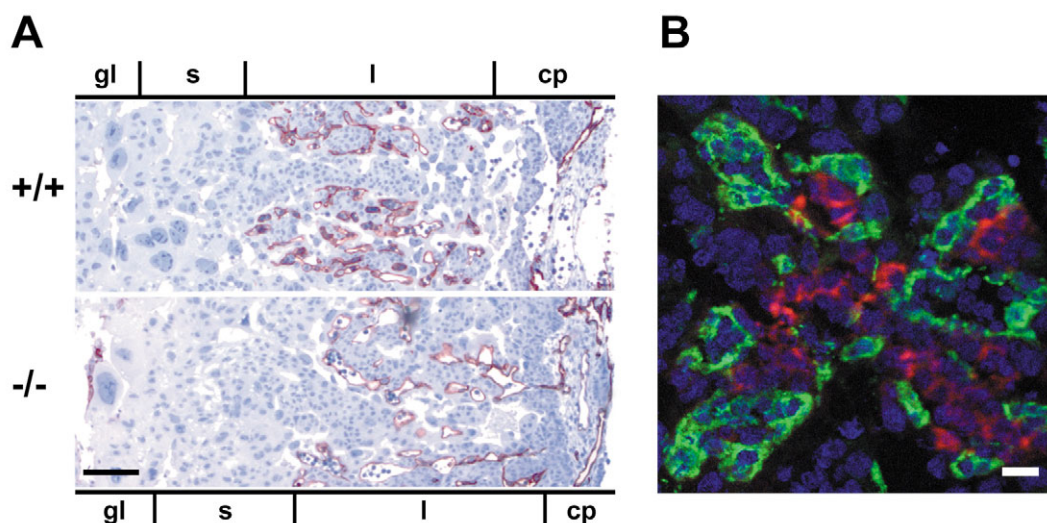
Observed number of progeny of each genotype are shown in parenthesis; p.c., post coitum.



**Fig. 2.** Analysis of the vascularization of CAR-deficient embryos. Wild type (+/+) and CAR-deficient (-/-) embryos of stage E10.5 (top) or E11.5 (middle) were stained as whole mounts with anti-PECAM-1 antibodies. The head region is shown for E10.5, limb buds are shown for E11.5. No obvious differences in overall vascularization were observed. Bottom panel: paraffin sections of E11.5 embryos. Sections were taken from the posterior limb region, and stained with hematoxylin and eosin (H+E). Note: aortae and cardinal veins were enlarged in CAR<sup>-/-</sup> embryos. Key: a, left and right aorta; c, left and right cardinal vein. Bar, 200  $\mu$ m. In each case, CAR<sup>+/+</sup> and CAR<sup>-/-</sup> littermates were compared.

**Fig. 3.** Analysis of placental expression of CAR and of overall placenta organization of CAR-deficient embryos.

(A) Paraffin sections of wild type (+/+) and CAR-deficient (-/-) placentae (E11.5) were stained with antibodies against the endothelial marker endomucin and counterstained with hematoxylin. Since maternal vessels are devoid of endothelial cells the endothelial staining shows selectively only blood vessels of the fetus. The different zones of the placenta are marked: gl, giant layer; s, spongiotrophoblast layer; l, labyrinthine layer; cp, chorionic plate. Note: the extension of the labyrinthine layer was not altered in CAR<sup>-/-</sup> embryos. Bar, 100  $\mu$ m. (B) Cryostat section of a placenta of E11.5 wild-type embryo double stained with anti-CAR polyclonal antibodies (red) and an anti-PECAM-1 mAb (green) visualized by indirect immunofluorescence. Nuclei were visualized with Sytox (blue). CAR was found on trophoblast cells of the labyrinth zone, but not on endothelial cells. Bar, 20  $\mu$ m.

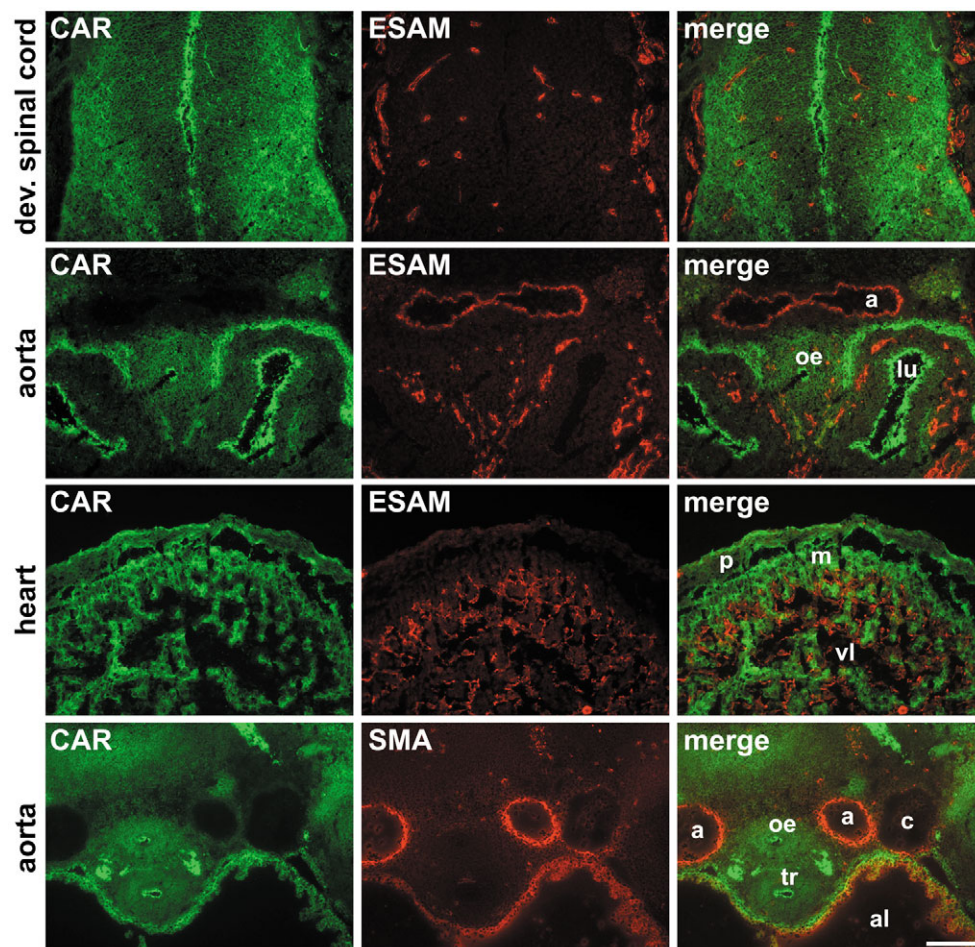


embryos and controversial results have been published on the expression of CAR on endothelium, we analyzed the expression of CAR in sections of E11.5 embryos. For comparison, the same sections were stained for the endothelial junctional protein ESAM. We found CAR to be strongly expressed in cells of the developing spinal cord and the ependyma, in epithelial cells of esophagus and the developing lung, and in the myocard and the pericard of the heart (Fig. 4). We did not detect any endothelial staining for the marker ESAM that showed co-localization with the staining for CAR. Importantly, double staining for CAR and smooth muscle actin showed that smooth muscle cells of large vessels were also devoid of CAR expression (Fig. 4). The lack of CAR expression in cells of the blood vessel wall renders it unlikely that disruption of the expression of CAR in mouse embryos would directly cause blood vessel dilatation. Therefore, we assume that blood vessel dilatation is a secondary effect of deficits caused by the lack of CAR elsewhere.

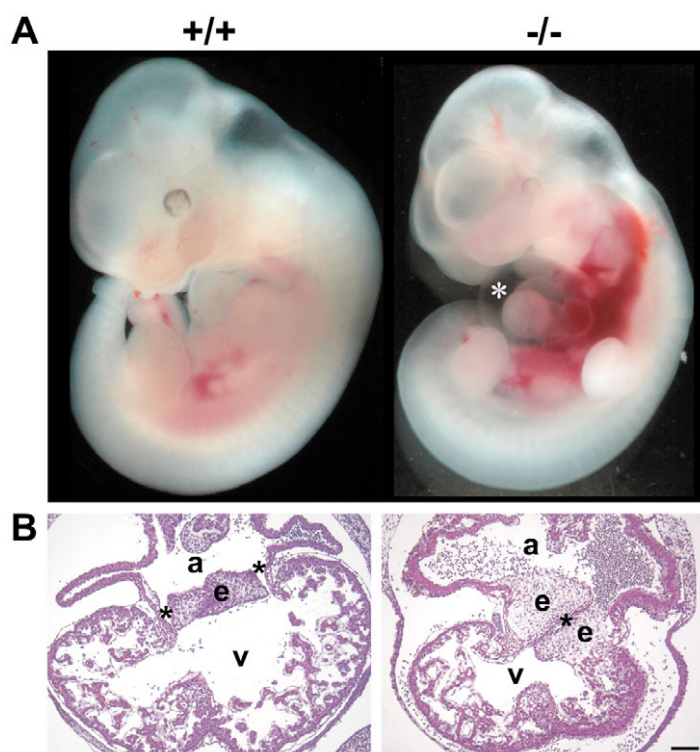
#### CAR deficiency causes abnormal heart formation

Preparations of E11.5 CAR<sup>-/-</sup> embryos usually revealed enlarged pericards due to edema formation (Fig. 5A). Since pericard swelling is usually a sign for insufficient heart function, we analyzed the morphology of the heart in more detail. Cross-sections of the heart of E11.5 embryos of CAR<sup>-/-</sup> and CAR<sup>+/+</sup> littermates revealed that the lumen of the ventricles in CAR<sup>-/-</sup> embryos was smaller (Fig. 5B). Trabeculation was not significantly altered. However, the heart of E11.5 CAR<sup>-/-</sup> embryos contained clearly enlarged cushions and only a single atrioventricular





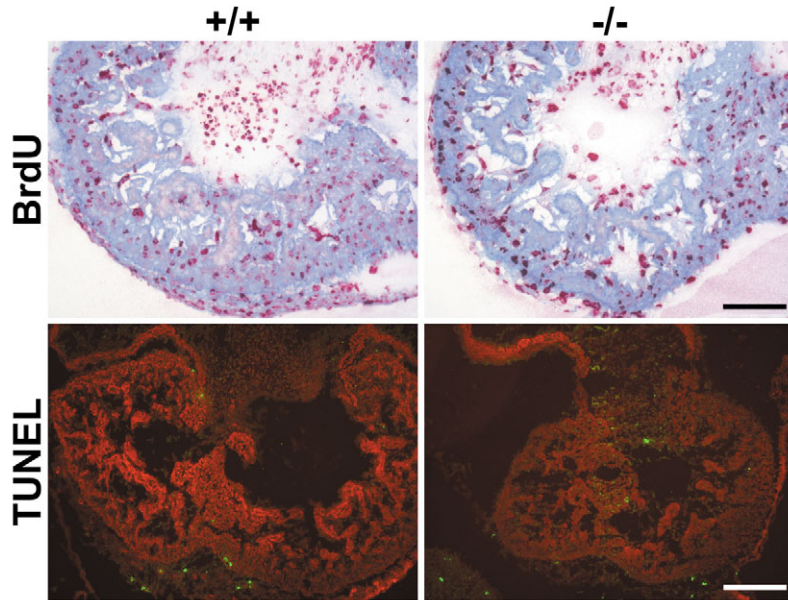
**Fig. 4.** CAR is not expressed on endothelial cells or vascular smooth muscle cells of E11.5 embryos. Cryostat sections of E11.5 embryos were double stained with antibodies against CAR (green), and either ESAM or smooth muscle actin (SMA) as indicated (red). The expression patterns of the endothelial marker protein ESAM and of SMA were non-overlapping with the distribution pattern for CAR. Key: a, aorta; lu, developing lung buds; p, pericard; m, myocard; c, cardinal vein; al, atrial lumen; vl, ventricular lumen; oe, oesophagus; tr, trachea. Bar, 100  $\mu$ m.



canal was observed whereas  $CAR^{+/+}$  embryos of the same developmental stage (littermates) already contained the two atrioventricular canals connecting the developing right atrium with the right ventricle and the left atrium with the left ventricle (Fig. 5B). This clear difference between the hearts of  $CAR^{-/-}$  and  $CAR^{+/+}$  embryos, which was observed in five out of six embryo pairs, represented a clear sign for delayed heart development in  $CAR^{-/-}$  embryos.

To see whether the reduction in ventricle size or the delay of morphological development of the heart would be affected by a reduction in cell proliferation of cardiomyocytes, sections of the hearts of in utero BrdU-labeled embryos of  $CAR^{-/-}$  and  $CAR^{+/+}$  littermates were

**Fig. 5.** Abnormalities of the heart of CAR-deficient embryos. (A) Whole mounts of wild type (+/+) and CAR-deficient (-/-) E11.5 embryos of the same litter are shown. The asterisk marks the enlarged pericard in the  $CAR^{-/-}$  embryo. Hemorrhages resulting from preparing the embryos were more often observed in  $CAR^{-/-}$  embryos than in wild-type embryos. (B) Paraffin sections of E11.5 embryos of the same litter were counterstained with hematoxylin. The micrographs show the heart region.  $CAR^{-/-}$  embryos showed enlarged endocardial cushions and a single persistent atrioventricular canal instead of two canals in the wild-type embryo. Key: a, atrium; v, ventricle; e, endocardial cushion; \*, atrioventricular canal. Bar, 100  $\mu$ m.



**Fig. 6.** No signs for altered proliferation or apoptosis activity in cardiomyocytes of CAR-deficient embryos. Upper row: 2 hours before preparing of E11.5 embryos, the pregnant mother was injected with BrdU. Cryostate sections of  $CAR^{+/+}$  and  $CAR^{-/-}$  littermate embryos were stained for incorporated BrdU (red) and counterstained for sarcomeric  $\alpha$ -actinin (blue) to mark cardiomyocytes. Micrographs show the area of the heart ventricles. For better visualization of the red staining, the lightness of the blue staining was enhanced. Bar, 100  $\mu$ m. Bottom row: detection of apoptotic cells by TUNEL staining (green) of cryostate sections of E11.5 littermate embryos. Cardiomyocytes were stained for sarcomeric  $\alpha$ -actinin (red). No significant signs of apoptosis were detected independently of the genotype. Bar, 200  $\mu$ m.

analyzed by visualizing incorporated BrdU with antibodies. Cardiomyocytes were marked with antibodies against sarcomeric  $\alpha$ -actinin. As shown in Fig. 6, no obvious difference in the density of proliferating cells was observed between CAR deficient and wild-type embryos. Investigating heart sections of E11.5 embryos of  $CAR^{-/-}$  and  $CAR^{+/+}$  littermates for the presence of apoptotic cells by means of TUNEL staining did not show significant signs of apoptosis in either of the two genotypes (Fig. 6). We conclude that neither proliferation nor apoptosis were abnormal in hearts of this stage in  $CAR^{-/-}$  embryos.

#### Ultrastructural analysis of cardiomyocytes of CAR-deficient embryos reveals fewer myofibrils organized in a parallel fashion

Since cardiomyocytes are the major cell type in the heart that express CAR, we analyzed these cells on the ultrastructural level. Again, E11.5  $CAR^{-/-}$  and  $CAR^{+/+}$  embryos of the same litter were compared. Transmission electron microscopy revealed that cardiomyocytes of ventricular trabeculae of  $CAR^{-/-}$  embryos showed consistently myofibrils with reduced diameters (Fig. 7). On average, the diameters of myofibrils were 0.3  $\mu$ m in mutant cells and ranged from 1 to 1.5  $\mu$ m in wild-type cells. Numerous micrographs also showed a reduced number of myofibrils and a lack of close arrangement beside each other. In addition, myofibrils were shorter and contained a smaller number of sarcomers (not shown). We observed on average 12–15 sarcomers consecutively linked to each other in longitudinal sections of wild-type cardiomyocytes, whereas sections of mutant cells contained only 4–6 consecutive sarcomers. In addition, myofibrils were often found to be isolated within the cytosol. Occasionally, Z-discs were lacking. These alterations were never observed in cardiomyocytes of wild-type embryos that always contained well-organized thick myofibrils. It is likely that these defects in myofibril organization and content in  $CAR^{-/-}$  cardiomyocytes would affect the capacity of these cells to contract.

Other changes in  $CAR^{-/-}$  cardiomyocytes concerned the mitochondria that were grossly enlarged and often shaped in a ring-like fashion. In addition, some mitochondria displayed very high electron density, which was never observed in wild-type cardiomyocytes. Besides the mitochondrial abnormalities, cardiomyocytes of the mutant embryos contained large accumulations of glycogen granules, which could be visualized in the light microscope by periodic acid Schiff staining for polysaccharides (Fig. 7). These changes in mitochondrial morphology and in glycogen granule content in cardiomyocytes of the mutated embryos are reminiscent of similar effects that can be observed in cardiomyocytes of infarcted areas of the heart. Possibly, these changes could be secondary effects caused by suboptimal functioning of the cardiomyocyte contraction machinery.

#### Isolated cultured cardiomyocytes of $CAR^{-/-}$ embryos are defective in organizing myofibrils

To analyze further the deficits in myofibril organization in  $CAR^{-/-}$  embryos, we isolated cardiomyocytes of E11.5  $CAR^{-/-}$  and  $CAR^{+/+}$  littermates and visualized their myofibrils by immunofluorescence microscopy after 3 days of culture. As shown in Fig. 8, we found that the organization of myofibrils in  $CAR^{-/-}$  cells was chaotic. Whereas wild-type cardiomyocytes occasionally also displayed a lower grade of perfect parallel organization of myofilament bundles and intracellular areas devoid of myofibrils, a large fraction of wild-type cells showed perfect myofibril organization and close packing of most of the intracellular space with parallel myofibrils (Fig. 8). Such cells with perfectly organized and densely packed myofilament bundles were very rarely seen among cultured  $CAR^{-/-}$  cardiomyocytes.

Cultured cardiomyocytes of wild-type embryos expressed CAR at cell contacts (Fig. 9A). Since N-cadherin was recently reported to be involved in myofibril continuity across plasma membranes and thus in the spatial organization of myofilament bundles, we tested whether its expression or subcellular



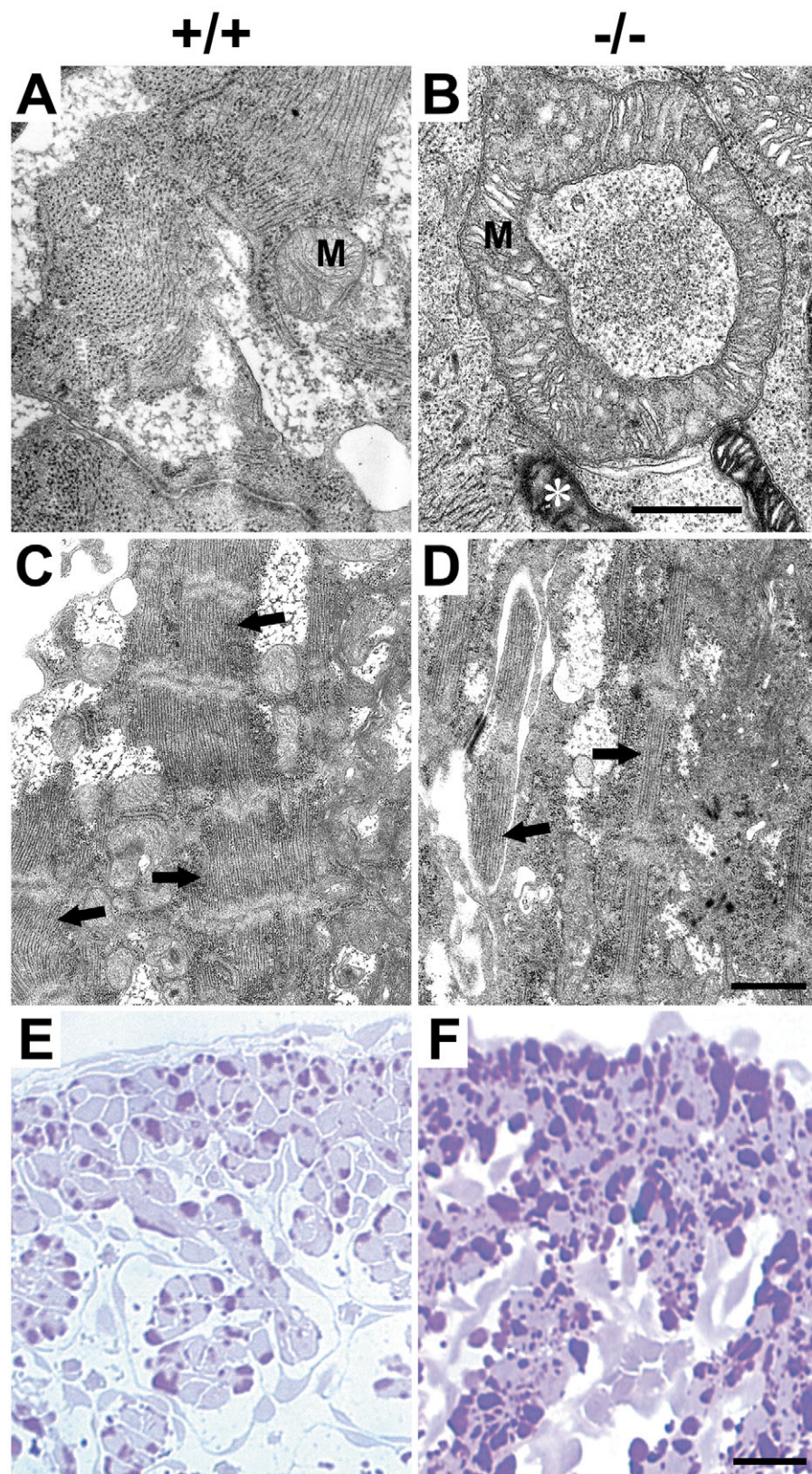
localization would be affected in cultured  $CAR^{-/-}$  cardiomyocytes. As shown in Fig. 9B, no difference was observed in N-cadherin cell contact staining between  $CAR^{-/-}$  or  $CAR^{+/+}$  cells. Likewise, the intracellular binding partners  $\beta$ -

catenin (Fig. 9B) and the junction protein ZO-1 (Fig. 9A) were normally localized at cell contacts in cardiomyocytes of both genotypes. In addition, double staining of cultured embryonal cardiomyocytes for  $\beta_1$ -integrin and  $\alpha$ -actinin (Fig. 9C) revealed, that the  $\alpha$ -actinin banding pattern aligned in register with  $\beta_1$ -integrin-based membrane structures, suggesting that the absence of CAR did not affect this association. Double staining for N-cadherin and  $\alpha$ -actinin of  $CAR^{+/+}$  cardiomyocytes showed that myofibrils were correctly anchored at cell contacts and 'continued' into the neighboring cells across the plasma membrane (Fig. 9D). This continuation of orientation was dramatically disturbed in  $CAR^{-/-}$  cells, although cell contact regions were not devoid of myofibrils (Fig. 9D). However, some contact sites of  $CAR^{-/-}$  cells stained for N-cadherin were close to Z-disks detected by  $\alpha$ -actinin staining. Thus despite the defects in proper organization and alignment and in continuation across membrane contacts, myofibrils in  $CAR^{-/-}$  cells were still able to associate with N-cadherin and  $\beta_1$ -integrin-containing membrane structures.

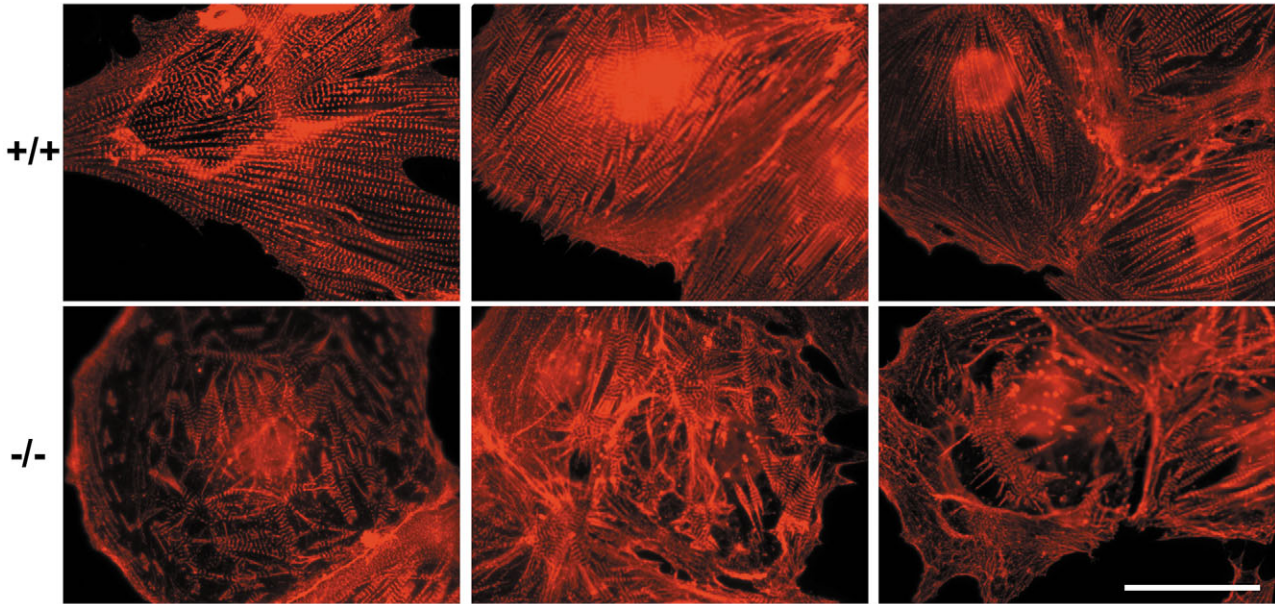
## Discussion

In the present study, we demonstrate that CAR, a membrane protein with cell adhesion properties but unknown physiological function, is essential for embryonal development and formation of the cardiovascular system. Mice lacking CAR expression due to targeted gene disruption die during mid-gestation between E11.5 and E13.5. We found that

**Fig. 7.** Alterations of cardiomyocytes in CAR-deficient embryos. Ultrathin sections of the heart of E11.5  $CAR^{+/+}$  (left) and  $CAR^{-/-}$  (right) littermate embryos (A-D) were analyzed by transmission electron microscopy. The micrographs show alterations within cardiomyocytes, such as enlarged mitochondria (M), mitochondria with increased electron density (\*). Most importantly large areas of the cytosol of  $CAR^{-/-}$  cardiomyocytes were devoid of myofilament bundles and the thickness of these bundles was much reduced (arrows). Bar in A and B represents 0.5  $\mu$ m, and in C and D 1  $\mu$ m. (E,F) Semi-thin sections of E11.5 embryos of both genotypes were prepared and the heart region was stained for the accumulation of glycogen granula by periodic acid Schiff staining. Note that  $CAR^{-/-}$  embryos showed a strong increase in glycogen storage. Bar, 25  $\mu$ m.







**Fig. 8.** Comparison of in vitro cultured wild type and  $CAR^{-/-}$  embryonal cardiomyocytes. Cardiomyocytes of E11.5 embryos isolated and cultured for 3 days were stained by indirect immunofluorescence with antibodies against sarcomeric  $\alpha$ -actinin. Three examples of typical wild-type cells (+/+) and  $CAR$ -deficient cells (-/-) are depicted. Note: the myofibrils were disorganized and shortened in  $CAR^{-/-}$  cells.

cardiomyocytes showed a diminished density of myofibrils and that these myofibrils were reduced in thickness and showed defects in their arrangement and organization into parallel structures. In addition, abnormalities in endocardial cushion and atrioventricular canal formation in the heart and dilatations of blood vessels in various tissues were observed. Our results suggest that the lack of  $CAR$  expression leads to defects in cardiac function that cause embryonal lethality.

Since the placenta is the first organ to form during mammalian development, many genes that affect the survival of the embryo do this by disturbing the development of this vital organ. Placental defects that affect the exchange of nutrients and oxygen between the maternal and fetal circulation often lead to alterations of the labyrinth zone of the placenta that represents the interphase between both circulatory systems (Rossant and Cross, 2001). Indeed,  $CAR$  is expressed in the placenta. We detected  $CAR$  on fetus-derived labyrinthal trophoblasts, and  $CAR$  has been described on extravillous trophoblasts of the human placenta (Koi et al., 2001). However, histologic examination of the placenta of  $CAR^{-/-}$  embryos did not reveal any major alterations of the placental structure, especially no signs for a reduction of the size of the labyrinth zone or for a reduction of the density of embryonal blood vessels in this zone were observed. Modest blood vessel dilatations that were frequently observed possibly represent secondary effects (see below). Thus, the  $CAR^{-/-}$  deficiency does not seem to cause obvious structural derangements of the placenta.

The expression of  $CAR$  on endothelial cells is controversial and has been described in some reports (Carson et al., 1999; Nasuno et al., 2004; Vincent et al., 2004), yet others found no expression on endothelial cells (Noutsias et al., 2001) or endothelial expression only on capillary-like structures in infarcted areas of the heart (Fechner et al., 2003). Increasing

endothelial cell density was found to upregulate  $CAR$  expression (Carson et al., 1999) and  $TNF-\alpha$  was reported to down regulate it (Vincent et al., 2004). We analyzed the expression of  $CAR$  systematically throughout the embryo and compared it with the endothelial protein ESAM and a smooth muscle cell marker. Based on this we found no evidence for the expression of  $CAR$  on endothelial cells or vascular smooth muscle cells during those embryonal stages of development that we analyzed. In line with this we found no general defect in vascularization. Dilatations of larger blood vessels were the only changes that were frequently observed. Since  $CAR$  was not expressed in blood vessels it is unlikely that vessel dilatations represented a direct consequence of the  $CAR$  mutation.

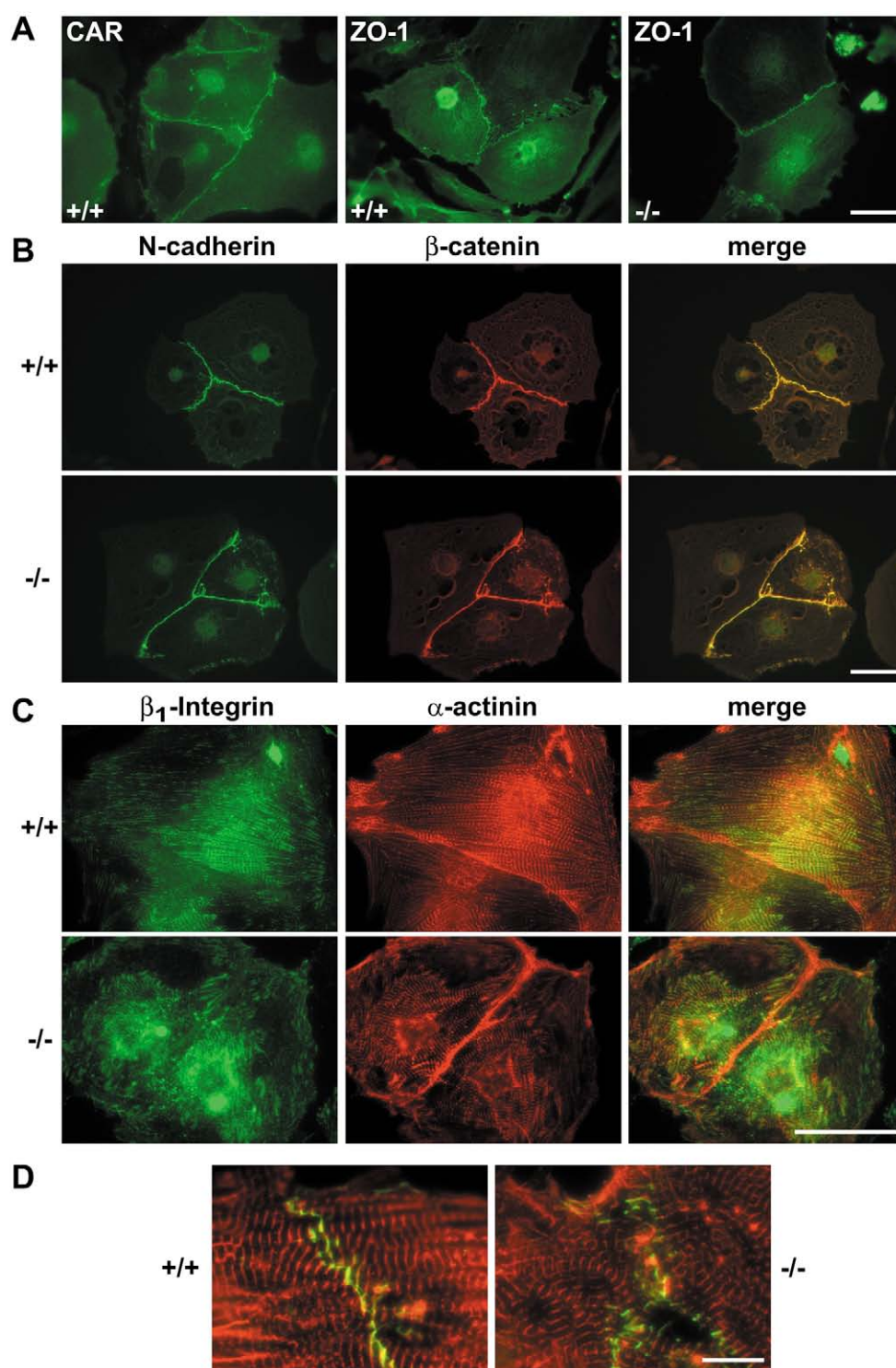
Defects more likely to be primary effects of the  $CAR$  deficiency are those we found in the subcellular organization of cardiomyocytes, cells that strongly express  $CAR$  during normal embryonal development. The disturbance of myofibril organization and their reduced thickness suggest that cardiomyocytes of  $CAR^{-/-}$  embryos are not fully functional. In line with this, hearts of E11.5  $CAR^{-/-}$  embryos usually showed edema formation and distended pericards, which are clear signs for insufficient heart function. Additional abnormalities in cardiomyocytes, such as enlargement of mitochondria and accumulation of glycogen granules, could possibly be the result of compensatory mechanisms, induced to balance insufficient cardiomyocyte function. Similar changes of mitochondrial shape and glycogen content have been observed in the so-called 'hibernating' myocard that describes myocard of reduced function in ischemic areas of the heart (Rahimtoola, 1985).

Another major abnormality we observed in the hearts of E11.5  $CAR^{-/-}$  embryos were enlarged endocardial cushions and a single atrioventricular canal instead of two. Again neither

the endocard in these areas nor the mesenchymal cells of the cardiac cushions expressed CAR in normal wild-type embryos. Thus, the lack of CAR expression would probably not directly affect these cells. The absence of two atrioventricular canals at this stage of embryonal development points towards a delay of normal heart development. It is well documented that a malfunctioning blood circulation and insufficient blood flow can indirectly affect the normal process of remodeling of the heart during development (Hogers et al., 1999; Berdougo et al.,

2003; Huang et al., 2003). In addition, it has been shown that normal blood flow is necessary for vessel development and insufficient flow can cause blood vessel dilatations (Conway et al., 2003). Thus it is conceivable that the defects we observed in cardiomyocytes could lead to heart insufficiency as documented by the enlarged pericardium and that this could be the reason for the morphological malformations in the region of the endocardial cushion and the dilatations in larger vessels.

It is remarkable that the defects in myofibril organization



**Fig. 9.** Orientation and localization of myofibrils in the vicinity of cell contacts and focal contacts in embryonal CAR<sup>+/+</sup> and CAR<sup>-/-</sup> cardiomyocytes. (A) Indirect immunofluorescence of CAR<sup>+/+</sup> cells (+/+) for CAR and of CAR<sup>+/+</sup> cells and CAR<sup>-/-</sup> cells (-/-) for ZO-1. (B-D) Double staining of CAR<sup>+/+</sup> and CAR<sup>-/-</sup> cells for: (B) N-cadherin (green) and β-catenin (red); (C) β<sub>1</sub>-integrin (green) and α-actinin (red); (D) N-cadherin (green) and α-actinin (red). In B and C the single stainings and the merge are shown (as indicated) whereas in D only the merged picture is depicted. Bars in A-C represent 50 μm, bar in D is 10 μm.



could even be observed in primary isolated cardiomyocytes that had been cultured for 3 days. This suggests that the myofibril defects observed in heart tissue were most likely not indirectly caused by a developmental delay in heart formation, but by a more cell autonomous effect related to the lack of CAR on these cells. CAR was reported to support homotypic cell adhesion of transfected cells (Honda et al., 2000; Cohen et al., 2001) and we found it to be expressed at cell contacts between cultured cardiomyocytes (Fig. 9) and to be recruited to cell contacts of transfected CHO cells, only if the neighboring cell expressed CAR too (not shown). It is possible that CAR could play a role in cell contact formation of cardiomyocytes. Nascent myofibrils are thought to initiate assembly by interacting with the actin cytoskeleton at the plasma membrane (Lin et al., 1989). Thus, cell adhesion proteins are likely to be involved in the organization of myofibrils. Indeed N-cadherin that associates via catenins to the actin cytoskeleton has been described as being involved in myofibrillogenesis, since antibodies blocking N-cadherin function and dissociating cell contacts between myocytes also disrupted myofibril organization (Goncharova et al., 1992; Soler and Knudsen, 1994; Wu et al., 1999). More recently, however, it was found that the overall myofibrillogenesis was normal in N-cadherin deficient cultured cardiomyocytes, whereas the organization of myofibrils and the continuity of the orientation of myofibrils across the plasma membrane to the neighboring cell were disturbed (Luo and Radice, 2003). Interestingly the absence of CAR leads to misalignment of myofibrils across the plasma membrane suggesting that N-cadherin and CAR may both be required for proper myofibril orientation. In this context it may be interesting that CAR was recently reported to co-immunoprecipitate with  $\beta$ -catenin (Walters et al., 2002). Cell substratum adhesion receptors are also likely to be involved since it was found that lack of the  $\beta_1$ -integrin chain affects normal sarcomeric architecture in cardiomyocytes differentiated from  $\beta_1$ -integrin deficient embryonic stem cells (Fassler et al., 1996).

The association of CAR to intracellular binding partners will probably be key to understanding how the lack of CAR expression on cardiomyocytes could lead to the defects in myofibril organization. CAR was recently found to associate with the intracellular membrane and junction associated PDZ-scaffold proteins MAGI-1b, PICK and PSD-95 (Excoffon et al., 2004), MUPP-1 (Coyné et al., 2004) and LNX (Sollerbrant et al., 2003). Other CAR related members of the CTX subfamily of the Ig-SF proteins are also adhesion molecules and bind to PDZ domain proteins. JAM-A has been shown to be important for the establishment of epithelial cell polarity and binds to PAR-3, ZO-1 and AF-6 (Ebnet et al., 2000; Ebnet et al., 2001; Itoh et al., 2001). PAR-3 is known to be essential for the formation of epithelial cell polarity (Joberty et al., 2000; Johansson et al., 2000; Lin et al., 2000). The endothelial specific ESAM binds to MAGI 1c (Wegmann et al., 2004) and was found to be involved in tumor angiogenesis (Ishida et al., 2003). JAM-C can bind to similar PDZ proteins as JAM-A (Ebnet et al., 2003). In testis, two other PDZ proteins that are important for cell polarity, PAR-6 and PATJ, were found to associate with JAM-C, and JAM-C was found to be essential for the formation of cell polarity during spermatid differentiation, leading to male infertility (Gliki et al., 2004). In addition, the lack of JAM-C leads to postnatal lethality

among 60% of newborn mice (Gliki et al., 2004). All three proteins (JAM-A, JAM-C and ESAM) are associated with epithelial and/or endothelial tight junctions and also CAR was found close to tight junctions in epithelial cells. Whereas targeted gene disruptions for JAM-A and ESAM have not caused any defects in embryonal development, with JAM-C (Gliki et al., 2004) and CAR (this study) now two members of the CTX family have been defined to be essential for the development of the mouse.

Besides the property to support cell adhesion, an inhibitory activity on cell growth has been attributed to CAR based on experiments with in vitro cultured bladder carcinoma cells (Okegawa et al., 2001) and CAR transfected L cells (Excoffon et al., 2004). Based on in utero BrdU incorporation experiments with wild-type and CAR<sup>-/-</sup> embryos we could not detect changes in the proliferation of cardiomyocytes within the heart. Thus the developmental defects we have observed are probably not related to growth regulatory functions.

In conclusion, our results show that CAR is required for the normal development of the heart, and for the formation of normal myofibrils and their parallel organization as a basis of normal sarcomeric architecture within cardiomyocytes. Given that CAR is upregulated in the adult heart upon ischemia induced infarction or dilated cardiomyopathy, it is possible that CAR might also be involved in repair mechanisms in the adult heart.

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## References

- Aurrand-Lions, M. A., Duncan, L., Du Pasquier, L. and Imhof, B. A. (2000). Cloning of JAM-2 and JAM-3: an emerging junctional adhesion molecular family? *Curr. Top. Microbiol. Immunol.* **251**, 91-98.
- Berdougo, E., Coleman, H., Lee, D. H., Stainier, D. Y. and Yelon, D. (2003). Mutation of weak atrium/atrial myosin heavy chain disrupts atrial function and influences ventricular morphogenesis in zebrafish. *Development* **130**, 6121-6129.
- Bergelson, J. M., Cunningham, J. A., Droguett, G., Kurt-Jones, E. A., Krithivas, A., Hong, J. S., Horwitz, M. S., Crowell, R. L. and Finberg, R. W. (1997). Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science* **275**, 1320-1323.
- Carson, S. D., Hobbs, J. T., Tracy, S. M. and Chapman, N. M. (1999). Expression of the coxsackievirus and adenovirus receptor in cultured human umbilical vein endothelial cells: regulation in response to cell density. *J. Virol.* **73**, 7077-7079.
- Cohen, C. J., Shieh, J. T., Pickles, R. J., Okegawa, T., Hsieh, J. T. and Bergelson, J. M. (2001). The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. *Proc. Natl. Acad. Sci. USA* **98**, 15191-15196.
- Conway, S. J., Kruzynska-Frejtak, A., Kneer, P. L., Machnicki, M. and Koushik, S. V. (2003). What cardiovascular defect does my parental mouse mutant have, and why? *Genesis* **35**, 1-21.
- Coyné, C. B., Voelker, T., Pichla, S. L. and Bergelson, J. M. (2004). The coxsackievirus and adenovirus receptor interacts with the multi-PDZ domain protein-1 (MUPP-1) within the tight junction. *J. Biol. Chem.* **279**, 48079-48084.
- Du Pasquier, L., Courtet, M. and Chretien, I. (1999). Duplication and MHC linkage of the CTX family of genes in Xenopus and in mammals. *Eur. J. Immunol.* **29**, 1729-1739.
- Ebnet, K., Schulz, C. U., Meyer-zu-Brickwedde, M.-K., Pendl, G. G. and Vestweber, D. (2000). Junctional Adhesion Molecule (JAM) interacts with

- the PDZ domain containing proteins AF-6 and ZO-1. *J. Biol. Chem.* **275**, 27979-27988.
- Ebnet, K., Suzuki, A., Horikoshi, Y., Hirose, T., Meyer-zu-Brickwedde, M.-K., Ohno, S. and Vestweber, D. (2001). The cell polarity protein ASIP/Par-3 directly associates with junctional adhesion molecule (JAM). *EMBO J.* **20**, 3738-3748.
- Ebnet, K., Aurrand-Lions, M., Kuhn, A., Kiefer, F., Butz, S., Zander, K., Brickwedde, M. K., Suzuki, A., Imhof, B. A. and Vestweber, D. (2003). The junctional adhesion molecule (JAM) family members JAM-2 and JAM-3 associate with the cell polarity protein Par-3: a possible role for JAMs in endothelial cell polarity. *J. Cell Sci.* **116**, 3879-3891.
- Ebnet, K., Suzuki, A., Ohno, S. and Vestweber, D. (2004). Junctional adhesion molecules (JAMs): more molecules with dual functions? *J. Cell Sci.* **117**, 19-29.
- Excoffon, K. J., Hruska-Hageman, A., Klotz, M., Traver, G. L. and Zanner, J. (2004). A role for the PDZ-binding domain of the coxsackie B virus and adenovirus receptor (CAR) in cell adhesion and growth. *J. Cell Sci.* **117**, 4401-4409.
- Fassler, R., Rohwedel, J., Maltsev, V., Bloch, W., Lentini, S., Guan, K., Gullberg, D., Hescheler, J., Addicks, K. and Wobus, A. M. (1996). Differentiation and integrity of cardiac muscle cells are impaired in the absence of beta 1 integrin. *J. Cell Sci.* **109**, 2989-2999.
- Fechner, H., Noutsias, M., Tschoepe, C., Hinze, K., Wang, X., Escher, F., Pauschinger, M., Dekkers, D., Vetter, R., Paul, M. et al. (2003). Induction of coxsackievirus-adenovirus-receptor expression during myocardial tissue formation and remodeling: identification of a cell-to-cell contact-dependent regulatory mechanism. *Circulation* **107**, 876-882.
- Fedrowitz, M., Westermann, J. and Loscher, W. (2002). Magnetic field exposure increases cell proliferation but does not affect melatonin levels in the mammary gland of female Sprague Dawley rats. *Cancer Res.* **62**, 1356-1363.
- Gliki, G., Ebnet, K., Aurrand-Lions, M., Imhof, B. A. and Adams, R. H. (2004). Spermatid differentiation requires the assembly of a cell polarity complex downstream of junctional adhesion molecule-C. *Nature* **431**, 320-324.
- Goncharova, E. J., Kam, Z. and Geiger, B. (1992). The involvement of adherens junction components in myofibrillogenesis in cultured cardiac myocytes. *Development* **114**, 173-183.
- Hirata, K., Ishida, T., Penta, K., Rezaee, M., Yang, E., Wohlgemuth, J. and Quertermous, T. (2001). Cloning of an immunoglobulin family adhesion molecule selectively expressed by endothelial cells. *J. Biol. Chem.* **276**, 16223-16231.
- Hogan, B., Beddington, R., Costantini, F. and Lacy, E. (1994). *Manipulating the Mouse Embryo: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.
- Hogers, B., DeRuiter, M. C., Gittenberger-de Groot, A. C. and Poelmann, R. E. (1999). Extraembryonic venous obstructions lead to cardiovascular malformations and can be embryolethal. *Cardiovasc. Res.* **41**, 87-99.
- Honda, T., Saitoh, H., Masuko, M., Katagiri-Abe, T., Tominaga, K., Kozakai, I., Kobayashi, K., Kumanishi, T., Watanabe, Y. G., Odani, S. et al. (2000). The coxsackievirus-adenovirus receptor protein as a cell adhesion molecule in the developing mouse brain. *Mol. Brain Res.* **77**, 19-28.
- Huang, C., Sheikh, F., Hollander, M., Cai, C., Becker, D., Chu, P. H., Evans, S. and Chen, J. (2003). Embryonic atrial function is essential for mouse embryogenesis, cardiac morphogenesis and angiogenesis. *Development* **130**, 6111-6119.
- Ishida, T., Kundu, R. K., Yang, E., Hirata, K., Ho, Y. D. and Quertermous, T. (2003). Targeted disruption of endothelial cell-selective adhesion molecule inhibits angiogenic processes in vitro and in vivo. *J. Biol. Chem.* **278**, 34598-34604.
- Ito, M., Kodama, M., Masuko, M., Yamaura, M., Fuse, K., Uesugi, Y., Hirono, S., Okura, Y., Kato, K., Hotta, Y. et al. (2000). Expression of coxsackievirus and adenovirus receptor in hearts of rats with experimental autoimmune myocarditis. *Circ. Res.* **86**, 275-280.
- Itoh, M., Sasaki, H., Furuse, M., Ozaki, H., Kita, T. and Tsukita, S. (2001). Junctional adhesion molecule (JAM) binds to Par-3: a possible mechanism for the recruitment of Par-3 to tight junctions. *J. Cell Biol.* **154**, 491-497.
- Joberty, G., Petersen, C., Gao, L. and Macara, I. G. (2000). The cell-polarity protein Par6 links Par3 and atypical protein kinase C to Cdc42. *Nat. Cell Biol.* **2**, 531-539.
- Johansson, A., Driessens, M. and Aspenstrom, P. (2000). The mammalian homologue of the *Caenorhabditis elegans* polarity protein Par-6 is a binding partner for the Rho GTPases Cdc42 and Rac1. *J. Cell Sci.* **113**, 3267-3275.
- Joyner, A. L. (1993). *Gene Targeting*. Oxford: Oxford University Press.
- Kashimura, T., Kodama, M., Hotta, Y., Hosoya, J., Yoshida, K., Ozawa, T., Watanabe, R., Okura, Y., Kato, K., Hanawa, H. et al. (2004). Spatiotemporal changes of coxsackievirus and adenovirus receptor in rat hearts during postnatal development and in cultured cardiomyocytes of neonatal rat. *Virchows Arch.* **444**, 283-292.
- Koi, H., Zhang, J., Makrigiannakis, A., Getsios, S., MacCalman, C. D., Kopf, G. S., Strauss, J. F. and Parry, S. (2001). Differential expression of the coxsackievirus and adenovirus receptor regulates adenovirus infection of the placenta. *Biol. Reprod.* **64**, 1001-1009.
- Kubalak, S. W., Doevendans, P. A., Rockman, H. A., Hunter, J. J., Tanaka, N., Ross, J. and Chien, K. R. (1995). Molecular analysis of cardiac muscle disease based on mouse genetics. In *Methods in Molecular Genetics*, Vol. 8 (ed. K. W. Adolph), pp. 470-487. Orlando, FL, USA: Academic Press.
- Kuhn, A., Brachtendorf, G., Kurth, F., Sonntag, M., Samulowitz, U., Kopf, G. S. and Vestweber, D. (2002). Expression of endomucin, a novel endothelial sialomucin, in normal and diseased human skin. *J. Invest. Dermatol.* **119**, 1388-1393.
- Kuhn, R., Rajewsky, K. and Muller, W. (1991). Generation and analysis of interleukin-4 deficient mice. *Science* **254**, 707-710.
- Lenter, M., Uhlig, H., Hamann, A., Jenö, P., Imhof, B. and Vestweber, D. (1993). A monoclonal antibody against an activation epitope on mouse integrin chain  $\beta_1$  blocks adhesion of lymphocytes to the endothelial integrin  $\alpha_6\beta_1$ . *Proc. Natl. Acad. Sci. USA* **90**, 9051-9055.
- Lin, D., Edwards, A. S., Fawcett, J. P., Mbamalu, G., Scott, J. D. and Pawson, T. (2000). A mammalian Par-3-Par-6 complex implicated in Cdc42/Rac1 and aPKC signalling and cell polarity. *Nat. Cell Biol.* **2**, 540-547.
- Lin, Z. X., Holtzer, S., Schultheiss, T., Murray, J., Masaki, T., Fischman, D. A. and Holtzer, H. (1989). Polygons and adhesion plaques and the disassembly and assembly of myofibrils in cardiac myocytes. *J. Cell Biol.* **108**, 2355-2367.
- Luo, Y. and Radice, G. L. (2003). Cadherin-mediated adhesion is essential for myofibril continuity across the plasma membrane but not for assembly of the contractile apparatus. *J. Cell Sci.* **116**, 1471-1479.
- Ma, Q., Chen, Z., del Barco Barrantes, I., de la Pompa, J. L. and Anderson, D. J. (1998). neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. *Neuron* **20**, 469-482.
- Martin-Padura, I., Lostaglio, S., Schneemann, M., Williams, L., Romano, M., Fruscella, P., Panzeri, C., Stoppacciaro, A., Ruco, L., Villa, A. et al. (1998). Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *J. Cell Biol.* **142**, 117-127.
- Morgan, S. M., Samulowitz, U., Darley, L., Simmons, D. L. and Vestweber, D. (1999). Biochemical characterization and molecular cloning of a novel endothelial-specific sialomucin. *Blood* **93**, 165-175.
- Nasdale, I., Wolburg-Buchholz, K., Wolburg, H., Kuhn, A., Ebnet, K., Brachtendorf, G., Samulowitz, U., Kuster, B., Engelhardt, B., Vestweber, D. et al. (2002). A transmembrane tight junction protein selectively expressed on endothelial cells and platelets. *J. Biol. Chem.* **277**, 16294-16303.
- Nasuno, A., Toba, K., Ozawa, T., Hanawa, H., Osman, Y., Hotta, Y., Yoshida, K., Saigawa, T., Kato, K., Kuwano, R. et al. (2004). Expression of coxsackievirus and adenovirus receptor in neointima of the rat carotid artery. *Cardiovasc. Pathol.* **13**, 79-84.
- Noutsias, M., Fechner, H., de Jonge, H., Wang, X., Dekkers, D., Houtsmuller, A. B., Pauschinger, M., Bergelson, J., Warraich, R., Yacoub, M. et al. (2001). Human coxsackie-adenovirus receptor is colocalized with integrins  $\alpha(v)\beta(3)$  and  $\alpha(v)\beta(5)$  on the cardiomyocyte sarcolemma and upregulated in dilated cardiomyopathy: implications for cardiotropic viral infections. *Circulation* **104**, 275-280.
- Okegawa, T., Pong, R. C., Li, Y., Bergelson, J. M., Sagalowsky, A. I. and Hsieh, J. T. (2001). The mechanism of the growth-inhibitory effect of coxsackie and adenovirus receptor (CAR) on human bladder cancer: a functional analysis of car protein structure. *Cancer Res.* **61**, 6592-6600.
- Philipson, L. and Pettersson, R. F. (2004). The coxsackie-adenovirus receptor—a new receptor in the immunoglobulin family involved in cell adhesion. *Curr. Top. Microbiol. Immunol.* **273**, 87-111.
- Rahimtoola, S. H. (1985). A perspective on the three large multicenter randomized clinical trials of coronary bypass surgery for chronic stable angina. *Circulation* **72**, V123-V35.
- Rossant, J. and Cross, J. C. (2001). Placental development: lessons from mouse mutants. *Nat. Rev. Genet.* **2**, 538-548.



- Soler, A. P. and Knudsen, K. A.** (1994). N-cadherin involvement in cardiac myocyte interaction and myofibrillogenesis. *Dev. Biol.* **162**, 9-17.
- Sollerbrant, K., Raschperger, E., Mirza, M., Engstrom, U., Philipson, L., Ljungdahl, P. O. and Pettersson, R. F.** (2003). The Coxsackievirus and adenovirus receptor (CAR) forms a complex with the PDZ domain-containing protein ligand-of-numb protein-X (LNX). *J. Biol. Chem.* **278**, 7439-7444.
- Tomko, R. P., Xu, R. and Philipson, L.** (1997). HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. *Proc. Natl. Acad. Sci. USA* **94**, 3352-3356.
- Tomko, R. P., Johansson, C. B., Totrov, M., Abagyan, R., Frisen, J. and Philipson, L.** (2000). Expression of the adenovirus receptor and its interaction with the fiber knob. *Exp. Cell Res.* **255**, 47-55.
- Vecchi, A., Garlanda, C., Lampugnani, M. G., Resnati, M., Matteucci, C., Stoppacciaro, A., Schnurch, H., Risau, W., Ruco, L., Mantovani, A. et al.** (1994). Monoclonal antibodies specific for endothelial cells of mouse blood vessels. Their application in the identification of adult and embryonic endothelium. *Eur. J. Cell Biol.* **63**, 247-254.
- Vincent, T., Pettersson, R. F., Crystal, R. G. and Leopold, P. L.** (2004). Cytokine-mediated downregulation of coxsackievirus-adenovirus receptor in endothelial cells. *J. Virol.* **78**, 8047-8058.
- Walters, R. W., Freimuth, P., Moninger, T. O., Ganske, I., Zabner, J. and Welsh, M.** (2002). Adenovirus fiber disrupts CAR-mediated intercellular adhesion allowing virus escape. *Cell* **110**, 789-799.
- Wegmann, F., Ebnet, K., Du Pasquier, L., Vestweber, D. and Butz, S.** (2004). Endothelial adhesion molecule ESAM binds directly to the multidomain adaptor MAGI-1 and recruits it to cell contacts. *Exp. Cell Res.* **300**, 121-133.
- Wu, J.-C., Chung, T.-H., Tseng, Y.-Z. and Wang, S.-M.** (1999). Ncadherin/catenin-based costameres in cultured chicken cardiomyocytes. *J. Cell. Biochem.* **75**, 93-104.