Correction: Mice humanised for the EGF receptor display hypomorphic phenotypes in skin, bone and heart

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In Fig. 6, owing to an error in figure assembly during preparation of the proofs, the incorrect image (a duplication of panel D) was displayed in panel G. The corrected figure appears below.

We apologise to the authors and readers for this mistake.
Mice humanised for the EGF receptor display hypomorphic phenotypes in skin, bone and heart

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Summary

Mice lacking the epidermal growth factor receptor (EGFR) develop epithelial defects and a neurodegenerative disease and die within the first month of birth. By employing a conditional knock-in approach using the human EGF cDNA mice humanised for EGFR (hEGFRKI/KI) were generated. Homozygous hEGFRKI/KI mice are viable and live up to six months. However, these mice are growth retarded and show skin and hair defects similar to Egfr+/− mutants. Interestingly, the neurodegeneration is fully rescued in hEGFRKI/KI mice, however, they develop a severe heart hypertrophy with semilunar valve abnormalities. Moreover, hEGFRKI/KI mice display accelerated chondrocyte and osteoblast differentiation, a phenotype that is also present in Egfr+/− mice and has not been previously described. The severity of the phenotypes correlates with the expression levels of the hEGFRKI allele, which is not efficiently expressed in epithelial and bone cells, but is expressed at similar and even higher levels as the endogenous Egfr in brain and heart. These results demonstrate that mice humanised for EGFR display tissue-specific hypomorphic phenotypes and describe a novel function for EGFR in bone development.

Key words: Bone, Hair growth, Heart hypertrophy, Humanised EGFR knock-in mice, Skin

Introduction

The epidermal growth factor receptor (EGFR/Erbb1) belongs to a family of structurally related tyrosine kinase receptors, including Erbb2/neu, Erbb3 and Erbb4, which upon ligand binding can form homo- and heterodimers (Olayioye et al., 2000; Schlissinger, 2000; Yarden and Sliwkowski, 2001). Several growth factors such as epidermal growth factor (EGF), transforming growth factor α (TGFα), amphiregulin, heparin-binding EGF (HB-EGF), β-cellulin and epiregulin can bind the EGFR and induce receptor dimerisation. Subsequent activation of the intrinsic tyrosine kinase induces complex downstream signalling pathways, which can instruct cells either to proliferate, differentiate and/or survive. Little is known about how these cellular responses are regulated in different cell types in vivo (Olayioye et al., 2000; Yarden and Sliwkowski, 2001).

Most of the current knowledge about the function of the EGFR and its family members during normal development derives from the analysis of mutant mice. Mice lacking Erbb2, Erbb3 and Erbb4 die at midgestation, because of cardiac dysfunction associated with lack of ventricular trabeculation, and display abnormal development of the peripheral nervous system (Gassmann et al., 1995; Lee et al., 1995; Riethmacher et al., 1997). Inactivation of the Egfr in mice reveals that mutant mice die at midgestation (129/Sv), birth (C57BL/6) or can live up to postnatal day 20 (MF1, C3H and CD1) depending on their genetic background. They exhibit epithelial and neural phenotypes as well as craniofacial malformations (Miettinen et al., 1995; Miettinen et al., 1999; Sibilia and Wagner, 1995; Threadgill et al., 1995). Death in utero results from a placental defect, as the embryonic lethality can be rescued by generating aggregation chimeras between Egfr+/− and tetraploid wild-type embryos, the latter contributing exclusively to extra-embryonic tissues (Sibilia et al., 1998). Independent of the genetic background, surviving Egfr+/− mice develop a progressive neurodegeneration in the olfactory bulbs and frontal cortex, which is characterised by massive apoptotic cell death affecting both neurones and astrocytes (Kornblum et al., 1998; Sibilia et al., 1998). Ectopic neurones are always detected in the white matter of the hippocampus, suggesting that EGFR signalling might be important for neuronal migration in response to FGF (Tropepe et al., 1999). However, EGFR is crucial for astrocyte development, as cerebral cortices from Egfr+/− mice contain lower numbers of astrocytes, which...
display a severe proliferation defect in vitro (Kornblum et al., 1998; Sibilia et al., 1998). Therefore, the EGFR is most probably required for the proliferation/differentiation of astrocytes and for preserving brain integrity after birth.

In addition to the neural defects, Egfr–/– mice also display several abnormalities in epithelial tissues. They are born with open eyes and show impaired epidermal as well as hair follicle differentiation and fail to develop a hairy coat most likely because EGFR signalling is necessary for maintenance of hair follicle integrity (Miettinnen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). However, Egfr–/– mice do not survive beyond the termination of the first hair cycle and, therefore, a careful analysis of EGFR function during skin and hair follicle development could not be performed. Grafting experiments of Egfr–/– skin explants on athymic nude mice suggest that Egfr–/– follicles cannot proceed from the anagen to telogen phase of the hair cycle (Hansen et al., 1997). These alterations eventually lead to necrosis and disappearance of the follicles, accompanied by strong infiltration of the skin with inflammatory cells (Hansen et al., 1997). A similar but more severe skin and hair phenotype could be detected in transgenic mice expressing a dominant negative (DN) Egfr (CD533) from the keratin 5 (K5) promoter (Murillas et al., 1995). However, in these mice it could not be excluded that other Erbb receptors were also inhibited by the DN EGFR, thereby exacerbating the skin defects. Milder phenotypes characterised by a ‘wavy’ coat and curly whiskers are observed in mice deficient for the gene encoding TGFα and in mice homozygous for the hypomorphic Egfr allele waved 2 (wa2), which carry a point mutation in the kinase domain of the EGFR resulting in a drastically reduced kinase activity (Fowler et al., 1995; Luetteke et al., 1994; Luetteke et al., 1993; Mann et al., 1993).

Numerous studies have documented alterations in EGFR signalling pathways in the development of human neoplasms (Olayioye et al., 2000; Yarden, 2001). Amplifications, rearrangements and overexpression of the EGFR gene have been shown to occur at high frequency in human squamous cell carcinomas and glioblastomas (Olayioye et al., 2000; Yarden, 2001). The first in vivo evidence for a direct involvement of the EGFR in epithelial tumour development stems from the analysis of transgenic mice expressing an activated form of the Ras activator son of sevenless (Sos) from the K5 promoter (Sibilia et al., 2000). All K5-Sos transgenic mice develop skin tumours only in the presence of a functional EGFR. K5-Sos transgenic skin papillomas in a EGFR mutant background show increased apoptosis and are more differentiated indicating that signalling through the EGFR enhances the survival and inhibits differentiation of epidermal cells (Sibilia et al., 2000). This is supported by the fact that Egfr is most strongly expressed in the proliferating compartments of the basal layer of the epidermis and in the outer root sheath of the hair follicles, and the number of receptors decreases as keratinocytes enter the pathway of terminal differentiation and migrate to the suprabasal layers of the epidermis (Peus et al., 1997; Sibilia and Wagner, 1995; Stoll et al., 2001).

The function of the EGFR in adult mice could not be addressed because of the early postnatal lethality of EGFR-deficient mice. Here, we employed a knock-in strategy to generate mice in which the endogenous mouse Egfr is replaced by a human EGFR cDNA that is flanked by loxP sites (hEGFRKI allele). This approach enables the generation of humanised EGFR mice (hEGFRKI/KI mice) to study the functional homologies between the mouse and human EGFR in a conditional way. In addition, the phenotypic consequences of knocking-in well characterised human EGFR mutants can also be addressed. Homozygous hEGFRKI/KI mice are growth retarded but can survive for up to 6 months after birth. However, they display skin and hair growth defects similar to surviving Egfr–/– mice. Interestingly, the neurodegeneration is fully rescued, although hEGFRKI/KI mice develop a severe heart hypertrophy with abnormalities in semilunar valve development. Accelerated chondrocyte and osteoblast differentiation are detected in both hEGFRKI/KI and Egfr–/– mice, suggesting that EGFR signalling negatively regulates bone cell differentiation. The severity of the phenotypes correlates with the expression levels of the hEGFRKI allele in various tissues. In bone cells and epithelial tissues the expression of the hEGFRKI allele is severely reduced, whereas the hEGFRKI allele is expressed at similar levels as the endogenous mouse gene in the brain thereby rescuing the neurodegeneration. Moreover, higher levels of expression of the hEGFRKI allele are detected in the heart and are likely responsible for the development of the heart hypertrophy. These results demonstrate that mice humanised for EGFR display tissue-specific hypomorphic phenotypes thereby uncovering novel functions of the EGFR in bone and heart development.

Materials and methods

Generation of targeting construct and hEGFRKI/KI mice

To generate the EGFR knock-in vector the pGNA-based knock-out vector described previously (Sibilia and Wagner, 1995) was modified as follows. The lacZ gene was removed and replaced by a multiple cloning site. A loxP-flanked human EGFR (hEGFR) cDNA was inserted into the multiple cloning site and the vector was linearised with XbaI before electroporation into feeder-dependent GS-1 ES cells. Selection and screening of ES cell colonies by PCR and Southern blot analysis was performed as previously described (Sibilia and Wagner, 1995). Two correctly targeted ES cell clones were injected into C57BL/6 blastocysts and germline male chimeric mice were obtained with both clones. Chimeras were mated to 129/Sv and/or C57BL/6 females to obtain hEGFRKI/KI mice of inbred 129/Sv and mixed 129/Sv × C57BL/6 background, which were used for further breeding and generation of hEGFRKI/KI mice. Genotyping of the mice was performed by PCR as previously described (Sibilia and Wagner, 1995).

Histology

Mice were sacrificed and tissues were fixed in 4% PBS-buffered formaldehyde and embedded in paraffin wax. Sections (5 µm) were cut and stained with Haematoxylin and Eosin (Sigma Immunochemicals) according to standard procedures. Immunohistochemical staining for Ki67 (Novocastra, NCL-Ki67p, 1:1000) was performed using the ABC staining kit (Vector Laboratories) according to the manufacturer’s recommendations. For histomorphometric analysis, Haematoxylin and Eosin stained cross-sections of hearts from 2.5- to 3.5-month-old hEGFRKI/KI mice (n=4) and age-matched controls (n=4) were evaluated using an Axioskop microscope (Carl Zeiss, Oberkochen, Germany) with integrated morphometric device at 400x magnification. The size of perpendicularly cut cardiomyocytes was determined in the subendocardial layers of the left ventricle.

RNase protection assay

Total RNA was extracted and purified from various organs using
TRIzol reagents (Gibco) and subjected to RNAse protection analysis as previously described (Fleischmann et al., 2000). For the generation of the EGFR riboprobe, the region encompassing the 3’ end of the Egfr mouse promoter, the loxP site and the 5’ end of the hEGFR cDNA were cloned into SP64 plasmid, linearised and transcribed in vitro using the in vitro translation Kit from Stratagene.

Western blot analysis and in vitro EGFR autophosphorylation assay
Protein lysates were prepared from various tissues, cleared by centrifugation and either processed for western blot analysis or subjected to immunoprecipitation employing the rabbit anti EGFR antibody #1001 (Santa Cruz) as previously described (Sibilia et al., 2000; Sibilia and Wagner, 1995). Immunoprecipitated EGFR complexes were resuspended in 20 μl HNTG (20 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% Glycerol) containing 0.4 μl γ³²PdATP (10 μC/μl, Amersham) and incubated for 15 minutes on ice. Kinase reactions were stopped and protein complexes separated on 10% SDS-PAGE. Gels were dried at 80°C under vacuum and exposed to X-ray films (Amersham). Western blot analysis was performed with the anti EGFR antibody #06-129 (Upstate, 1:1000).

Astrocyte cultures
Primary astrocyte cultures were prepared from brains of newborn mice as previously described (Sibilia et al., 1998). Cells were plated in DMEM/F12 (1:1, GIBCO) containing 10% foetal calf serum (PAA), 1998). Cells were plated in DMEM/F12 (1:1, GIBCO) culture medium containing 10% foetal calf serum (PAA), and after reaching confluency passaged at a 1:3 split ratio. Before replating, viable cell numbers were determined using a Neubauer-type haemocytometer with Trypan Blue.

Osteoblast culture
Primary osteoblasts were isolated from calvariae of newborn mice as previously described (Sibilia et al., 2000; Sibilia and Wagner, 1995). Immunoprecipitated EGFR complexes were resuspended in 20 μl HNTG (20 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% Glycerol) containing 0.4 μl γ³²PdATP (10 μC/μl, Amersham) and incubated for 15 minutes on ice. Kinase reactions were stopped and protein complexes separated on 10% SDS-PAGE. Gels were dried at 80°C under vacuum and exposed to X-ray films (Amersham). Western blot analysis was performed with the anti EGFR antibody #06-129 (Upstate, 1:1000).

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Results
Generation of hEGFRKI/KI mice
To generate mice humanised for EGFR a gene-targeting vector was constructed, which after homologous recombination replaces parts of the first exon of the mouse Egfr gene with the loxP-flanked human EGFR cDNA (referred to as hEGFRKI allele, Fig. 1A). This vector is a modified version of the one previously employed to generate Egfr knockout (EgfrKI) mice (Sibilia and Wagner, 1995). The construct was electroporated into ES cells and correctly targeted clones were identified by PCR and Southern blot analysis (data not shown). Two independent ES clones were injected into blastocysts and both formed germine chimeras. Heterozygous hEGFRKI/+ mice of inbred 129/Sv or mixed 129/SvxC57BL/6 background were intercrossed to generate hEGFRKI/KI mice, which were identified by PCR analysis

Fig. 1. Generation of hEGFRKI/KI mice. (A) Schematic representation of the targeting strategy employed to insert a floxed human EGFR cDNA (hEGFR) into the mouse Egfr locus. The full-length hEGFR expression cassette (grey box) contains its own ATG and polyA (pA) site and is flanked by loxP sites (grey triangles). After homologous recombination, the hEGFR cDNA will be inserted into the first exon of the mouse Egfr gene (black box) and placed under the control of the endogenous mouse Egfr promoter. This correctly targeted allele will be referred to as the hEGFR knock-in allele (hEGFRKI). The neomycin resistance gene (RSV-neo) and the thymidine kinase gene (HSV-tk) are shown. The broken lines delineate the homology regions in the targeting vector, the horizontal bar indicates the Southern blot probe, and the black arrowheads indicate the PCR primers employed for genotype analysis. X, XbaI; BX, BstXI; N, NdeI. (B) PCR analysis of genomic DNA isolated from the progeny of hEGFRKI/+ intercrosses. (C) photograph of 6-week-old hEGFRKI/KI and control littermate mice showing that hEGFRKI/KI mice are smaller and display short fur hair. (D) Weight representative of a hEGFRKI/KI and control littermate mouse at different postnatal ages. At all stages, hEGFRKI/KI mice are significantly smaller than their littermates. w, weeks; m, months
(Fig. 1B). Although no viable hEGFR<sup>KI/KI</sup> mice were obtained in a 129/Sv background (0/68), ~7% of hEGFR<sup>KI/KI</sup> mice were born alive in a mixed 129/SvxC57BL/6 background (Fig. 1C). When bred with Egfr<sup>+/−</sup> mice, no viable hEGFR<sup>KI</sup> mice could be obtained, even in the mixed 129/SvxC57BL/6 background, indicating that one copy of the hEGFR<sup>KI</sup> allele is not sufficient to rescue the lethality of Egfr<sup>−/−</sup> mice (data not shown). Therefore, the phenotypic analysis was performed on surviving hEGFR<sup>KI/KI</sup> mice of mixed 129/SvxC57BL/6 background, which at birth could be identified by their open eyes and curly whiskers (data not shown). The development of the first coat hair was delayed and the hair was generally much shorter and sparse (Fig. 1C). Some of the hEGFR<sup>KI/KI</sup> mice survived up to 7 months after birth, but were always significantly smaller than their control littermates (Fig. 1C,D). These observations strongly suggest that the hEGFR<sup>KI</sup> allele is most probably not efficiently expressed leading to a hypomorphic phenotype.

**Expression analysis in hEGFR<sup>KI/KI</sup> mice**

To compare the expression levels of the hEGFR<sup>KI</sup> allele to the endogenous mouse Egfr, total RNA was extracted from various organs of a hEGFR<sup>KI/+</sup> heterozygous mouse. Ribonuclease (RNase) protection assay was performed using a riboprobe, which recognises both the endogenous and the hEGFR<sup>KI</sup> transcripts, but the respective protected bands are different in size. Surprisingly, the hEGFR<sup>KI</sup> allele was not expressed at lower levels than the endogenous Egfr in every tissue (Fig. 2A). Organs, which are mostly composed of epithelial cells such as liver, lung, skin and stomach expressed significantly lower levels of the hEGFR<sup>KI</sup> allele compared with the endogenous allele (Fig. 2A). By contrast, in brain regions such as cortex and hippocampus, in kidney and in thymus the hEGFR<sup>KI</sup> allele was expressed at similar levels as the endogenous mouse allele (Fig. 2A). Interestingly, in the heart the hEGFR<sup>KI</sup> allele seemed to be expressed at even higher levels than the endogenous one (Fig. 2A). These results suggest that the hEGFR<sup>KI</sup> allele behaves like a hypomorph particularly in epithelial tissues.

To investigate the expression of the hEGFR<sup>KI</sup> allele at the protein level, lysates were prepared from brain, liver and heart of hEGFR<sup>KI/KI</sup>, hEGFR<sup>KI/+</sup>, Egfr<sup>+/+</sup> and Egfr<sup>−/−</sup> mice. Western blot analysis employing an anti-EGFR antibody recognising both the mouse and the human EGFR protein showed that EGFR protein of similar size was produced in the brain and liver of wild-type and hEGFR<sup>KI/KI</sup> mice (Fig. 2B). The levels of EGFR protein expressed in the brain were similar to controls, but severely reduced in the liver of hEGFR<sup>KI/KI</sup> mice correlating with the mRNA expression results (Fig. 2A,B). An in vitro EGFR autophosphorylation assay, which is usually more sensitive than western blot analysis, was performed on immunoprecipitated EGFR complexes. Consistent with the previous results, EGFR protein levels were similar to control in the brain of hEGFR<sup>KI/KI</sup> mice, whereas they were lower and almost absent in the liver (Fig. 2C). Using both assays, EGFR protein could not be detected in the hearts of all mice analysed, most probably owing to very low EGFR levels (Fig. 2C and data not shown).

**Rescue of the brain phenotypes in hEGFR<sup>KI/KI</sup> mice**

In the brain, the hEGFR<sup>KI</sup> allele is expressed at similar levels...
than the endogenous Egfr (Fig. 2A). In order to investigate whether in hEGFRKI/KI mice the brain phenotypes were rescued, brains were isolated at different stages after birth and compared to Egfr+/− and controls. At all stages analysed, brains of hEGFRKI/KI mice appeared structurally normal and comparable to controls (Fig. 3A,B). Histological sections through the frontal cortex of a 3-month-old hEGFRKI/KI mouse did not reveal any signs of degeneration and apoptosis and the cortical tissues appeared normal and comparable to controls (Fig. 3C,E). By contrast, in the cortex of Egfr−/− mice nests of ectopic neurones were always present (Fig. 3D,F,H) (Sibilia et al., 1998). In addition, other brain regions such as the olfactory bulb, thalamus and cerebellum of hEGFRKI/KI mice appeared normal and comparable with controls (data not shown).

We next isolated astrocytes from the frontal cortex of newborn hEGFRKI/KI mice and analysed their proliferation potential in vitro. As shown in Fig. 3I, the proliferation rate of hEGFRKI/KI primary astrocytes was comparable with hEGFRKI/+ and Egfr+/− astrocytes. As the hEGFRKI allele is flanked by loxP sites, the hEGFR was deleted by breeding hEGFRKI/KI mice to GFAP-cre transgenic mice, which express the Cre recombinase in astrocytes (Marino et al., 2000). Removal of the EGFR severely compromised the proliferation capacity of primary astrocytes and after 42 days in culture their cumulative cell number reached only about 15% of the values obtained with hEGFRKI/KI and control astrocytes (Fig. 3I). Overall, the proliferation rate of hEGFRKI/KI GFAP-cre astrocytes was reduced to the same extent as in Egfr−/− astrocytes (Fig. 3I) (Sibilia et al., 1998). Southern blot analysis confirmed the absence of the Egfr gene in hEGFRKI/KI GFAP-cre astrocytes (Fig. 3J). These results indicate that expression of the hEGFRKI allele is fully rescuing the brain and astrocyte defects of Egfr−/− mice.

**Severe hair follicle and hair cycle defects in hEGFRKI/KI mice**

The hEGFRKI allele is not efficiently expressed in the skin based on the RNAse protection analysis (Fig. 2A). Moreover, hEGFRKI/KI mice displayed curly whiskers and the development of the first coat hair was impaired (Fig. 1C). These phenotypes greatly resemble the ones observed in Egfr−/− mice (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). However, in contrast to Egfr−/− mice, which do not survive longer than postnatal day 20, hEGFRKI/KI mice survive up to 6 months after birth and, therefore, represent a useful model to analyse how reduced EGFR expression affects hair development. The skin of hEGFRKI/KI mice was isolated postnatally at different stages of the hair cycle, after 18 days (end of first cycle, catagen/early telogen), after 1 month (second cycle, end of anagen/early catagen) and after 3 months (resting phase, telogen). Histological examination of hEGFRKI/KI skins showed...
Severe fibrosis with infiltration of inflammatory cells can be detected, as follicles stuck in the subdermal fat tissue have degenerated (arrows). Follicles are present in the upper dermis (arrowheads) and most of the dermal layers (arrows). (F) In mutant skins, only a few aberrant follicles were present in the upper dermis and most of the follicles, which had remained stuck in the subdermal fat layers, were degenerated (Fig. 4F). The cell layers composing the hair follicles were very thin and partly destroyed (Fig. 4F). Severe fibrosis of the dermis and subdermal fat tissue with massive infiltration of inflammatory cells was observed and had most likely been triggered by the exposure of naked hair shafts from the degenerating follicles (Fig. 4F and data not shown). The inflammatory infiltrate mainly contained macrophages, lymphocytes, neutrophils and multinucleated giant cells (data not shown). The progressive degeneration and inflammation led to the loss of most follicles over time and the majority of hEGFR$^{KI/KI}$ mice were completely bald at the age of 5-7 months (data not shown). These results indicate that EGFR signalling is absolutely required for hair cycle progression. Moreover, EGFR is also essential for proper hair follicle differentiation, orientation and migration, as well as to preserve hair follicle integrity during the hair cycle.

Bone cell defects in hEGFR$^{KI/KI}$ mice

hEGFR$^{KI/KI}$ and Egfr$^{−/−}$ mice are significantly smaller than their control littermates. Impaired skeletal development is one of several defects that can be responsible for growth retardation. Therefore, we investigated whether bone development was defective in hEGFR$^{KI/KI}$ mice. Histological analysis of the long bones at birth revealed that the hypertrophic chondrocyte zone of the growth plate was significantly increased in hEGFR$^{KI/KI}$ mice (Fig. 5A,B). This phenotype might be caused either by increased or reduced expression of the hEGFR$^{KI}$ allele in the bone compartment. To discriminate between these two possibilities, bone sections from Egfr$^{−/−}$ newborn mice of mixed genetic background were analysed. Interestingly, in Egfr$^{−/−}$ long bones the zone of hypertrophic chondrocytes was similarly or even more increased as in hEGFR$^{KI/KI}$ bones, indicating that this phenotype is most probably caused by the absence of EGFR expression in cartilage (Fig. 5B,C). The proliferation of chondrocytes measured by Ki67 staining on sections was not affected and comparable in all three genotypes (Fig. 5E). The expression of EGFR in chondroblasts was analysed by X-galactosidase staining in Egfr$^{−/−}$ E14.5 foetuses, as the knockout allele harbours an E. coli lacZ allele inserted in-frame downstream of the first exon of the mouse Egfr gene (Sibilia and Wagner, 1995). In control foetuses, EGFR expression was detected in chondroblasts of all ossification centres (Fig. 5D). These results show that EGFR is expressed in chondroblasts during
Skin, bone and heart defects in \textit{hEGFR}^{KI/KI} mice

To test whether loss of the EGFR also influences osteoblastic differentiation and proliferation, calvarial osteoblasts were prepared from newborn \textit{hEGFR}^{KI/KI}, \textit{Egfr}^{–/–} and control mice. The proliferation potential of \textit{hEGFR}^{KI/KI} and \textit{Egfr}^{–/–} primary calvarial osteoblasts was decreased towards the end of the culture period (Fig. 5F,G). Moreover, calvarial osteoblasts derived from \textit{hEGFR}^{KI/KI} and \textit{Egfr}^{–/–} mice showed increased differentiation compared to controls, as assessed by an in vitro mineralisation assay. Compared with controls, \textit{hEGFR}^{KI/KI} and \textit{Egfr}^{–/–} osteoblast cultures formed approximately twice and three times as many mineralised bone nodules, respectively (Fig. 5H,I). Western blot analysis revealed a severe reduction of EGFR expression in calvarial osteoblasts of \textit{hEGFR}^{KI/KI} mice (Fig. 5J). Although differentiation and proliferation defects were observed in cultured osteoblasts, the bone thickness of newborn \textit{hEGFR}^{KI/KI} and \textit{Egfr}^{–/–} mice was comparable with controls and no overt bone remodelling defects could be detected (Fig. 5A-C). These results suggest that EGFR signalling normally supports proliferation, but inhibits differentiation of osteoblastic cells, keeping them in an undifferentiated, pre-osteoblastic state.

\textbf{\textit{hEGFR}^{KI/KI} mice develop heart hypertrophy}

\textit{hEGFR}^{KI/KI} mice can survive up to 6 months after birth, but often sudden death is observed at earlier times. To investigate the possible cause of lethality, \textit{hEGFR}^{KI/KI} mice were sacrificed and different organ systems were analysed. An increase in heart size was consistently observed, which was already visible at 3 weeks after birth and became more pronounced with age (Fig. 6A). Comparison of heart weights at 3 months of age showed that \textit{hEGFR}^{KI/KI} hearts were about twice as heavy as control \textit{hEGFR}^{KI/+} and \textit{Egfr}^{+/–} hearts (Fig. 6B). This difference was even more dramatic when the heart weights were corrected for the body weights considering that \textit{hEGFR}^{KI/KI} mice are smaller and only about half of the size of control mice (Fig. 6B). Histological cross-sections through the hearts of \textit{hEGFR}^{KI/KI} mice revealed a severe hypertrophy with dramatically increased thickness of the left ventricular wall and the interventricular septum at 3 weeks after birth (Fig. 6D). These defects became more severe with increasing age (Fig. 6G). Cardiomyocytes of 2.5- to 3.5-month-old \textit{hEGFR}^{KI/KI} mice were hypertrophic and measurements of their mean cross-sectional areas revealed a 1.9-fold increase compared with controls and histological sections did not reveal signs of hypertrophy (Fig. 6E; data not shown). Cardiac hypertrophies can occur as primary myocardial diseases or as a consequence of other conditions such as valve defects leading to valvular stenosis and/or regurgitation (Katz, 1990). When compared with controls, histological examinations of \textit{hEGFR}^{KI/KI} hearts revealed that the cusps of the pulmonary and aortic valves were thickened and hypercellular, a condition that most likely resulted from the development and suggest that EGFR signalling negatively regulates the maturation of hypertrophic chondrocytes, because the zone of hypertrophic chondrocytes is enlarged in its absence.
Discussion

In this study, a knock-in approach was employed to generate humanised EGFR mice, which harbour a human EGFR cDNA flanked by loxP sites. hEGFR\textsuperscript{KI/KI} mice display tissue-specific hypomorphic phenotypes, which correlate with the expression levels of the hEGFR\textsuperscript{KI} allele in various tissues. This is surprising because other genetically engineered alleles that displayed hypomorphic behaviour showed reduced expression in most of the tissues analysed (Vivian et al., 1999). Often hypomorphic alleles still harbour the neomycin resistance cassette with its regulatory elements in the locus and it has been shown for several genes that this can interfere with their proper expression in many tissues (Meyers et al., 1998; Partanen et al., 1996). This is a likely explanation for the low expression of the hEGFR\textsuperscript{KI} allele as it also contains a neomycin cassette in the first intron downstream of the human EGFR cDNA. The surprising finding, however, is that the hEGFR\textsuperscript{KI} allele is not hypomorphic in every tissue, but only in bone and epithelial cells. In other organs such as the brain the expression levels of the hEGFR\textsuperscript{KI} allele were comparable with the endogenous mouse Egfr gene. This suggests that there might be elements regulating Egfr expression in a tissue-specific manner. It is possible that the controlling elements of the neomycin gene interfere and compete with transcription factors, which would otherwise direct correct EGFR expression in the affected organs. Alternatively, the insertion of the human EGFR cDNA into the mouse locus might alter its chromatin conformation, thereby rendering it inaccessible to tissue-specific transcription factors.

In all the brain regions analysed, the hEGFR\textsuperscript{KI} allele was expressed at similar levels to the endogenous mouse gene. In fact, in brains of homozygote hEGFR\textsuperscript{KI/KI} mice none of the defects observed in \textit{Egfr}\textsuperscript{−/−} mice could be detected. hEGFR\textsuperscript{KI/KI} mice do not develop the cortical neurodegeneration and astrocyte proliferation in vitro is comparable with controls. Only after Cre-mediated deletion of the floxed hEGFR\textsuperscript{KI} allele a severe proliferation defect was observed in astrocytes cultured in vitro, demonstrating that the EGFR is required for proper proliferation of these cells. hEGFR\textsuperscript{KI/KI} mice did also not contain ectopic neurones in the hippocampus, a phenotype that is always observed in \textit{Egfr}\textsuperscript{−/−} mice (Sibilia et al., 1998). Because all the brain defects observed in \textit{Egfr}\textsuperscript{−/−} mice were rescued in hEGFR\textsuperscript{KI/KI} mice, it can be assumed that the amount of EGFR protein present in brain cells of hEGFR\textsuperscript{KI/KI} mice is similar to that in wild-type controls. Moreover, it appears that the binding affinity of the mouse EGFR ligands to the human EGFR is not significantly altered, suggesting that impaired receptor activation is not contributing to the hypomorphic defects seen in other tissues.

The observed phenotypes are probably determined by the level of transcription of the hEGFR\textsuperscript{KI} allele in various tissues. In epithelial tissues such as the skin and hair follicles where the hEGFR\textsuperscript{KI} allele is poorly transcribed, severe defects were observed that resemble those observed in \textit{Egfr}\textsuperscript{−/−} mice (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). Because \textit{Egfr}\textsuperscript{−/−} mice do not survive longer than P20, the skin and hair phenotypes could not be properly analysed directly in mutant mice. The hEGFR\textsuperscript{KI/KI} mice proved...
to be extremely useful to analyse how the absence of the EGFR affects hair follicle differentiation, migration and cycling. After the first hair cycle, hair follicles of hEGFR<sup>KI/KI</sup> mice fail to enter into catagen and remain in aberrant anagen, indicating that EGFR signalling is needed to regulate hair cycle progression. The EGFR also seems to be required to preserve that EGFR signalling is needed to regulate hair cycle progression. The aberrant localisation of hEGFR<sup>KI/KI</sup> hair follicles in the skin might have triggered an immunological response, which results in the destruction of the follicles. Alternatively, as the EGFR has been shown to inhibit differentiation and promote survival of epithelial cells, it is possible that in the absence of EGFR expression, hair follicles might be impaired in their survival capacity and/or undergo premature differentiation. With time, this would lead to hair follicle destruction and release of follicle material into the dermis, which in turn would trigger the immunological response. Similar hair abnormalities have also been observed in grafting experiments with Egfr<sup>−/−</sup> skin onto immunodeficient mice and in a skin targeted DN Egfr transgenic mouse model (Hansen et al., 1997; Murillas et al., 1995). However, in the latter model it could not be excluded that the severity of the phenotypes observed was determined not only by inhibition of the EGFR itself but also by the inhibition of the other Erbb family members which are also expressed in the epidermis (Stoll et al., 2001). As the skin and hair follicle defects observed in hEGFR<sup>KI/KI</sup> and the DN EGFR transgenic mice are very similar, it can be concluded that these phenotypes mainly result from direct EGFR inhibition.

EGFR signalling does not only seem to inhibit epithelial cell differentiation, but also the differentiation of bone cells. Bone development starts with the formation of mesenchymal condensations that first differentiate into chondrocytes, which become hypertrophic and are then invaded by blood vessels, bone-forming osteoblasts and bone-resorbing osteoclasts (Karsenty and Wagner, 2002). Active remodelling ultimately gives rise to a bone with growth plate and a central marrow cavity (Karsenty and Wagner, 2002). Numerous extracellular factors, such as hormones, growth factors and cytokines, modulate bone remodelling via differentiation and proliferation of bone cells, and this process has to be tightly regulated in order to prevent bone diseases (Karsenty and Wagner, 2002). The EGFR is expressed in chondroblasts of the developing ossification centres and the EGFR has also been shown to be expressed in osteoblasts and osteocytes in vivo (Daveideau et al., 1995). However, no bone defects have been reported so far for mice defective in EGFR signalling. A recent report has shown that transgenic mice expressing EGF ubiquitously are growth retarded, display defective chondrocyte development in the growth plate and osteoblasts accumulate in the endosteme and periostium (Chan and Wong, 2000). Controversial in vitro data regarding the role of EGFR signalling in bone cells have been reported, although two studies suggest that EGFR might stimulate osteogenic cell proliferation and suppress the differentiation into mature osteoblasts and chondrocytes (Chien et al., 2000; Yoon et al., 2000). In Egfr<sup>−/−</sup> and hEGFR<sup>KI/KI</sup> mice both osteoblasts and chondrocytes display increased differentiation in vitro and in vivo, respectively. As the hEGFR<sup>KI</sup> allele was poorly expressed in the affected cell types, it is likely that these defects result from the lack of Egfr expression. In the long bones of hEGFR<sup>KI/KI</sup> mice, the region of hypertrophic chondrocytes in the growth plate was significantly increased, suggesting that chondrocytes had undergone premature differentiation. This phenotype was even more severe in Egfr<sup>−/−</sup> mice, where Egfr expression is completely absent, indicating that EGFR signalling prevents differentiation of chondrocytes. Although no overt differences in bone mass could be detected in newborn Egfr<sup>−/−</sup> and hEGFR<sup>KI/KI</sup> mice, calvarial osteoblasts from these mice proliferate more slowly and display an increased differentiation capacity in vitro. It is likely that EGFR signalling is important to keep osteoblasts and chondrocytes in a proliferative state and inhibit their differentiation, thus allowing proper development of long bones. In the absence of EGFR expression, accelerated differentiation of chondrocytes and possibly decreased proliferation of osteoblasts can disturb longitudinal growth of long bones leading to severe growth retardation in the mice.

hEGFR<sup>KI/KI</sup> mice develop a severe heart hypertrophy with dramatically increased thickness of the ventricular walls and the interventricular septum, which was visible already at 3 weeks after birth and became more pronounced as age proceeded. Interestingly, Egfr<sup>−/−</sup> mice did not display signs of hypertrophy at this age, suggesting that this phenotype was most probably not due to the lack of EGFR expression. Cardiac hypertrophy is an adaptive response to many forms of disorders such as hypertension, myocardial infarction and valve defects aimed to augment the cardiac output (Katz, 1990). However, sustained hypertrophy usually leads to ventricular dilatation with consequent heart failure and sudden death (Katz, 1990). The response of cardiomyocytes to hypertrophic signals involves an increase in cell size and protein synthesis, induction of immediate-early genes such as AP-1 and re-expression of several foetal myocardial structural proteins (Sadoshima and Izumo, 1997). It has been shown that treatment of rat cardiomyocytes with EGF can trigger a hypertrophic response resulting in increased protein synthesis and transcriptional activation of Fos and Jun (Rebsamen et al., 2000). Similarly, G-protein-coupled receptor (GPCR) agonists were shown to transactivate the EGFR via shedding of HB-EGF by the metalloproteinase ADAM12 thereby inducing heart hypertrophy (Asakura et al., 2002; Prenzel et al., 1999). Moreover, EGF increases adenyl cyclase activity and cAMP accumulation, thus enhancing the heart contractility and beating rate (Nair et al., 1993). As heart-specific expression of the hEGFR<sup>KI</sup> allele seemed to be slightly higher than the endogenous wild-type allele, we speculate that increased EGFR signalling in cardiomyocytes is contributing to the development of the heart hypertrophy.

hEGFR<sup>KI/KI</sup> mice also display semilunar valve defects, which are known to induce aortic stenosis and regurgitation, and as a consequence can lead to heart hypertrophy. Heart valves develop from the endocardium, a specialised endothelium that undergoes complex epithelial-mesenchymal transformations leading to the formation of mesenchymal outgrowths (also called the cardiac cushions), from which the mature valve leaflets originate (Towbin and Belmont, 2000). The pulmonary and aortic, but not the atroventricular valves of hEGFR<sup>KI/KI</sup> hearts were thickened and hypercellular, which most probably results from the accumulation of mesenchymal cells. As similar valve defects were also present in Egfr<sup>−/−</sup> hearts, it is likely that
the hEGFR\textsuperscript{KI} allele is not expressed in the developing valves. Although we were unable to detect EGFR expression in wild-type valve structures by immunohistochemistry, we believe that the valve defects of hEGFR\textsuperscript{KI/KI} and Egfr\textsuperscript{−/−} hearts result from lack of EGFR expression in these structures. The other EGFR family members Erbb2 and Erbb4 are expressed in the endocardium and have been shown to be involved in the formation of the mesenchymal cushions (Camenisch et al., 2002; Erickson et al., 1997). It still remains to be determined how EGFR alone or in combination with these receptors regulates mesenchyme development and its differentiation into mature valve structures.

By studying the genetic interaction between EGFR and the protein-tyrosine phosphatase Shp2, it was shown that EGFR is required for semilunar valve development (Chen et al., 2000). Compound mutants between Shp2 and the hypomorphic EGFR\textsuperscript{wa2/wa2} mouse strains showed signs of aortic stenosis and regurgitation with subsequent development of myocardial hypertrophy (Chen et al., 2000). Although these defects were most severe in compound mutants, thickened semilunar valves were also observed in EGFR\textsuperscript{wa2/wa2} and Egfr\textsuperscript{−/−} mice, whereas no cardiac dilatation were reported for the single EGFR mutants (Chen et al., 2000). Similarly, we could not detect any signs of myocardial hypertrophy in 3-week-old Egfr\textsuperscript{−/−} mice, although semilunar valve thickening was observed. By contrast, the heart of hEGFR\textsuperscript{KI/KI} mice at this stage was already enlarged and displayed a severe thickening of the myocardial walls. Therefore, it is likely that the severe hypertrophy observed in hEGFR\textsuperscript{KI/KI} mice results from the malformations of the valves and from the enhanced hypertrophic response of cardiomyocytes to increased EGFR signalling, as the hEGFR\textsuperscript{KI} allele is expressed at higher levels in the myocardium. These defects can lead to a severe heart condition, which is probably responsible for the lethality of hEGFR\textsuperscript{KI/KI} mice, whereas the valve defects alone, as seen in Egfr\textsuperscript{wa2/wa2} mice do not seem to increase the mortality of these mice. Moreover, as hEGFR\textsuperscript{KI/+} or hEGFR\textsuperscript{KI/+} mice do not develop heart hypertrophy, it seems unlikely that enhanced expression of the EGFR in the myocardium alone can lead to the development of this heart condition. The conditional hEGFR\textsuperscript{KI/KI} mice may provide a useful model not only to study aortic valve diseases and developmental aspects of bone differentiation, but also to test novel therapies aimed to inhibit EGFR signalling for the treatment of cardiac hypertrophies and hyperproliferative bone diseases.

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