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The collagen receptor DDR2 regulates proliferation and its elimination leads to dwarfism

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The discoidin domain receptor 2 (DDR2) is a member of a subfamily of receptor tyrosine kinases whose ligands are fibrillar collagens, and is widely expressed in postnatal tissues. We have generated DDR2-deficient mice to establish the *in vivo* functions of this receptor, which have remained obscure. These mice exhibit dwarfism and shortening of long bones. This phenotype appears to be caused by reduced chondrocyte proliferation, rather than aberrant differentiation or function. In a skin wound healing model, DDR2–/– mice exhibit a reduced proliferative response compared with wild-type littermates. *In vitro*, fibroblasts derived from DDR2–/– mutants proliferate more slowly than wild-type fibroblasts, a defect that is rescued by introduction of wild-type but not kinase-dead DDR2 receptor. Together our results suggest that DDR2 acts as an extracellular matrix sensor to modulate cell proliferation.

INTRODUCTION

One defining characteristic of metazoans is the ability of their cells to communicate with one another and with the extracellular matrix (ECM) to co-ordinate their functions. This communication is mediated to a large extent by the family of receptor tyrosine kinases (RTKs) and their ligands. Most RTKs bind soluble growth factors, and thereby mediate diverse cellular responses, including proliferation, differentiation, migration and survival. There are two receptors of the discoidin domain RTK subfamily that bind and are activated by collagen, in particular the fibril-forming collagens (Shrivastava *et al.*, 1997; Vogel *et al.*, 1997).

Little is known about the possible downstream events mediated by discoidin domain receptors (DDRs). DDR1 is able to bind the adaptor protein Shc, however, the MAP kinase pathway is apparently not activated under these conditions (Vogel *et al.*, 1997, 2000). *In vitro* activation of DDR2 has been reported to induce expression of the matrix metalloproteinase 1 (MMP-1) (Vogel *et al.*, 1997), one of a group of enzymes which are involved in remodelling the ECM during morphogenesis and tissue repair (Werb, 1997). The mitogenic response of several growth factors, is regulated by metalloproteinases that control their availability to the receptor (Dong *et al.*, 1999; Manes *et al.*, 1999; Martin *et al.*, 1999; Prenzel *et al.*, 1999). This raises the interesting possibility that collagen-induced activation of DDR2 results in degradation of the ECM and allows crosstalk with other signalling systems.

The physiological functions of the widely expressed DDRs are not well understood. Recently, it was shown that DDR1 signalling is essential for cerebellar granule cell differentiation (Bhatt *et al.*, 2000). Interestingly, DDR1 is overexpressed in fast-growing tumours (Alves *et al.*, 1995), suggesting a possible role for DDRs as collagen sensors on the surface of tumour cells (Vogel *et al.*, 1997).

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To establish the physiological role of DDR2, we generated DDR2-deficient mice. Lack of DDR2 signalling is compatible with prenatal development, yet leads to reduced chondrocyte proliferation and bone growth during postnatal development. Wounded skin of DDR2–/– mice contains fewer proliferating cells than skin of wild-type mice. These physiological studies, combined with *in vitro* proliferation assays, establish a requirement for DDR2 in the proliferation of chondrocytes and fibroblasts.

RESULTS

DDR2–/– mice are smaller and exhibit defects in bone growth

To disrupt the *ddr2* gene in embryonic stem (ES) cells, the exon K1, coding for the ATP binding region of the kinase domain, was replaced by a neomycin resistance cassette (Figure 1A). Homologous recombination in ES cells was confirmed by Southern blot analysis (Figure 1B) and several targeted ES cell clones were microinjected into C57BL/6J blastocysts to generate chimeras. Chimeric males were mated to 129/Sv females and offspring were screened for germline transmission. Germline targeted mice were obtained from one ES cell clone only. Loss of DDR2 protein in DDR2–/– mice was demonstrated by western blot analysis, demonstrating that the mice are protein null mutants (Figure 1C). RT–PCR analysis failed to detect DDR2 mRNA in DDR2–/– mice (data not shown). These data and the successful rescue of the proliferation defect of DDR2–/– fibroblasts with a DDR2 cDNA (see below), strongly suggest that disruption of the *ddr2* gene is solely responsible for the observed defects.

Breeding of heterozygous animals gave rise to the expected Mendelian ratios of the different genotypes, and the homozygous null mice survived for at least 12 months on mixed 129/Sv × C57BL/6 or inbred C57BL/6 backgrounds. The external appearance of newborn DDR2–/– pups was indistinguishable from control littermates although the mutants failed to thrive, resulting in proportionate smaller body size and reduced weight (obvious 10 days after birth). Adult mutant mice showed on average 30–40% reduced weight compared with wild-type or heterozygous littermates (35.3% mean ± 7.7% SEM at 5 weeks, $n = 20$) (Figure 1D).

DDR2–/– mice developed a progressive skeletal phenotype characterized by shortening of long bones, irregular growth of flat bones and a shorter snout. The long bones of the appendicular skeleton of 4-month-old DDR2–/– mice showed a reduction in length of 12 to 15% (Figure 1E; $P < 0.001$; $n = 6$ animals per genotype). The flat bones of the skull were also stunted, with a variable reduction of up to 20% in length; axial skeleton was also affected (data not shown). These results suggest a requirement for DDR2 signalling in postnatal bone growth.

DDR2 mRNA is expressed in proliferating chondrocytes in long bones

We analysed DDR2 expression in the tibial growth plates of 1-week-old wild-type mice and found low yet specific levels of DDR2 mRNA (Figure 2A and B) whereas no signals were detected when serial sections were hybridized with a DDR2 sense probe (Figure 2C). DDR2 shows a characteristic pattern of expression corresponding to the chondrocyte columns in the proliferative region of the growth plate. Type II collagen, one of its putative ligands, is expressed in a similar pattern consistent with an interaction between fibril-forming collagens and DDR2 in bone (Figure 3A and B). DDR2 mRNA is also present, although dispersed, at the areas of calcified cartilage in the cartilage–bone junction, as well as in the trabecular bone surface (Figure 2A).

DDR2–/– mice display shortened growth plates in long bones

We next attempted to characterize the cellular mechanism underlying the reduced long bone growth in the DDR2 null mutants. Using *in situ* hybridization analysis with a panel of specific markers, we compared tibias, femurs and metatarsals of wild-type and DDR2–/– mutants at several postnatal stages (1, 2, 4 and 6 weeks; Figure 3 and data not shown). Type II collagen (Figure 3A and B) and type X collagen (Figure 3C and D) are markers for chondrocytes at different stages of differentiation. Alkaline phosphatase and osteocalcin, osteoblast markers, were also analyzed (data not shown). All markers used were correctly expressed and no differences in the expression patterns between wild-type and DDR2–/– mutant bones were found, suggesting that chondrocyte and osteoblast differentiation is not impaired in DDR2–/– mice.

Several metalloproteinases (MMPs) MMP-9 (Figure 3E and F), MMP-13 (Figure 3G and H) and MT-MMP (data not shown) were also examined since they have key functions during bone development and DDR2 has been shown to control MMP secretion *in vitro* (Vogel *et al.*, 1997). The expression pattern of these metalloproteinases was normal in DDR2–/– mice. We did, however, observe a 16% reduction in growth-plate height of 2-week-old metatarsals in DDR2–/– mutants compared with wild-type control littermates ($n = 6$ +/+ and $n = 8$ DDR2–/– mice; $P < 0.025$; data not shown). In summary, DDR2 does not appear to be essential for differentiation of the cells involved in bone growth. The reduction in growth-plate height may explain the observed bone growth defect and be associated with aberrant chondrocyte apoptosis and/or proliferation.

Impaired chondrocyte proliferation is responsible for shortened growth plates in DDR2–/– mice

Growth within the cartilage element is largely dependent on chondrocyte proliferation, subsequent hypertrophy, and finally, death by apoptosis. We therefore compared chondrocyte proliferation in wild-type and mutant growth plates. The number of proliferating cells in DDR2–/– metatarsals at 2 or 3 postnatal weeks was markedly reduced compared with wild-type control littermates (Figure 4A and B and data not shown). The reduction was more pronounced at 2 weeks postnatally (Figure 4B; 23% compared with 56%, $P < 0.005$) than at 3 weeks (data not shown, 16% compared with 32%, $P < 0.05$).

In both wild-type and DDR2–/– littermates, apoptotic chondrocytes were only found in the region adjacent to the ossification front, and the number of apoptotic cells was comparable between different genotypes (Figure 4C). We therefore conclude that reduced chondrocyte proliferation, but not aberrant apoptosis within the growth plate, is the likely cause of reduced bone growth in DDR2–/– mice.

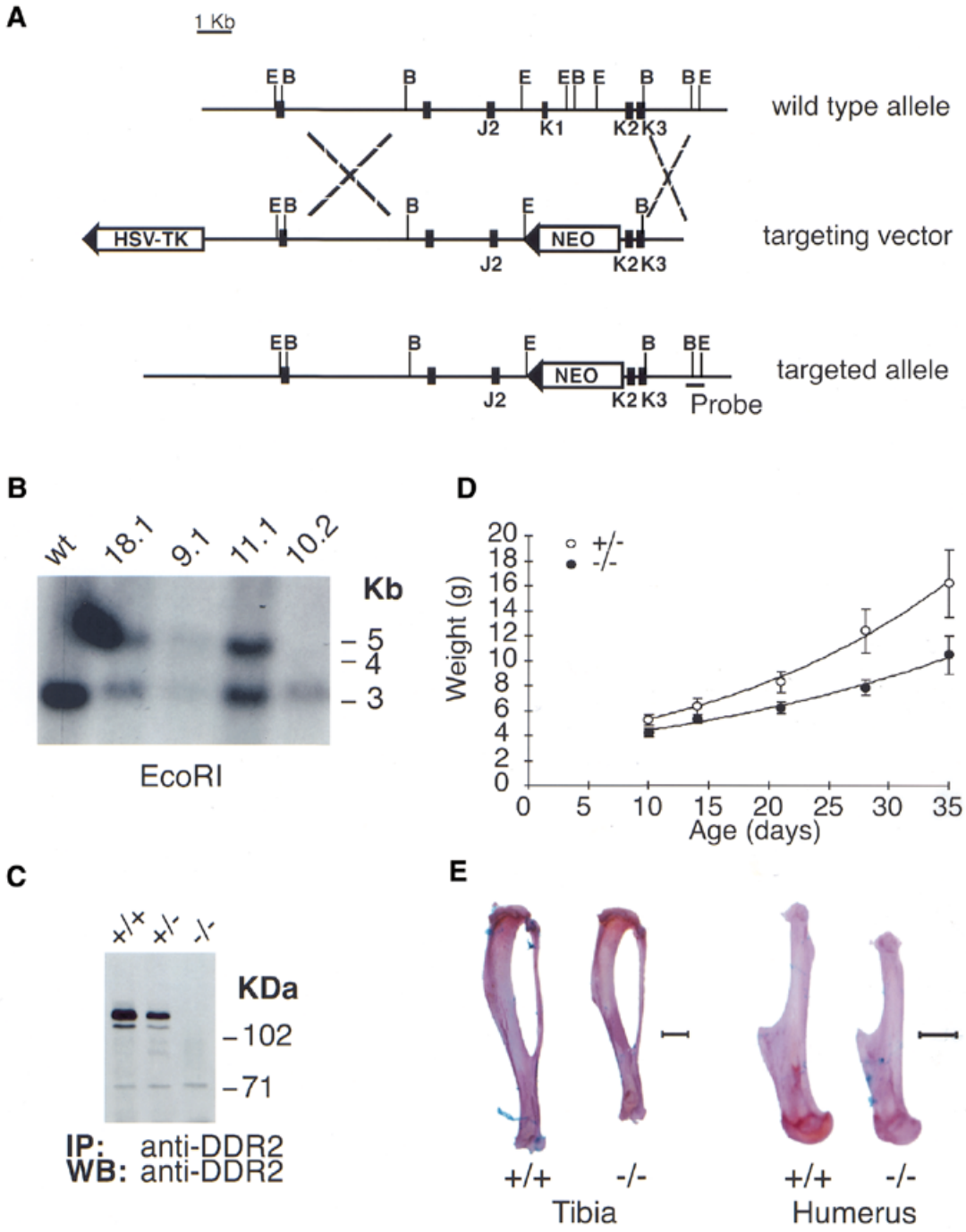

Fig. 1. Generation of DDR2 null mice. (**A**) Genomic organization of the DDR2 wild-type allele, targeting vector and targeted allele after homologous recombination. (B, *Bam*HI; E, *Eco*RI). (**B**) Southern blot analysis of genomic DNA from ES cell clones. EcoRI fragments corresponding to wild-type (3 kb) and targeted (5 kb) alleles are detected. (**C**) Detection of DDR2 protein in muscle extracts by immunoprecipitation (IP) and western blot analysis (WB) using DDR2-specific antiserum. (**D**) Growth curve of DDR2–/– mutant versus heterozygous littermates (129/Sv × C57BL/6J background). Each point represents the mean (± SEM) of at least nine individuals. (**E**) Representative images of Alizarin red-stained bones from wild-type (+/+) and homozygous mutant (–/–) mice. Scale bar = 2 mm.

DDR2 is required for proliferation *in vivo* and *in vitro*

To define the sites of its *in vivo* action, DDR2 protein expression and activity was analysed by immunoprecipitation and western blot analysis in tissues from 3-week-old mice (Figure 5A). DDR2 protein was found in most tissues (Figure 5A, top), but the highest levels of phosphorylation were found in lung, ovary and skin (Figure 5B, bottom). Interestingly, DDR2 expression did not necessarily correlate with its phosphorylation levels, and given

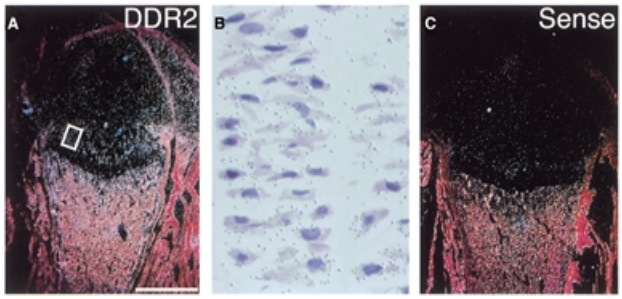

Fig. 2. DDR2 expression in bone. *In situ* hybridization analysis of sections from 1-week-old tibia from wild-type mice, hybridized with DDR2 antisense (**A**, **B**) or sense (**C**) cRNA probes. (B) High power bright field image of DDR2 mRNA expression (black silver grains) in a hematoxylin/eosin-stained section from proliferating chondrocytes. The area shown corresponds to the white square in (A). Scale bar = 0.5 mm.

the high level of DDR2 phosphorylation in the skin we analysed the role of the receptor in this tissue. The skin wound healing model is widely used to study cell migration and proliferation as well as ECM degradation (Johnsen *et al.*, 1998). Proliferation was observed mainly in epidermal keratinocytes and, to a lesser extent, in dermal mesenchymal cells (Figure 5B–E). Bromodeoxyuridine (BrdU) incorporation in wounded skin of DDR2–/– mice was reduced by ~50% at 2.5 days post-wounding (Figure 5B and C; $P < 0.001$) and by 60% 4.5 days after wounding (Figure 5D and E; $P < 0.001$). These results indicate a requirement for DDR2 in the proliferative response during skin wound healing.

We also assayed *in vitro* proliferation of fibroblasts obtained from ear skin of adult mice. Primary fibroblasts were immortalized and infected with recombinant retroviruses expressing GFP alone or GFP and either wild-type DDR2 or kinase-dead DDR2 (K608E). Adult skin fibroblasts from DDR2–/– mice proliferated more slowly than their wild-type counterparts (Figure 6). Re-introduction of the receptor into mutant cells restored the normal proliferation rate, whereas kinase-dead DDR2 receptor-expressing cells showed no improvement over DDR2–/– fibroblasts. Similar results were obtained with mouse embryonic fibroblasts (data not shown). In summary, these results demonstrate that DDR2 kinase activity is required for cell proliferation *in vivo* and *in vitro*.

DISCUSSION

In this study we show that lack of DDR2 in knockout mice is compatible with embryonic development, but results in postnatal growth defects and reduced proliferative responses in DDR2-expressing cells. Our analysis of DDR2–/– bone development suggests that the underlying defect is in chondrocyte proliferation, rather than in bone cell differentiation. Bone growth depends mainly on chondrocyte proliferation and differentiation, together with the balance between bone deposition by the osteoblast and resorption by the osteoclast lineages. Development of the different lineages appeared normal in DDR2–/– mice. However, proliferating chondrocytes in the growth plates of DDR2–/– mice, where DDR2 should be expressed, show a reduction in the proliferation index, which accounts for the small size of bones. In contrast, the number of apoptotic cells remained unchanged in the absence of DDR2. Furthermore, epidermal fibroblasts and

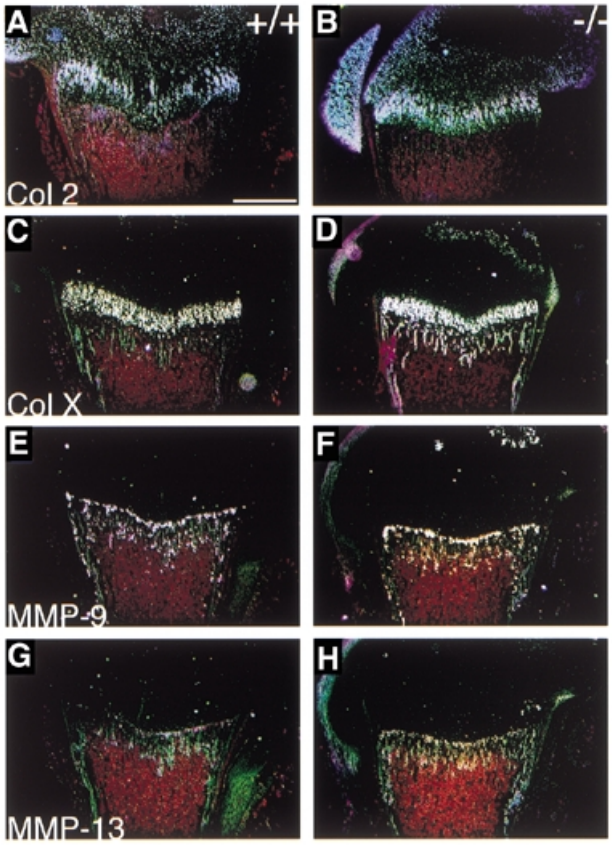

Fig. 3. Differentiation markers in DDR2–/– growth plates. *In situ* hybridization analysis of sections from 2-week-old wild-type (**A**, **C**, **E**, **G**) and homozygous mutant (**B**, **D**, **F**, **H**) tibias. Antisense cRNA probes for type II collagen (A and B), type X collagen (C and D), MMP-9 (E and F), and MMP-13 (G and H). Lack of differences between +/+ and DDR2–/– material suggests normal differentiation in mutant bones. Scale bar = 0.5 mm.

keratinocytes of DDR2-deficient mice incorporate less BrdU in a wound healing model. *In vitro* experiments using DDR2–/– fibroblasts demonstrate that absence of DDR2 causes a significant reduction in the proliferation rate of DDR2–/– fibroblasts compared with wild-type. Finally, only wild-type DDR2, but not kinase-dead DDR2, was able to rescue the fibroblast proliferation defect, indicating that DDR2 exerts a cell autonomous role in fibroblast proliferation.

Skeletal malformations such as those observed in DDR2–/– mice could be caused by abnormal bone development or malnutrition. The latter is unlikely to be the major cause, as there was no prominent reduction in subcutaneous fat, bone mineralization or muscle mass of DDR2–/– mice (data not shown). Bone growth depends mainly on chondrocyte proliferation and differentiation, together with the balance between bone deposition by the osteoblast and resorption by the osteoclast lineages (Kember and Walker, 1971). Development of the latter lineages appeared normal in DDR2–/– mice, pointing to a role for DDR2 in chondrocyte development. DDR2 is expressed in proliferating chondrocytes of the growth plate together with type II collagen, its putative ligand. Chondrocytes in the growth plates of DDR2–/– mice show a reduction in the proliferation index, that accounts

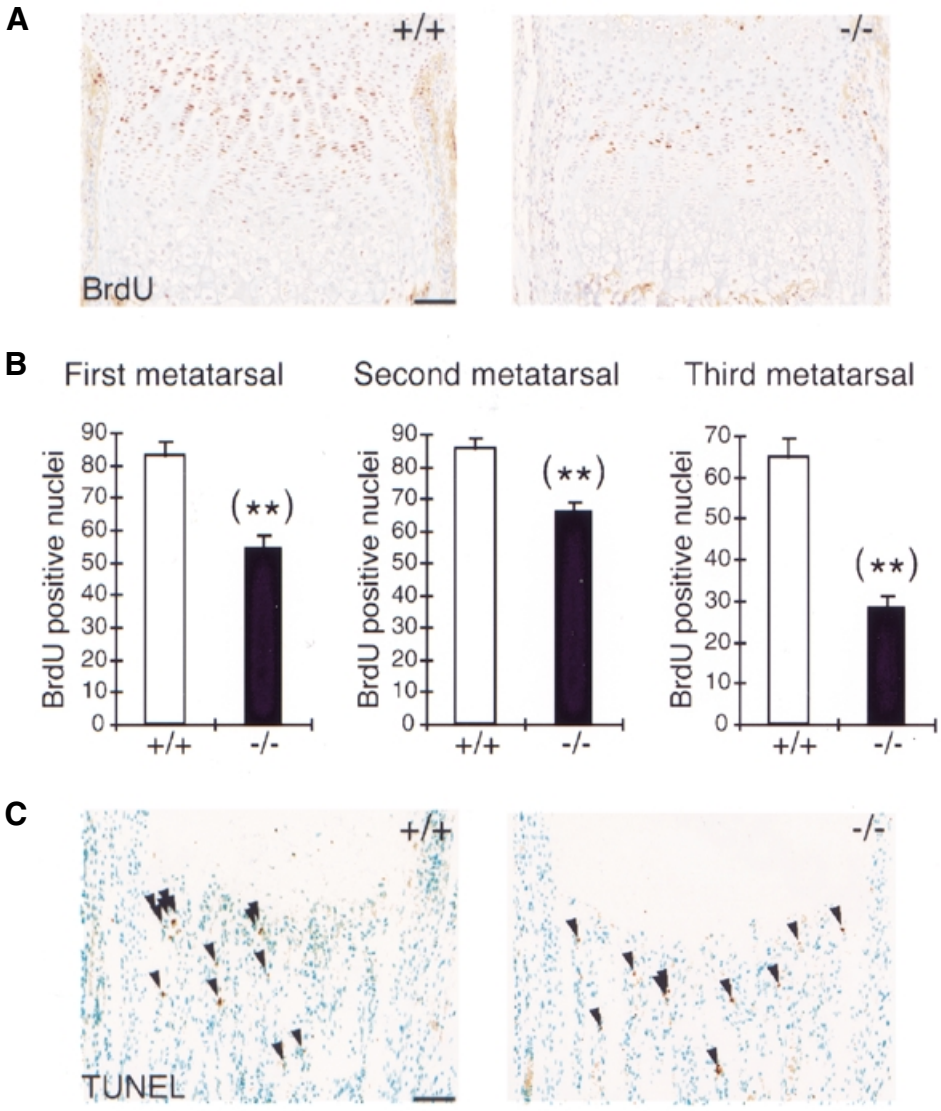

Fig. 4. Reduced chondrocyte proliferation in DDR2–/– mice. (**A**) *In vivo* BrdU labelling (brown) in metatarsals of wild-type (+/+) and DDR2–/– mice (–/–) at 2 weeks of age. (**B**) Quantification of BrdU incorporation. Data represent mean ± SEM (** P <0.001). (**C**) Apoptotic cells in metatarsals from 2-week-old wild-type (+/+) and homozygous mutant (–/–) mice, stained by TUNEL. Arrowheads indicate apoptotic hypertrophic chondrocytes. There is no apparent difference in the number of TUNEL-positive cells in DDR2–/– or wild-type bones. Scale bar = 0.5 mm.

for the small size of bones. In contrast, the number of apoptotic cells remained unchanged in the absence of DDR2. Available evidence thus suggests that reduced chondrocyte proliferation contributes to the skeletal defec ts in DDR2–/– mice.

The fact that DDR2 regulates proliferation in situations associated with extreme matrix deposition and degradation, such as bone growth or skin wound healing, suggests an important role for DDR2 in pathological scenarios where the ECM provides a signal for increased proliferation. In this respect DDR2 is dramatically upregulated in hepatic wound healing by activated (proliferative) stellate cells (Ankoma-Sey *et al.*, 1998), the key mesenchymal element in liver fibrosis (Friedman, 2000). Rheumatoid arthritis is also characterized by increased proliferation and increased MMP production. Fibroblast-like synoviocyte (FLS) proliferation is induced by growth factors such as platelet-derived growth factor (PDGF), but is dependent on a coactivation signal from the ECM since attachment to collagen enhances PDGF-induced FLS proliferation (Sarkissian and Lafyatis, 1999). In a similar manner DDRs may also be implicated in tumour progression, as they are overexpressed in tumours (Barker *et al.*, 1995). The data presented here indicate that the role of DDRs in tumour formation and metastasis may extend beyond regulation of ECM degradation.

DDR2 induces expression of MMP-1 when overexpressed in the human fibrosarcoma cell line HT 1080, suggesting a regulatory function in collagen degradation (Vogel *et al.*, 1997; Vogel, 1999). Several metalloproteinases are expressed in growing bone and have very important roles in ECM turnover, vascularization,

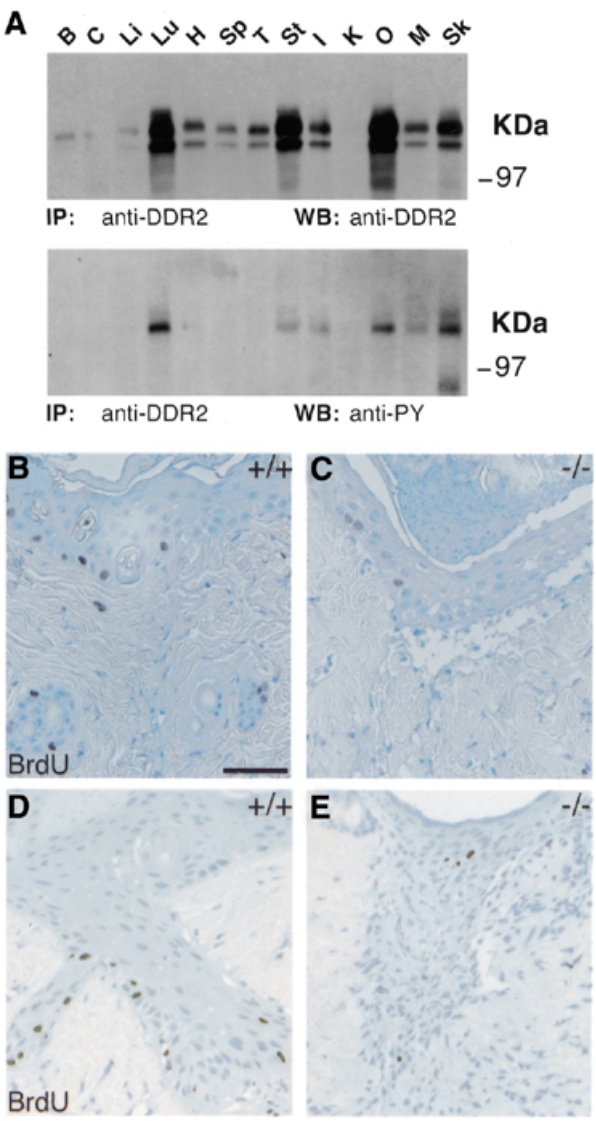

Fig. 5. Reduced BrdU incorporation in skin. (**A**) Broad tissue distribution of DDR2 (top panel) shown by immunoprecipitation (IP) and western blotting (WB) with anti-DDR2 antibodies. WB with anti-phosphotyrosine specific antibodies (bottom panel) reveals high levels of phosphorylated DDR2 protein in the skin. (**B**, **C**, **D**, **E**) *In vivo* BrdU labelling in skin wounds of wild-type (B and D) and DDR2–/– mice (C and E), 2.5 days (B and C) and 4.5 days after wounding (D and E). Skin sections were labelled with PO-conjugated anti-BrdU antibody (brown nuclei) and counterstained with hematoxylin. Abbreviations: B, brain; C, cerebellum; H, heart; I, intestine; K, kidney; Li, liver; Lu, lung; M, skeletal muscle; O, ovary; Sk, skin; Sp, spleen; St, stomach; T, thymus.

ossification and apoptosis (Vu *et al.*, 1998; Holmbeck *et al.*, 1999, Tuckermann *et al.*, 2000). We found no obvious change by *in situ* hybridization in the pattern of expression of these metalloproteinases between DDR2–/– and wild-type mice in the growing bones or in wounded skin (data not shown). However this technique only allows us to compare large changes in the level of MMP expression and we have evidence that DDR2 can regulate MMP-2 levels in skin fibroblasts (E. Olaso *et al.*, submitted).

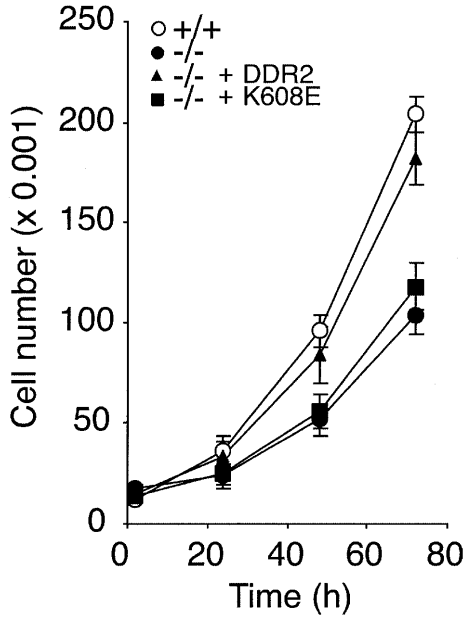

Fig. 6. *In vitro* rescue of DNA synthesis and proliferation defects of DDR2–/– fibroblasts. Proliferation rate of adult skin fibroblasts derived from wild-type (+/+) or DDR2–/– mice. Mutant cells were infected with recombinant retroviruses expressing GFP alone, wild-type, myc-tagged DDR2 or a kinase-dead version of DDR2 (K608E). Wild-type, but not kinase-dead DDR2 rescues the proliferation defect of DDR2–/– fibroblasts.

In summary, we suggest a cell-to-matrix attachment-dependent function for DDR2. These properties make DDR2 a likely candidate to play a major role in pathological situations where increased proliferation and matrix turnover are coupled.

METHODS

Construction of the targeting vector and generation of DDR2 null mice. A pPNT-based vector was used as a replacement-type targeting vector. The targeting construct consisted of 6.7 kb of *ddr2* genomic sequence upstream and 1.4 kb downstream of the *neo* cassette. The deleted 1.2 kb region included one entire exon (K1) of the kinase domain and the flanking intronic sequences, spanning amino acids 578 to 619 from the published Tyro 10 mouse sequence (Lai and Lemke, 1994). Murine R1 ES cells were targeted following standard protocols.

Skeletal preparations, histology and *in situ* hybridization. For skeletal preparations, adult mice were skinned, eviscerated and fixed in acetone for 5 days, cleared for 24 h in 2% KOH, stained overnight in 1 volume 0.1% Alizarin red S (Sigma): 19 volumes 1% KOH, then cleared in 1% KOH. Bone length was measured at the midline and the data analyzed using the student's *t*-test. For histological analysis, bones were fixed overnight in 4% paraformaldehyde at 4°C and decalcified at room temperature in 0.5 M EDTA. For *in situ* hybridization, 6 µm paraffin sections from decalcified bone were treated as described using the following [^{35}S]UTP-labelled sense and antisense probes: a 1kb fragment PCR amplified from the extracellular domain of DDR2 cloned into PCR-TOPO2. Col2, ColX, MMP-9 and MMP-13 probes were described previously (Tuckermann *et al.*, 2000).

Detection of BrdU-labelled and apoptotic nuclei. Mice received BrdU (100 µg/g body weight; Sigma) intraperitoneally 90 min prior to being killed. Bones were fixed and processed for paraffin sections as described above (2 weeks, $n = 3$ +/+ and $n = 3$ DDR2–/– ; 3 weeks, $n = 3$ +/+ and $n = 2$ DDR2–/–). An average of 10 sections from each animal was counted. Apoptotic nuclei were identified by the TUNEL method using a kit (Oncor) and developed with a PO-conjugated anti-digoxygenin antibody (1:400 dilution; Boehringer). Sections were counterstained with methyl green.

Wound healing assay. For wound healing experiments, the dorsal skin of the animals was shaved and full thickness cutaneous dorsal incisions (0.5 cm in length) were made under anesthesia. The wounds were immediately closed with a suture. After 57 or 106 h, three adult heterozygous and three DDR2–/– littermates or two heterozygous and two DDR2–/– littermates, respectively, received a BrdU pulse 90 min before being killed.

Immunoprecipitation and western blotting. For immunoprecipitations and western blots established procedures were followed (Brückner *et al.*, 1999). The DDR2 antiserum (#203) was raised against the DDR2 juxtamembrane domain expressed as a glutathione *S*-transferase fusion protein in bacteria.

***In vitro* cell proliferation assays.** Adult skin fibroblasts were immortalized by SV40 large T antigen expression. DDR2–/– fibroblasts were infected by recombinant virus coding for DDR2 or the K608E mutant together with EGFP. Cells were sorted after 48 h using fluorescence-activated cell sorting (FACS). Cells were allowed to grow to near confluence, then sorted a second time. Immediately after the second sort, 1.5×10^4 cells were plated onto 35 mm culture dishes in DMEM medium supplemented with 5% fetal calf serum. At indicated time points, cells were treated with 0.025% trypsin (W/V) and 0.75 mM EDTA in phosphate-buffered saline, and counted.

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