after RNase pretreatment. For quantitative autoradiography, average optical densities (OD) over the dorsal striatum were measured on X-ray film autoradiographs using an IBAS image analyser (Zeiss Kontron). The measurement field was interactively defined and 512×512 pixel, 256 grey-level images were generated using a MTI video camera with fixed black level and gain. The background level was subtracted for each section, and mean OD was determined for right and left striata. Five or more sections per mouse were analysed, and results were analysed by the Mann–Whitney *U*-test. Autoradiography binding assays have been described²⁷ and used [³H]CGS 21680 (NEN; $48.6 \text{ Ci mmol}^{-1}$) as ligand and $10 \mu\text{M}$ NECA as competitor.

Blood pressure and heart rate. Mice were anaesthetized using halothane inhalation (1–2% in oxygen). The left carotid artery was isolated and a PE-10 tubing catheter was inserted into the artery, secured with silk, and tunnelled subcutaneously to exit at the back of the neck. Mice were allowed to recover from surgery for at least 4 h. The arterial line was connected to a pressure transducer, and blood pressure and heart rate were measured using a computerized data acquisition system. For agonist study, a bolus of CGS 21680 (1 mg kg^{-1}) was administered through the arterial catheter.

Platelet aggregation. Blood was collected by cardiac puncture on sodium citrate (0.38%) and pooled from about 10 mice. Platelet-rich plasma was prepared by centrifugation and adjusted to $1.75 \times 10^8 \text{ ml}^{-1}$. Platelets were aggregated using a platelet-aggregation profiler (model PAP-4; Bio Data). Platelet-rich plasma ($100 \mu\text{l}$) was incubated for 5 min in siliconized cuvettes at 37°C , and aggregation initiated by addition of $5 \mu\text{M}$ ADP.

Behavioural studies. Male mice were kept on a 7 a.m.–7 p.m. light cycle. Exploratory activity (horizontal and vertical) was monitored in the $20 \times 20 \text{ cm}$ arena of a Digiscan actimeter (Omnitech Electronics) during a 60-min period. An elevated plus-maze, with $18 \times 6 \text{ cm}$ arms, was placed 50 cm above the floor. The mouse was placed at the centre of the maze, with head facing a closed arm. The number of entries, time spent and distance travelled in each arm and central area over a 5-min period was recorded by an automated image analysis system (Videotrack 512 system, Viewpoint). For the black and white compartments test, a 5-cm opening connected the compartments ($32 \times 22 \text{ cm}$, 18 cm height). Mice were placed in the black compartment (head facing a corner). The delay for first entering into the brightly lit white compartment, the time spent in the white compartment, and the number of entries were recorded for 5 min. The hot-plate test has been described²⁸; the tail-flick test was done by using a tail-flick apparatus. Two measurements were made on each mouse at 3 and 5 cm from the tail tip. Aggressive behaviour was assayed by the resident–intruder test¹⁵, with slight modifications. Resident male $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ mice (14 weeks old) were isolated in cages ($27 \times 13 \times 13 \text{ cm}$) without replacement of the litter for the four weeks preceding the test. 8-week-old outbred male CD1 mice, housed eight per cage, were used as intruders. A randomly chosen intruder, used only once in each session, was introduced in the resident cage, and aggressive behaviour recorded during a 5-min period.

Statistical analysis. Unless otherwise state, statistical analysis was done by the two-tailed unpaired Student's *t*-test (Instat program, GraphPad Software). The two-tailed non-parametric Mann–Whitney *U*-test was used when variances were not equal among series.

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Altered pain perception and inflammatory response in mice lacking prostacyclin receptor

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Prostanoids are a group of bioactive lipids working as local mediators¹ and include D, E, F and I types of prostaglandins (PGs) and thromboxanes. Prostacyclin (PGI₂) acts on platelets and blood vessels to inhibit platelet aggregation and to cause vasodilatation, and is thought to be important for vascular homeostasis². Aspirin-like drugs, including indomethacin,

which inhibit prostanoid biosynthesis, suppress fever, inflammatory swelling and pain, and interfere with female reproduction, suggesting that prostanoids are involved in these processes^{1,3}, although it is not clear which prostanoid is the endogenous mediator of a particular process. Prostanoids act on seven-transmembrane-domain receptors which are selective for each type⁴. Here we disrupt the gene for the prostacyclin receptor⁵ in mice by using homologous recombination. The receptor-deficient mice are viable, reproductive and normotensive. However, their susceptibility to thrombosis is increased, and their inflammatory and pain responses are reduced to the levels observed in indomethacin-treated wild-type mice. Our results establish that prostacyclin is an antithrombotic agent *in vivo* and provide evidence for its role as a mediator of inflammation and pain.

We disrupted the mouse gene encoding the prostacyclin receptor (*IP*) (Fig. 1a) and established two lines of embryonic stem cells.

Chimaeric mice were generated from each line. The mice were backcrossed with C57BL/6 mice and the resulting heterozygous littermates (*IP*^{+/-}) were bred to produce homozygous mice (Fig. 1b). Breeding of heterozygotes produced homozygous *IP*^{-/-} mice at a rate lower than expected from the mendelian frequency; 30 (+/+), 72 (+/-) and 19 (-/-) males and 48 (+/+), 80 (+/-) and 32 (-/-) females. This suggests that there may be prenatal mortality in *IP*-deficient mice, especially in males. A similar abnormal mendelian ratio of male F₂ progeny was reported for cyclooxygenase-2-deficient mice⁶. Homozygous mice that survived to birth grew normally, lived longer than one year, and were fertile. No morphological or histological abnormalities were detected. We analysed one line of *IP* mutant mice in detail.

Loss of the functional *IP* receptor in *IP*^{-/-} mice was verified by the inability of a prostacyclin analogue, cicaprost⁷, to inhibit ADP-induced platelet aggregation (Fig. 1c). In addition, aortic

Figure 1 Targeted disruption of the *IP* gene and loss of the functional receptor. **a**, Restriction maps for the targeting vector (top), the wild-type *IP* receptor allele (middle) and the targeted allele (bottom). Exons are shown by closed boxes. *NEO*, neomycin-resistant gene; *TK*, thymidine kinase gene; N, *Nco*I; E, *Eco*T221. **b**, Southern blot analysis of genomic DNA of heterozygous offspring. *Nco*I- and *Eco*T221-digested genomic DNA was hybridized with the external probe as shown in **a**. +/+, Wild type; +/-, heterozygote; -/-, homozygote. **c**, Platelet aggregation. Platelet-rich plasma was prepared and pretreated with (+) and without (-) 10 nM cicaprost (CP) for 1 min. Aggregation was initiated by addition of 5 µM ADP (arrow) and monitored by following the change in turbidity. **d**, Relaxation of aortic smooth muscle. The aortic ring from wild-type (+/+) and *IP*^{-/-} animals was contracted with 0.1 µM noradrenaline. The relaxation response to 1 µM cicaprost is shown. **e**, Blood pressure response to cicaprost in wild-type (+/+) (top) and *IP*^{-/-} (bottom) mice.

Figure 2 Arterial thrombosis in wild-type and IP-deficient homozygous mice. **a**, Thrombosis induced by ferric chloride. Five arterial preparations each from wild-type (left) and $IP^{-/-}$ mice (right) are shown. Middle, preparation from a sham-operated homozygous animal. **b**, Thrombotic scores in wild-type (+/+) (left) and $IP^{-/-}$ (right) mice. Thrombosis was scored as follows: 0, without apparent

thrombus; 1, small and isolated thrombi; 2, mural thrombi; 3, partially occlusive thrombi; 4, occlusive thrombi. Results from fifteen mice (a total of 30 arteries) are shown for each. There is a significant difference at $P < 0.01$ between wild-type and $IP^{-/-}$ mice by the Mann-Whitney U -test.

Figure 3 Inflammatory responses in IP-deficient mice. **a**, Vascular permeability to exogenous prostacyclin and PGE₂ in wild-type and $IP^{-/-}$ mice. Either prostacyclin ($n = 6$) or PGE₂ ($n = 5$) (1 nmol of each) was injected intradermally alone or with 0.1 nmol bradykinin. Vascular permeability was examined as described in Methods. **b**, Carrageenin-induced paw oedema. 30 μ l of 2% carrageenin was

injected into the right hind paw of mice and the increase in paw volume was monitored. Indomethacin (Ind) (10 mg kg⁻¹) was administered intraperitoneally 30 min before carrageenin treatment. White squares, wild-type ($n = 9$); white circles, $IP^{-/-}$ mice ($n = 10$); black squares, wild-type mice treated with indomethacin ($n = 8$); black circles, $IP^{-/-}$ mice treated with indomethacin ($n = 7$). *, $P < 0.01$.

rings isolated from IP-deficient homozygous mice did not relax in response to cicaprost, although the aorta from wild-type mice did (Fig. 1d). Furthermore, intravenous injection of cicaprost (1 μ g kg⁻¹) caused hypotension of ~30 mm Hg in anaesthetized wild-type mice, whereas there was no change in blood pressure in IP-deficient mice even at a cicaprost dose of 10 μ g kg⁻¹ (Fig. 1e). On the other hand, intravenous administration of the prostaglandin PGE₂ caused transient hypotension in both types of animals; a drop of 24.0 ± 2.8 ($n = 5$) and 23.2 ± 1.7 mm Hg ($n = 5$) at 10 μ g kg⁻¹ of PGE₂ for wild-type and IP-deficient mice, respectively. These results verified that specific disruption of the IP receptor had occurred, and further indicate that a single type of IP receptor mediates the actions of prostacyclin in both platelets and vascular smooth muscles. The basal blood pressure and heart rate of IP-deficient mice were identical with those of wild-type mice in the conscious state: the mean blood pressure and heart rate were 88 ± 3 mm Hg and 646 ± 23 beats per min ($n = 10$), and 85 ± 3 mm Hg and 645 ± 21 beats per min ($n = 10$) for wild-type and IP-deficient mice, respectively. The bleeding time in IP-deficient mice was normal, being 87 ± 10 and 90 ± 11 s for wild-type and $IP^{-/-}$ mice, respectively ($n = 12$ each). However, when a prostacyclin mimetic, beraprost⁸, was administered *per os* (3 mg kg⁻¹),

it prolonged the bleeding time of wild-type mice (>10 min; $n = 6$) but not that of IP-deficient mice (117 ± 14 s; $n = 6$). These results suggest that prostacyclin is not involved in the regulation of heart rate, blood pressure and bleeding under basal conditions. This is in contrast with the findings on another endothelium-derived vasorelaxant, nitric oxide; mice deficient in endothelial nitric oxide synthase are hypertensive under basal conditions⁹.

Endogenous prostacyclin has long been thought to be involved in the prevention of thrombosis and vasospasm². We therefore evaluated the thrombotic tendency of IP-deficient mice. We first confirmed that $IP^{-/-}$ mice could synthesize prostacyclin. Isolated aortas from $IP^{-/-}$ mice produced 126 ± 22 pmol ($n = 5$) of prostacyclin, determined as the stable metabolite 6-keto-PGF_{1 α} that was produced per mg wet weight in 20 min. These levels were similar to those of wild-type mice (177 ± 40 pmol; $n = 5$). The bilateral carotid arteries of wild-type and $IP^{-/-}$ mice were then exposed and topically painted with 7.5% FeCl₃ solution, a procedure known to injure the endothelium¹⁰. Occlusion of the arterial flow and thrombus formation were examined after 4 h. As shown in Fig. 2a, most of the arteries of wild-type mice showed only mural thrombi of yellowish colour. In contrast, about two-thirds showed obstructive

Figure 4 Nociceptive responses in IP-deficient mice. **a**, Acetic acid writhing. Acetic acid was injected intraperitoneally into wild-type mice ($n = 8$), wild-type mice treated with indomethacin (Ind) ($n = 8$), $IP^{-/-}$ mice ($n = 9$) and $IP^{-/-}$ mice treated with indomethacin ($n = 6$), and the writhing responses were counted for 30 min. Indomethacin was administered as for Fig. 3. *, $P < 0.01$. **b**, Production of prostacyclin and PGE₂ in the peritoneal cavity of mice treated with acetic acid. Diluted acetic acid was injected i.p. and the peritoneal fluid collected after 15 min

for measuring PGs. Results are means $\pm$ s.e.m. (n was 4–5). Ind, indomethacin-pretreatment. **c**, Writhing responses to exogenous prostacyclin and PGE₂. 2 μ g of each was administered intraperitoneally to wild-type (+/+) and IP-deficient (-/-) mice, and the writhing response was counted in affected animals for 15 min. The numbers of animals used were 20 for prostacyclin injection and 16 for PGE₂ injection.

thrombi with reddish tails (in $IP^{-/-}$ mice) (Fig. 2b). In another experiment monitoring the effects of the FeCl₃ treatment for a longer period, 30% of IP-deficient mice (4 out of 12) died within 1 day owing to bilateral occlusions of the carotid arteries and/or embolic stroke, whereas the wild-type mice ($n = 13$) all survived this period.

Acute inflammation is characterized by hyperaemia, oedema, leukocyte migration and pain. Prostanoids are thought to contribute to these processes by causing peripheral vasodilation and by sensitizing primary afferents to noxious stimuli³. PGE₂ has long been regarded as a principal mediator of these processes, although several prostanoids show similar effects^{11,12}. We therefore first compared the effects of exogenously added PGE₂ and prostacyclin on vascular permeability. As shown in Fig. 3a, both PGE₂ and prostacyclin potentiated the bradykinin-induced permeability to a similar extent in wild-type mice; $IP^{-/-}$ mice showed a response to PGE₂ only. We then examined the role of endogenous prostacyclin in inflammation by applying the carrageenin-induced paw-oedema model¹³ to $IP^{-/-}$ mice. The carrageenin injection caused a time-dependent increase in the volume of the injected paw in wild-type animals as a result of oedema (Fig. 3b). This increase is suppressed by 50% by pretreatment with indomethacin. Unexpectedly, the progress of oedema was reduced in IP-deficient mice to the same extent as in indomethacin-treated animals, indicating that prostacyclin is a major prostanoid eliciting the inflammatory response in this model. The proinflammatory role of prostacyclin was also tested in another model, carrageenin-induced pleurisy¹⁴, in which carrageenin was injected into the pleural cavity of mice and exudate was collected after 3 h. A significant reduction of the exudate volume was observed in IP-deficient mice ($P < 0.05$) (data not shown).

The role of prostacyclin in inflammatory pain was next examined in IP-deficient mice. Our interest in the involvement of IP in pain originates from our findings that IP receptor messenger RNA is abundantly expressed and coexists with substance-P mRNA in neurons of the dorsal root ganglion¹⁵. Prostanoids may produce hyperalgesia not only by peripheral sensitization³ but also by facilitating pain transmission at the spinal¹⁶ and supraspinal¹⁷ levels. IP involvement in the latter mechanisms was tested by the tail-flick method^{18,19} and by the hot-plate test¹⁸. The latent period before response in both tests was not significantly different in wild-type and IP-deficient mice (data not shown), suggesting that the receptor IP is not involved in nociceptive transmission at the spinal and supraspinal levels. We next used the acetic acid-induced wri-

thing test²⁰ to examine the involvement of IP in inflammatory pain. Injection of a dilute acetic acid solution intraperitoneally caused about forty writhing responses in wild-type animals; this reaction was significantly reduced by pretreatment with indomethacin (Fig. 4a). Surprisingly, the response of the IP-deficient mice was comparable to that of the indomethacin-pretreated animals and markedly reduced compared to wild-type mice, indicating that IP is involved in this nociceptive response. Under these conditions, acetic acid injection produced similar amounts of prostacyclin in wild-type and $IP^{-/-}$ mice (Fig. 4b). PGE₂ was also produced, but in about one quarter of the amount of prostacyclin. The same profile for PG production has been reported in the peritoneal cavity of zymosan-injected mice²¹. Because PGE₂ may be involved in pain response, we compared the writhing responses to prostacyclin and PGE₂ by injecting the same amount of each PG intraperitoneally into these animals (Fig. 4c). Prostacyclin caused the writhing response in 60% of the wild-type mice and in none of the IP-deficient animals, whereas PGE₂ induced the response in less than 25% animals in both types.

In conclusion, we have established the antithrombotic role of endogenous prostacyclin. As prostacyclin has also been implicated in atherosclerosis and diabetic angiopathy^{2,22}, IP-deficient mice may be useful for examining its role in these diseases. Our results indicate that prostacyclin is a mediator of inflammatory swelling and pain. Whether prostacyclin and PGE₂ have different, context-dependent roles in these processes is an important issue that could be tested by comparing the response to various stimuli at different sites in IP-deficient mice and in mice deficient in PGE receptors. □

Methods

Gene targeting. Murine IP genomic clones were isolated from a 129/Sv-strain genomic DNA library (Stratagene) by using its cDNA³ as a probe. The targeting vector was constructed by replacing a 2.4-kb fragment containing parts of the putative exons II and III, which encode the sixth transmembrane domain towards the C terminus of this seven-transmembrane-domain receptor⁵, with the neomycin-resistance gene (pMC1-neo; Stratagene). The herpes simplex virus thymidine kinase gene (TK) was inserted downstream (Fig. 1a). The targeting vector was linearized with Asp718 and introduced into E14-1 ES cells by electroporation. G418- and gancyclovir-resistant clones were isolated and screened by PCR for homologous recombination, which was then confirmed by Southern blot hybridization. The generation of chimaeras and mutant mice has been described²³.

Bioassays. Blood was drawn from the heart and mixed with an equal volume of 20 mM HEPES, pH 7.4. Sodium citrate (0.38%) was used as anticoagulant.

Platelet-rich plasma was obtained by centrifugation, and diluted to 5×10^5 platelets per μl with plasma. Platelet aggregation was assayed as described⁷. An aortic ring 4 mm long was prepared from thoracic aorta and mounted on a pair of wires in an organ bath filled with Krebs–Henseleit buffer gassed with 95% O_2 –5% CO_2 at 37 °C. Preload was 0.5 g. The ring was contracted with 0.1 μM noradrenaline and its relaxation response to various concentrations of cicaprost tested.

Blood pressure, heart rate and bleeding time. Basal blood pressure and heart rate were measured in conscious animals by using the tail-cuff method. In the analysis of cicaprost- or PGE_2 -induced hypotension, animals were anaesthetized with pentobarbital sodium (60 mg kg^{-1} ; i.p.), and arterial blood pressure was monitored through a cannula into the carotid artery. Bleeding time was measured as described²⁴.

Prostanoid production. Isolated aorta was preincubated in oxygenated Krebs–Henseleit buffer at 37 °C for 1 h and then incubated in fresh buffer for 20 min; the supernatant was assayed. Prostanoids in peritoneal lavage were extracted with Sep-Pak C18 columns and then assayed for 6-keto-PGF $_{1\alpha}$ and PGE_2 (ref. 25).

Analysis of thrombus formation. Mice (24–30 g) were anaesthetized with halothane and the carotid arteries exposed by a cervical incision. A piece of Parafilm 1 mm wide was inserted under the artery and 5 μl of a 7.5% FeCl_3 solution was dropped on the artery¹⁰. After 1 min the solution was wiped off, and the mice were allowed to recover from the anaesthetic. After 4 h, the arteries and blood flow were examined microscopically by cutting the distal end of the artery. A 5-mm long segment containing the affected region was excised and fixed with 10% formaldehyde. Thrombosis-induced death of animals treated with 5% FeCl_3 solution was recorded after 24 h.

Inflammation. Vascular permeability was tested using Pontamine skyblue as described²⁶. PGs alone, bradykinin alone, or both were intradermally injected. After 40 min, mice were killed. The exuded dye in the skin was extracted and the amounts determined. Permeability was expressed as a percentage of that induced by 10 nmol bradykinin alone in the same animals. Carrageenin-induced pleurisy was evoked by injecting 40 μl of 2% carrageenin into the right pleural cavity of mice¹⁴. After 3 h, they were killed and the exudate volume measured.

Nociceptive tests. The tail-flick response was evoked either by heat¹⁸ or by applying pressure¹⁹. Latencies were determined by using a tail-flick apparatus (Ugo Basile) and with an analgesy meter (Ugo Basile), respectively. In the hot-plate test, mice were placed on a plate (55 ± 0.1 °C) and the latency for jumping determined as described¹⁸. In the writhing test²⁰, mice were intraperitoneally injected with either 0.9% acetic acid (5 ml kg^{-1}) or PGs (2 μg). The frequency of stretch responses was counted for 30 min for acetic acid or for 15 min for PGs. Indomethacin (10 mg kg^{-1}) was administered intraperitoneally 30 min before acetic acid injection.

Data analysis. Data are expressed as means $\pm$ s.e.m.. The significance of difference between groups was evaluated using ANOVA with a subsequent Dunnett's test, except for thrombus formation (Fig. 2 legend).

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Crustacean appendage evolution associated with changes in Hox gene expression

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Homeotic (Hox) genes specify the differential identity of segments along the body axis of insects. Changes in the segmental organization of arthropod bodies may therefore be driven by changes in the function of Hox genes^{1–3}, but so far this has been difficult to demonstrate. We show here that changes in the expression pattern of the Hox genes *Ubx* and *AbdA* in different crustaceans correlate well with the modification of their anterior thoracic limbs into feeding appendages (maxillipeds). Our observations provide direct evidence that major morphological changes in arthropod body plans are associated with changes in Hox gene regulation. They suggest that homeotic changes^{1,4} may play a role in the normal process of adaptive evolutionary change.

Genetic manipulation of model organisms like *Drosophila* can reveal the potential of developmental systems to undergo particular types of morphological change: for example, patterns of segmental specialization may be altered by changing the function of Hox genes. This approach alone, however, cannot identify the actual genetic changes that take place over macro-evolutionary timescales. Comparative studies are required but these so far indicate that crustaceans and insects share almost identical complements of Hox genes⁵ and that the domains of Hox gene expression have been broadly conserved during insect evolution (reviewed in ref. 6). Initial comparisons of Hox gene expression between crustaceans and insects documented changes associated with the independent specialization of trunk regions (thorax and abdomen) in these