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Soluble dominant-negative receptor uncovers essential roles for fibroblast growth factors in multi-organ induction and patterning

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Despite a wealth of experimental data implicating fibroblast growth factor (FGF) signaling in various developmental processes, genetic inactivation of individual genes encoding specific FGFs or their receptors (FGFRs) has generally failed to demonstrate their role in vertebrate organogenesis due to early embryonic lethality or functional redundancy. Here we show that broad mid-gestational expression of a novel secreted kinase-deficient receptor, specific for a defined subset of the FGF superfamily, caused agenesis or severe dysgenesis of kidney, lung, specific cutaneous structures, exocrine and endocrine glands, and craniofacial and limb abnormalities reminiscent of human skeletal disorders associated with FGFR mutations. Analysis of diagnostic molecular markers revealed that this soluble dominant-negative mutant disrupted early inductive signaling in affected tissues, indicating that FGF signaling is required for growth and patterning in a broad array of organs and in limbs. In contrast, transgenic mice expressing a membrane-tethered kinase-deficient FGFR were viable. Our results demonstrate that secreted FGFR mutants are uniquely effective as dominant-negative agents *in vivo*, and suggest that related soluble receptor isoforms expressed in wild-type mouse embryos may help regulate FGF activity during normal development.

Keywords: dominant-negative/FGF/limb/organogenesis/transgenic mice

Introduction

Fibroblast growth factors (FGFs) belong to a superfamily of signaling molecules thought to regulate cellular proliferation, migration and differentiation by binding to and activating members of a family of tyrosine kinase receptors (FGFRs). Mutations in FGFRs are associated with heritable human skeletal disorders involving craniofacial and limb anomalies, including Apert, Crouzon, Pfeiffer, Jackson–Weiss and Beare–Stevenson cutis gyrata syndromes, and achondroplasia (Johnson and Williams, 1993; Mason, 1994; Muenke and Schell, 1995; Przylepa *et al*., 1996). FGFR proteins, encoded by four genes, are characterized by three immunoglobulin (Ig)-like domains (designated either as loops I, II and III, or D1, D2 and D3) within the extracellular region, a single transmembrane region and a split cytoplasmic tyrosine kinase domain. Although both loops II and III are involved in ligand binding, ligand specificity is determined by the C-terminal portion of loop III (Givol and Yayon, 1992; Johnson and Williams, 1993; Cheon *et al*., 1994). For example, alternative splicing of FGFR2 results in the creation of the differentially responsive receptor isoforms designated FGFR2IIIb and FGFR2IIIc (here abbreviated FGFR2b and FGFR2c, respectively) (Miki *et al*., 1992). FGFR2b binds efficiently to aFGF, FGF3, FGF7 and FGF10 (A.Blunt and D.Ornitz, personal communication) in cultured BaF3 cells, while FGFR2c binds to aFGF, bFGF, FGF4, FGF6 and FGF9 (Ornitz *et al*., 1996, and references therein). Ligand binding induces receptor dimerization and intermolecular transphosphorylation of the receptor subunits, thereby initiating FGFR signaling. The regulation of FGFR signaling is intricate and complex because the multiple ligand and receptor isoforms that exist share overlapping recognition and redundant specificity (Givol and Yayon, 1992; Johnson and Williams, 1993). Further complexity is contributed by the generation of membrane-bound and secreted receptor isoforms from the same gene, and by interactions of FGF ligands with heparan sulfate proteoglycans on the cell surface and extracellular matrix (Givol and Yayon, 1992; Johnson and Williams, 1993).

FGFs and their receptors have been implicated as regulators of vertebrate development based on analysis of embryonic expression patterns (Wilkinson *et al*., 1989; Orr-Urtreger *et al*., 1991, 1993; Stark *et al*., 1991; Niswander and Martin, 1992; Peters *et al*., 1993; Crossley and Martin, 1995; Yamasaki *et al*., 1996; Neubüser *et al*., 1997; Ohuchi *et al*., 1997). Gene targeting studies have provided compelling genetic evidence that FGFR signaling is involved in early developmental processes such as blastocyst growth, primary mesoderm induction and early pattern formation (Amaya *et al*., 1991; Deng *et al*., 1994; Yamaguchi *et al*., 1994; Feldman *et al*., 1995). A later developmental role in mediating mesenchymal–epithelial cell interactions during the formation of structures such as limb and lung has been suggested through use of FGF implants, antisense oligonucleotides and neutralizing antibodies (Represa *et al*., 1991; Niswander and Martin, 1993; Niswander *et al*., 1993; Cohn *et al*., 1995; Nogawa and Ito, 1995; Crossley *et al*., 1996; Post *et al*., 1996; Ohuchi *et al*., 1997). However, obtaining more definitive genetic data for a role in secondary inductive events and organogenesis from gene targeting approaches has been problematic due to early embryonic lethality prior to organ induction, as with null mutations in FGFR1, FGF4, FGF8 and FGFR2 (Deng *et al*., 1994; Yamaguchi *et al*., 1994; Feldman *et al*., 1995; C.Deng, personal communication; E.Meyers and G.Martin, personal communication), or conversely due to functional redundancy within both the

FGF and FGFR families, yielding little or no embryonic phenotype. It has been shown that inactivation of FGF3 perturbs the development of the ear and tail (Mansour *et al*., 1993), loss of FGF5 or FGF7 function results in abnormal hair growth and development (Hébert *et al*., 1994; Guo *et al*., 1996), FGFR3 null mutants demonstrate prolonged enchondral bone growth and inner ear defects (Colvin *et al*., 1996; Deng *et al*., 1996), FGF6 inactivation perturbs adult skeletal muscle regeneration (Floss *et al*., 1997), and bFGF null mice show no overt phenotypes (S.Ortega and C.Basilico, personal communication). While chimeric embryos demonstrate a partial rescue and a delay in the early lethality associated with FGFR1 inactivation (Ciruna *et al*., 1997; Deng *et al*., 1997), their phenotypes are strongly influenced by generally unpredictable competitive interactions between wild-type and null embryonic cells.

Dominant-negative transgenic strategies have been used with mixed success to overcome functional redundancies and early embryonic lethality. Truncated membrane-bound FGFRs that lack a functional tyrosine kinase domain have been shown *in vitro* to disrupt FGFR signaling of multiple receptor isoforms by competing for ligand binding and forming inactive heterodimers with endogenous FGFRs (Ueno *et al*., 1992). *In vivo*, this dominant-negative approach has been used to show that FGFs are required for *Xenopus* gastrulation (Amaya *et al*., 1991), and also for epidermal organization, differentiation and wound healing (Werner *et al*., 1993, 1994), branching morphogenesis of the lung (Peters *et al*., 1994), lens development (Robinson *et al*., 1995) and lobuloalveolar development of the mammary gland (Jackson *et al*., 1997). However, the efficacy of defective membrane-bound receptors is limited by the spatial and temporal expression properties of the transcriptional promoter directing expression of the transgene to specific cell types, and by the need to be greatly overexpressed in order to compete effectively for ligand binding with native receptors at the cell surface.

We have overcome many of these limitations through the use of a secreted soluble dominant-negative receptor (DNR) mutant which can bind a specific subset of FGFs extracellularly and disrupt signaling of virtually all FGFR isoforms. By using a metallothionein (MT) promoter to express this soluble DNR mutant broadly in mouse embryos, we could demonstrate that FGFR signaling is required for the normal development of multiple organs, including kidney, lung and specific endocrine and exocrine glands, as well as of a variety of craniofacial and cutaneous structures and limbs.

Results

Efficacy of dominant-negative FGFR2 mutants in vitro

The extracellular ligand-binding domain of the mouse FGFR2b cDNA, containing both D2 and D3 Ig-like domains, was used to generate two truncated DNR forms (Figure 1A). In one form, the transmembrane domain was retained, anchoring it to the cell membrane (dnFGFR-Tm), while in the other the transmembrane domain was replaced by the mouse Ig heavy chain hinge and Fc domains, thereby creating a stable chimeric protein (dnFGFR-HFc), secreted as a disulfide-linked dimer (Cheon *et al*., 1994) (Figure 1A). To test and compare their biological function *in vitro*, the two receptor mutants were overexpressed using the mouse MT I gene promoter (MMTneo vector) in Balb/MK cells, an epidermal cell line which expresses the native FGFR2b (Bottaro *et al*., 1990). Both dominant-negative receptor forms selectively reduced the mitogenic activity of aFGF and FGF7 (Figure 1B), but not that of epidermal growth factor (EGF), hepatocyte growth factor (HGF) or insulin-like growth factor-1 (IGF-1) (not shown), indicating that they were equally effective dominant-negative receptor forms *in vitro*.

The ligand-binding specificity of dnFGFR-HFc was tested in a mitogenic assay using serum-starved NIH 3T3 cells treated overnight with increasing concentrations of purified dnFGFR-HFc pre-incubated with 1 ng/ml aFGF, bFGF, FGF3 or FGF4. The mitogenic activity, measured as [^{3}H]thymidine incorporation, is shown in Figure 1C. As expected (Ornitz *et al*., 1996), dnFGFR-HFc inhibited the mitogenic response to aFGF completely and to FGF3 less effectively, but did not affect bFGF or FGF4 activity. To determine that the dnFGFR-HFc mutant was actually blocking signaling through endogenous FGFRs, quiescent NIH 3T3 mouse fibroblasts were exposed to aFGF or bFGF pre-incubated with increasing amounts of the purified soluble DNR. Endogenous FGFR1 was immunoprecipitated with either anti-phosphotyrosine or anti-FGFR1 antibodies, and analyzed for phosphorylation levels by immunoblot analysis. The dnFGFR2-HFc mutant blocked phosphorylation of FGFR1 in response to aFGF but not bFGF, indicating that the soluble chimera functions as a dominant-negative inhibitor by interfering with specific FGFR signaling (Figure 1D).

Soluble, but not tethered, dominant-negative FGFR2 mutant induces embryonic lethality

To determine the *in vivo* consequences of perturbing FGFR signal transduction, the mouse MT promoter with flanking locus control regions (LCRs) was employed to achieve broad reproducible expression (Takayama *et al*., 1996) of either the dnFGFR-HFc or dnFGFR-Tm mutant forms in transgenic mice. Attempts to establish MT-dnFGFR-HFc transgenic lines were unsuccessful (0/89 potential founders screened), raising the possibility that expression of the soluble chimera was incompatible with normal mouse development. Therefore, mouse zygotes micro-injected with the MT-dnFGFR-HFc transgene were allowed to develop to 18.5 days post-coitum (d.p.c.) and harvested for analysis. Fifteen percent of viable fetuses were grossly abnormal, all of which harbored the MT-dnFGFR-HFc transgene (Table I). All abnormal transgenic fetuses were small and displayed limb truncations of varying degrees of severity, ranging from shortening of the most distal phalangeal elements to complete loss of the appendage (Figure 2A). Typically, limb phenotypes were accompanied by craniofacial anomalies, curly tails, thin featureless skin and open eyes with opacities. Transgenic embryos were also identified at 12.5 d.p.c., about half of which were smaller with related limb and head phenotypes (Figure 2B; Table I). Transgenic embryos at 9.5 d.p.c were unremarkable.

Variability in the penetrance and expressivity of the phenotypes demonstrated in these ‘transient’ transgenic embryos could be explained by spatial and/or temporal differences in transgene expression caused by genomic

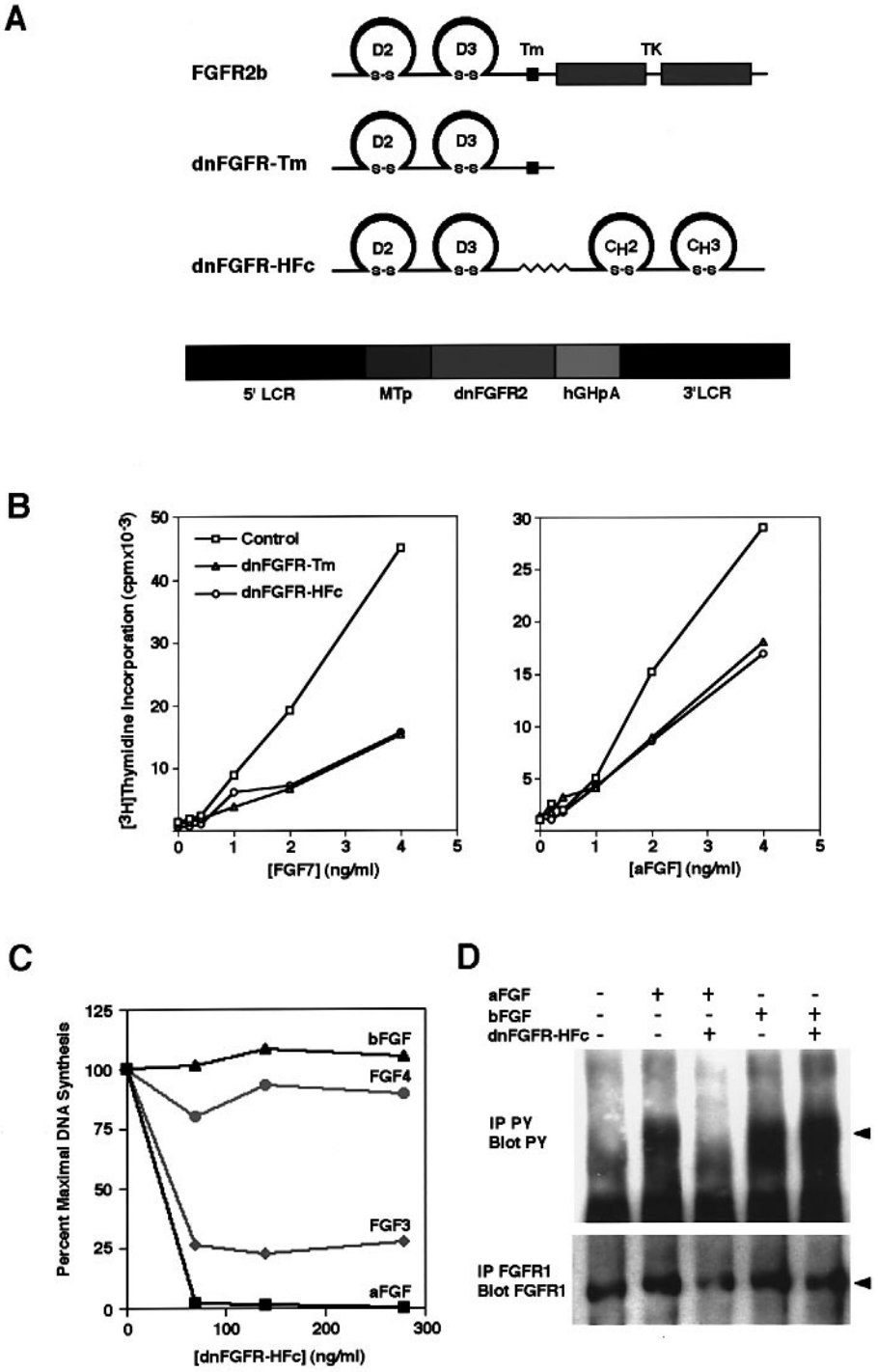

Fig. 1. Structure and *in vitro* activity of dominant-negative FGFR2 mutants. (**A**) Schematic diagram of native and dominant-negative receptors, and transgene construct. D2 and D3, Ig-like loops 2 and 3 of FGFR2b extracellular ligand-binding domain; Tm, transmembrane region; TK, split tyrosine kinase domain; C_H2 and C_H3, constant regions 2 and 3 of Ig heavy chain; and the hinge region (zigzag line). The transgene consists of the mouse metallothionein (MTp) promoter and its flanking locus control regions (LCRs), the human growth hormone polyadenylation signal (hGHpA) and the cDNA of either truncated or chimeric FGFR2b. (**B**) Balb/MK cells were transfected with either dnFGFR-Tm or dnFGFR-HFc, and the activity of each mutant was measured as inhibition of the mitogenic response to aFGF and FGF7. (**C**) The ligand-binding specificity of the soluble mutant was determined by the ability of increasing amounts of purified dnFGFR-HFc to block the proliferative response of NIH 3T3 cells to distinct FGFs. (**D**) Starved NIH 3T3 cells were treated with aFGF or bFGF in the presence or absence of purified dnFGFR-HFc. Cellular extracts were immunoprecipitated (IP) and subsequently probed with either anti-PY or anti-FGFR1 antibodies. Mouse FGFR1 (arrowheads) had an apparent mol. wt of 130–145 kDa.

sequences near the site of integration. *In situ* hybridization revealed that the MT-dnFGFR-HFc transgene was broadly expressed (not shown), and the protein was found to be localized extracellularly throughout the embryo (Figure 2D and F). To determine if the variability in embryonic phenotype was dependent on the level of transgene expression achieved by mid-gestation, protein lysates were prepared from two transgenic mid-gestation embryos, one

with and one without overt head and limb phenotypes, and quantified by Western blot analysis using an anti-mouse IgG (Fc) antibody. Figure 2G shows that the MT-dnFGFR-HFc transgene was highly expressed by 12.5 d.p.c. in the embryo displaying the severe phenotype, while the overtly normal embryo had little or no transgene expression at that time. To demonstrate that expression of native FGFRs was not affected by expression of the soluble chimera, the same blot was stripped and probed in turn with antibodies to FGFR1 and FGFR2. No detectable differences were observed in receptor protein levels between control and transgenic littermates (not shown), indicating that the soluble DNR mutant does not overtly alter native receptor physiology.

In striking contrast to results using the MT-dnFGFR-HFc transgene, three viable founder lines carrying the MT-dnFGFR-Tm transgene were generated readily (3/22 potential founders screened). These mice were characterized by strong transgene expression in many adult tissues, including liver, kidney and gastrointestinal tract and, in two of three lines, by expression in mid-gestation embryos, as well as by relatively mild changes in the skin (not shown), resembling those reported previously in mice in which a K14 promoter was used to target expression of a similar membrane-bound FGFR2 kinase-deficient mutant (Werner *et al*., 1994). Therefore, the soluble chimera was far superior to the membrane-bound form at perturbing development, and much more effective as a dominant-negative agent.

Table I. Genotype and gross morphology of soluble FGFR2 mutant transgenic embryos

Age (days)	No. of viable embryos	No. of TG embryos	Overt phenotypes[a]
9.5	26	3 (12%)	–
12.5	155	20 (13%)	9 (45%)
18.5	66	10 (15%)	9 (90%)

[a]Of 10 E18.5 transgenic (TG) embryos, nine exhibited head, eye and limb abnormalities, and eight had a curly tail. Of 20 E12.5 embryos, nine had morphologically aberrant head and limbs.

Soluble FGFR causes developmental abnormalities reminiscent of human skeletal disorders

Recently, mutations in genes encoding FGFRs have been detected in a cluster of clinically related human autosomal dominant skeletal disorders involving craniofacial and

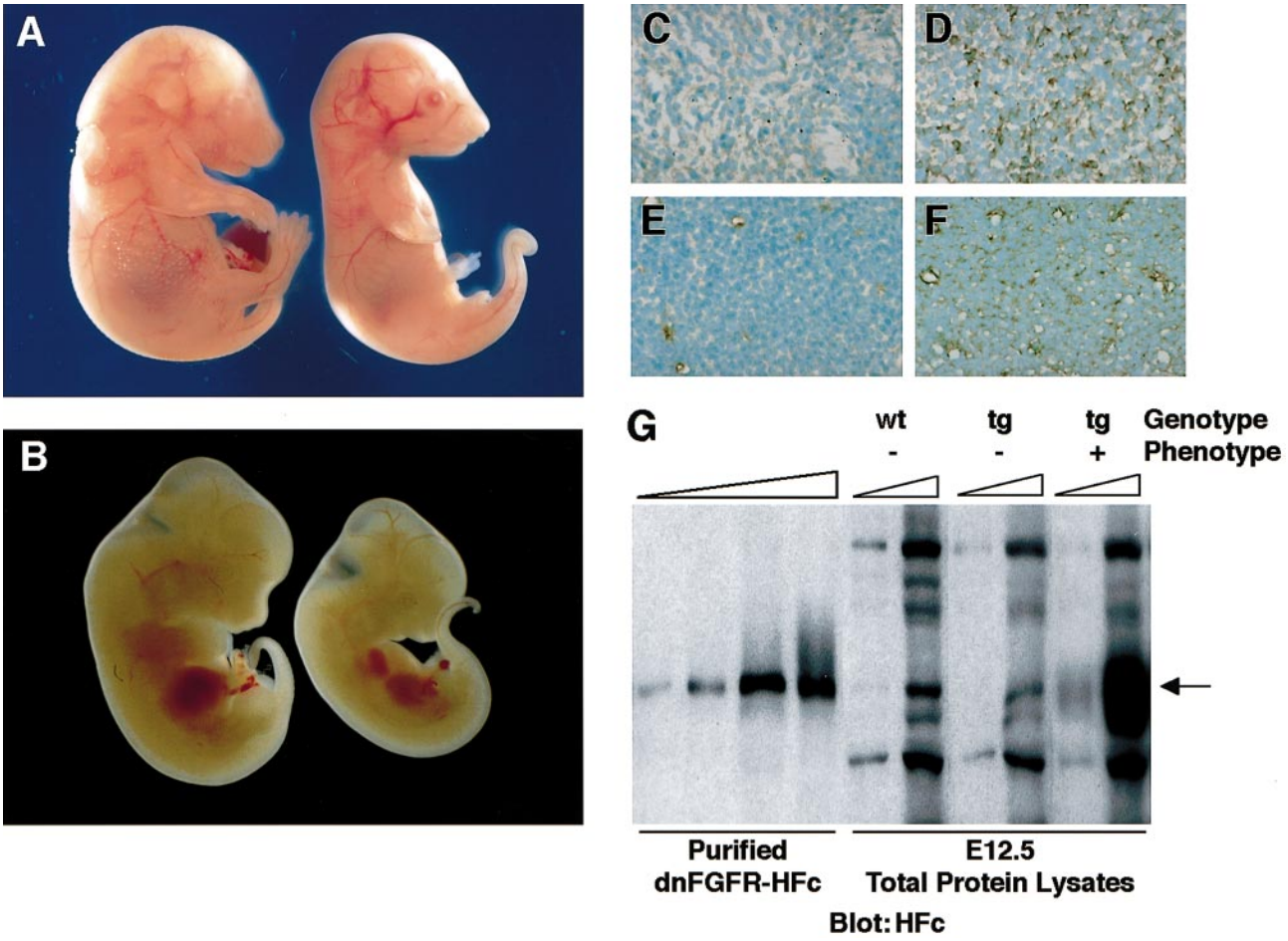

Fig. 2. Expression of a soluble FGFR2 mutant induces embryonic lethality. (**A**) E18.5 and (**B**) E12.5 transgenic (right) and control (left) mouse embryos. (**C–F**) Immunohistochemical detection of soluble FGFR2 mutant in transgenic (D and F) relative to control (C and E) E12.5 embryos using an anti-HFc antibody. Note the cytoplasmic and extracellular staining in transgenic muscle mass (D) and spinal cord (F). Magnification: 630×. (**G**) Immunoblot analysis of total protein lysates from wild-type (wt) and transgenic (tg) E12.5 embryos without (–) and with (+) an overt phenotype. The concentration of the purified FGFR2 chimera ranges from 5 to 100 ng per lane, while for each embryo, 5 and 100 μg were loaded per lane. The blot was probed with anti-mouse IgG (Fc). The chimeric protein (arrow) was 80 kDa.

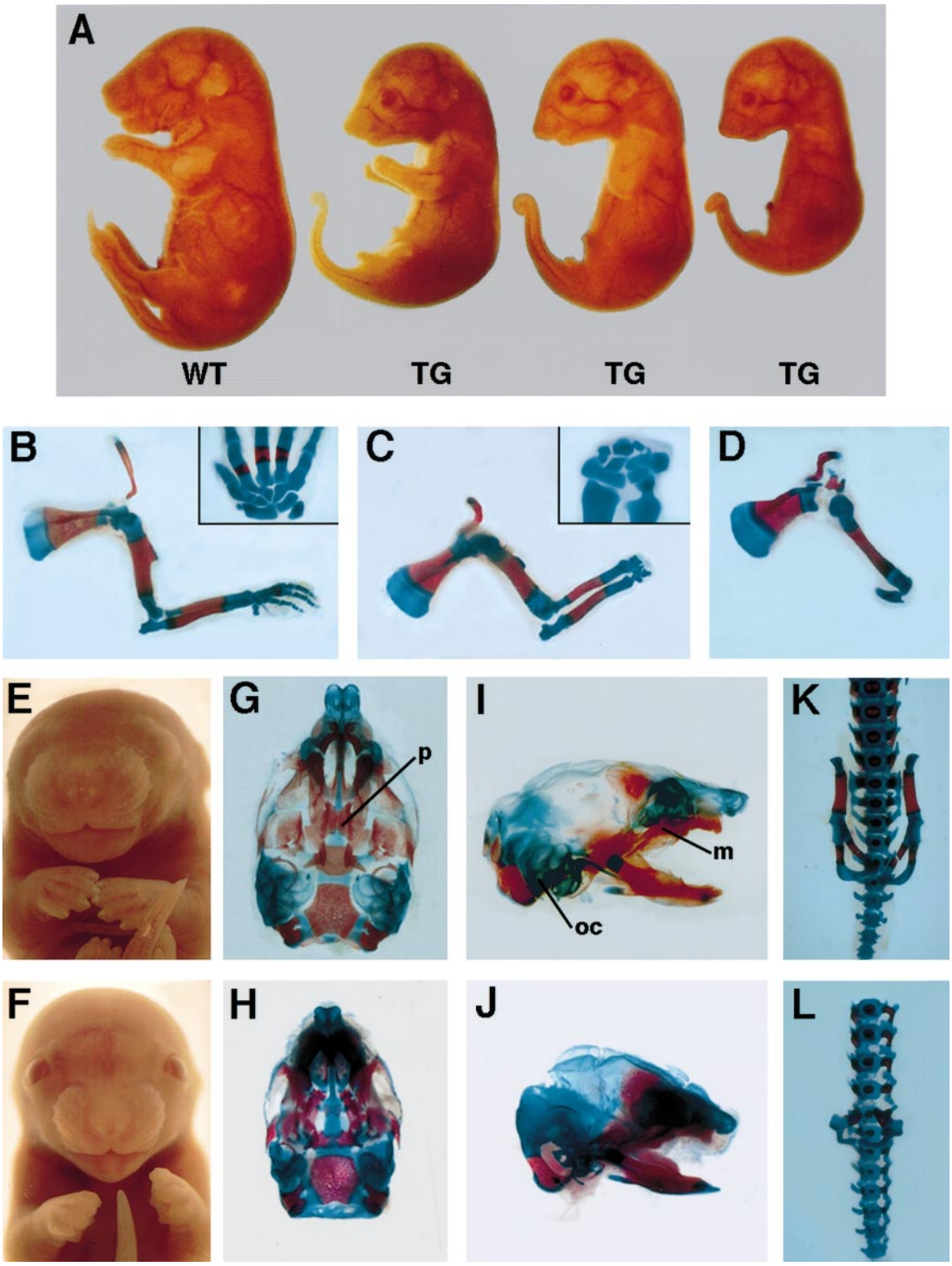

Fig. 3. Skeletal phenotypes in dnFGFR-HFc transgenic embryos. (**A**) Transgenic (TG) E18.5 embryos with increasingly severe limb phenotypes and a wild-type (WT) littermate. Note that the level of truncations is more proximal in hind than forelimb. (**B**) Normal forelimb skeleton stained with alizarin red and alcian blue compared with those of transgenic (**C** and **D**) embryos shown in (A). Inserts show normal carpal bones at the distal end of truncated limb compared with wild-type. Frontal view of normal (**E**) and transgenic (**F**) littermates. Note the open eyes, and the loss of toenails and the soft tissue syndactyly in the transgenic forelimb. Ventral (**G** and **H**) and side (**I** and **J**) views of skulls from control (G and I) and transgenic (H and J) embryos. Palantine bone (p), otic capsule (oc) and maxillary bone (m) are indicated. (**L**) Rudimentary pelvis from mutant embryo shown on the far right in (A), compared with that of a normal littermate (**K**).

limb anomalies (reviewed in Muenke and Schell, 1995; Mulvihill, 1995). Nine of 10 E18.5 MT-dnFGFR-HFc embryos displayed obvious anomalous limb and craniofacial features (Figure 3A and F). It was therefore of great interest to examine appendicular and craniofacial skeletal development in these transgenic embryos.

Table II. Incidence of histological abnormalities in E18.5 embryos expressing the soluble FGFR2 mutant

Organ	No. of embryos examined	Dysgenesis[a]	Agenesis[a]
Lung	8	–	8
Thyroid	5	–	5
Anterior pituitary	3	–	3
Tooth buds	6	–	6
Salivary glands	5	–	5
Kidney	8	3	5
Hair follicles	8	4	4
Inner ear	8	8	–
Thymus	8	8	–
Glandular stomach	8	8	–
Pancreas	8	8	–

[a]Dysgenesis is defined as malformation or reduction in number or size, and agenesis as absence of organ. See text for details.

Limb bud outgrowth and determination of limb elements in a proximal to distal sequence (i.e. hip to toe) depend upon continuous FGF signals from the apical ectodermal ridge, or AER (Niswander *et al*., 1993; Fallon *et al*., 1994, and references therein). In transgenic embryos, truncations of forelimb and hindlimb skeleton occurred at different levels along the proximodistal limb axis (Figure 3A–D) within a phenotypic range that recapitulated the results of AER removal at varying times in the chick wing bud (Saunders, 1948), and presumably reflected the time of onset of transgene expression in a given embryo. Development of the normal mouse hindlimb is delayed by about half a day relative to the forelimb and, accordingly, truncations were always seen at a more proximal level in the hindlimb compared with the forelimb (Figure 3A). Notably, in the most severely affected E18.5 embryos, only a rudimentary partial pelvis was formed (Figure 3L), clearly establishing a requirement for FGF signaling in pelvic development. The least affected embryos displayed absent toenails and very mild soft tissue syndactyly in distal appendages (Figure 3F).

Analysis of the skull of E18.5 transgenic fetuses revealed a widely cleft palate, reduced maxillary bone, absent otic capsule and rudimentary inner ear structures (Figure 3H and J). Therefore, the presence of the dnFGFR-HFc disrupted development of many skeletal tissues demonstrating abnormalities in FGFR-associated human syndromes.

Soluble FGFR dominant-negative mutant disrupts the formation of specific organs

Detailed histopathological analysis of E18.5 embryos expressing the soluble dnFGFR mutant revealed developmental anomalies in multiple organs (Table II), all of which demonstrate embryonic expression of FGFR1 and/or FGFR2 (Orr-Urtreger *et al*., 1991, 1993; Peters *et al*., 1992, 1993). In the most severely affected transgenic embryos, the kidneys failed to develop (Figure 4G). Occasionally a transgenic embryo demonstrated unilateral formation of a very small right kidney, which appeared disorganized and was characterized by small glomeruli and enlarged vacuolated tubules (Figure 4N). Although visceral and parietal pleura were present, the lungs routinely were absent (Figure 4B); the respiratory system consisted only of the trachea and rudimentary primary bronchi, which typically ended abruptly without further branching. However, in one of nine transgenic embryos analyzed, pulmonary branching morphogenesis had been initiated and then prematurely arrested, presumably due to a delay in transgene expression (Figure 4C).

Figure 4 also shows that in the head/neck region tooth buds did not form (Figure 4L), the thymus was small, hypoplastic and lacked a defined medulla and cortex (Figure 4P), the thyroid, salivary and pharyngeal serous glands were missing (Figure 4E, and not shown), the eyes were relatively small and never formed eyelids, and only a rudiment of the inner ear was present (Figure 4J). Furthermore, the central nervous system appeared hypocellular and disorganized, and the anterior pituitary was missing (not shown). In the abdomen, in addition to renal agenesis, the adrenal gland occasionally appeared hypoplastic and cytologically abnormal (Figure 4N). Of the gastrointestinal organs, the pancreas consisted of a greatly reduced number of morphologically aberrant acinar cells and no detectable islets (Figure 4T), the liver was smaller and exhibited increased red blood cell hematopoiesis (Figure 4G, H and T) and the glandular portion of the stomach was reduced or missing (Figure 4G and R). In contrast, the small and large intestine appeared overtly normal (Figure 4G and H), as did reproductive organs.

The most striking change in the transgenic E18.5 skin, which clearly expressed the soluble DNR protein (Figure 5D), was in hair follicle development. In some embryos, hair follicles were reduced in number by 40–60%, while in the most severely affected embryos they were absent (Figure 5B and F; Table II). The transgenic epidermis was very thin relative to wild-type. Keratin K14 staining in the transgenic epidermis was unremarkable, as was staining for K1, loricrin and neural cell adhesion molecule (N-CAM) (Figure 5F and H, and data not shown). In contrast, K6 expression, which in wild-type E18.5 skin was undetectable, was high throughout the transgenic epidermis (Figure 5J). Relative to age-matched controls, cellular proliferation was reduced by 55% (2.2 versus 1.0 mitotic figures/mm) and 61% (3.6 versus 1.4 figures/mm) in the E18.5 transgenic epidermis and dermis, respectively. Moreover, in the epidermis of transgenic embryos lacking hair follicles, apoptotic cells were reduced by 71% relative to controls (21 versus 6 cells/mm) (Figure 5L). These results suggest that the normal program of differentiation had been significantly altered in embryonic skin expressing the soluble DNR.

Inhibition of FGFR signaling blocks inductive interactions and outgrowth

To determine if dnFGFR-HFc expression disrupted mesenchymal–epithelial inductive interactions in affected appendages and organs, mid-gestational embryos were subjected to *in situ* hybridization using a variety of diagnostic molecular markers. In the normal developing limb, expression of the homeobox gene *Msx-1*, but not *Msx-2*, in the distal mesenchyme requires the presence of a functional AER (Wang and Sassoon, 1995, and references therein). The limb bud rudiment in a severely affected transgenic E12.5 embryo was identified by the presence of an anterior *Msx-2* domain (Figure 6D). However, transcripts for *Msx-1* were undetectable in mesenchyme

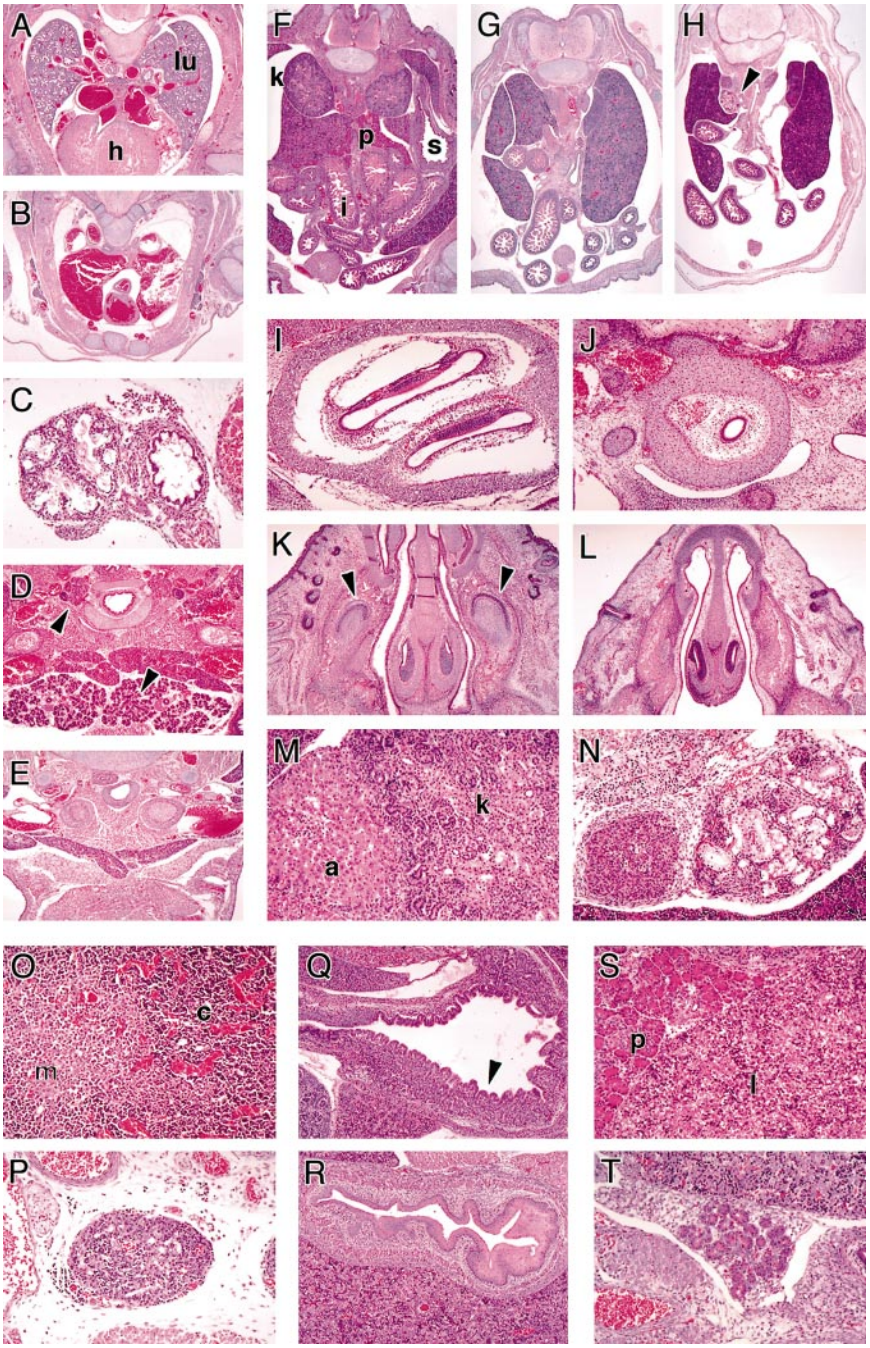

Fig. 4. Agenesis or dysgenesis of multiple organs in E18.5 embryos expressing the soluble FGFR2 dominant-negative mutant. (**A** and **B**) Transverse section through the chest cavity of wild-type (A) and transgenic (B) embryos. Note the absence of lungs and the enlarged heart in (B). (**C**) Prematurely arrested bronchial branching morphogenesis found in one transgenic embryo. (**D** and **E**) Thyroid and salivary glands (arrowheads) in wild-type embryo (D) are absent in mutant embryo (E). (**F–H**) Transverse section through the abdominal cavity of wild-type (F) and two transgenic embryos (G and H). The arrowhead in (H) points to a unilateral kidney rudiment of a transgenic embryo (see N). (**I** and **J**) Transverse section through the inner ear of control (I) and transgenic (J) embryos. (**K** and **L**) Tooth buds (arrowheads) in wild-type (K) are absent in transgenic embryo (L). (**M** and **N**) Higher magnification of normal embryonic E18.5 kidney and adrenal gland (M) compared with the dysgenic kidney found in some transgenic embryos (N). (**O** and **P**) Thymus gland of control (O) and transgenic (P) embryo; note the absence of distinct medulla and cortex. (**Q** and **R**) The glandular stomach (arrowhead) of a wild-type embryo (Q) appears to be replaced by non-glandular epithelium in a transgenic littermate (R). (**S** and **T**) Liver and exocrine pancreas of a non-transgenic embryo (S) compared with the same tissues of a mutant embryo (T). In the latter, the pancreas is limited to the hypoplastic and morphologically atypical gland shown (T). a, adrenal gland; c, thymic cortex; h, heart; k, kidney; l, liver; lu, lung; m, thymic medulla; p, pancreas; s, stomach. Original magnification: (A, B and F–H) 25×; (C, I, J, M–P, S and T) 200×; (D, E, K and L) 50×; (Q and R) 100×.

or ectoderm (Figure 6B), suggesting that a functional as well as morphological AER was lost by E12.5, or had never formed. In another E12.5 transgenic embryo, in which limb buds clearly were initiated and then arrested, distal 'progress zone' mesenchymal proliferation was virtually absent (Figure 6F). In contrast, in the more proximal pre-cartilaginous condensations, proliferation was still active at this time.

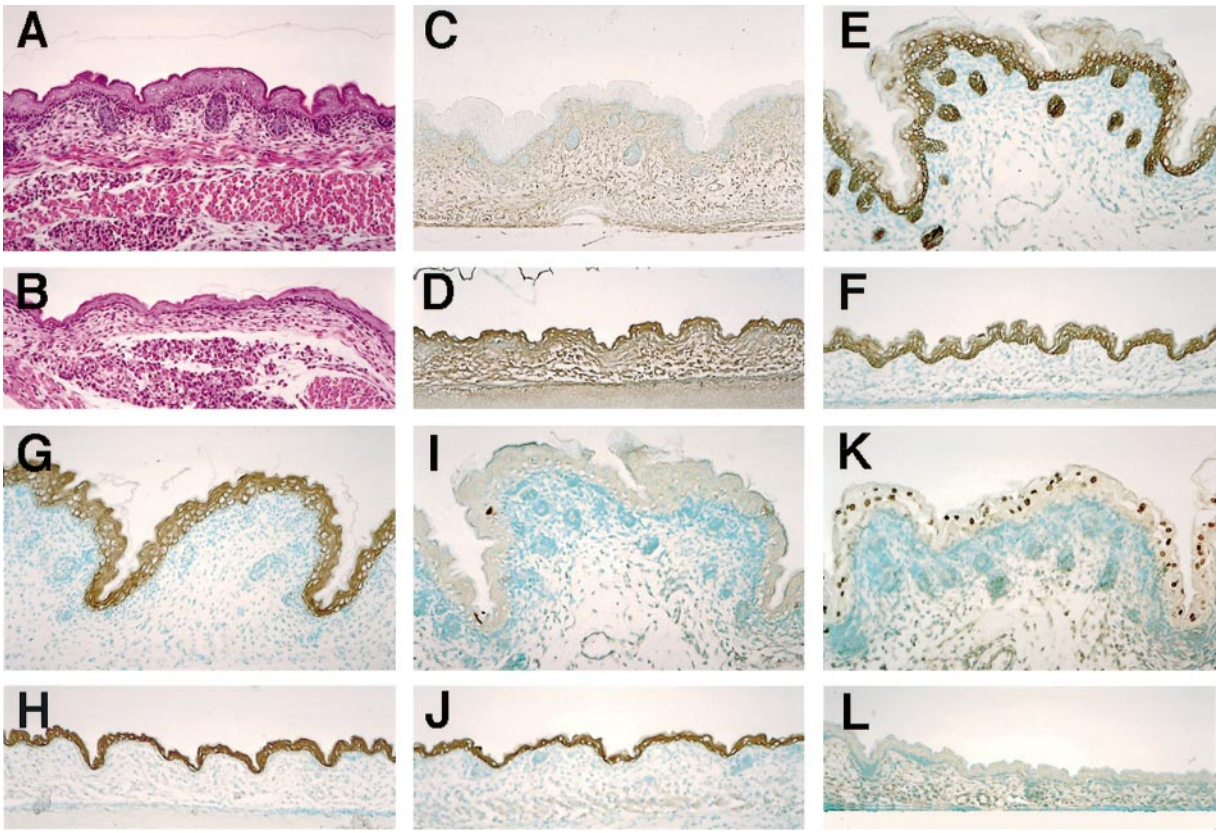

Fig. 5. Expression of keratin K6 and reduced apoptosis in epidermis of dnFGFR-HFc transgenic embryos reflects an altered program of differentiation. Dorsal skin of E18.5 wild-type (A, C, E, G, I and K) and transgenic (B, D, F, H, J and L) embryos stained with H&E (**A** and **B**) or subjected to immunohistochemistry to detect the transgene protein (**C** and **D**), keratin markers K14 (**E** and **F**), K1 (**G** and **H**) and K6 (**I** and **J**) or apoptotic cells (**K** and **L**). Original magnification: 200×.

Development of the definitive kidney proceeds through reciprocal inductive interactions between the metanephric blastema and the wolffian duct-derived ureteric bud, resulting in mesenchymal–epithelial conversion. During this conversion, the paired box gene *Pax-2* is expressed in condensed mesenchyme apparently in response to early epithelial signals (Eccles *et al*., 1995); however, *Pax-2* transcripts were not detectable in the blastema of overtly affected mid-gestational transgenic embryos (Figure 6H). Moreover, epithelium was not evident within the blastema, and no c-*met* expression was detected (not shown). These data strongly suggest that expression of dnFGFR-HFc disrupted reciprocal inductive signals that normally induce ureteric bud outgrowth and/or mesenchymal–epithelial conversion.

The lungs are derived from foregut endoderm via budding, outgrowth and subsequent branching morphogenesis. Mid-gestational transgenic embryonic lungs exhibited the initiation of one or two buds and very limited outgrowth, but no evidence of branching. Thyroid transcription factor 1 (TTF-1) is first expressed in epithelial cells at the onset of normal lung morphogenesis, is required for branching of the lower lung bronchial tree (Kimura *et al*., 1996) and acts as an upstream regulator of surfactant protein C (SPC) (Minoo *et al*., 1995; Kelly *et al*., 1996). *TTF-1* transcripts were detected in both lung buds of wild-type E12.5 embryos (Figure 6I) and in a single rudimentary truncated bud present in an age-matched transgenic embryo (Figure 6J), indicating that while a *TTF-1*-expressing lung primordium can form, it cannot be induced to grow and branch. Moreover, FGFs must act downstream of *TTF-1* or, alternatively, may function along a parallel pathway.

Discussion

In this report, we employ a novel dominant-negative loss-of-function approach, in which a secreted kinase-deficient FGFR2b mutant is broadly expressed in mid-gestation mouse embryos, to demonstrate that FGFR signaling is essential for inductive interactions and patterning associated with the formation of multiple organ systems. In some cases, phenotypic alterations induced by expression of this DNR constitute a dramatic confirmation of experimental studies implicating one or more FGFs as important developmental regulators, as in the limb, lung, tooth, skin, inner ear and skeleton. In other organs, such as the pancreas, kidney, pituitary, thyroid and thymus, our data provide new insights into the involvement of FGFs in organogenesis.

Soluble FGFR mutant is uniquely effective as a dominant-negative agent

Remarkably, while embryos broadly expressing soluble dnFGFR-HFc exhibited striking developmental abnormalities and died prior to birth, mice expressing dnFGFR-Tm, which equivalently inhibited FGF-mediated mitogenesis *in vitro*, were viable with few obvious phenotypes. Why was the dnFGFR-HFc form so much more

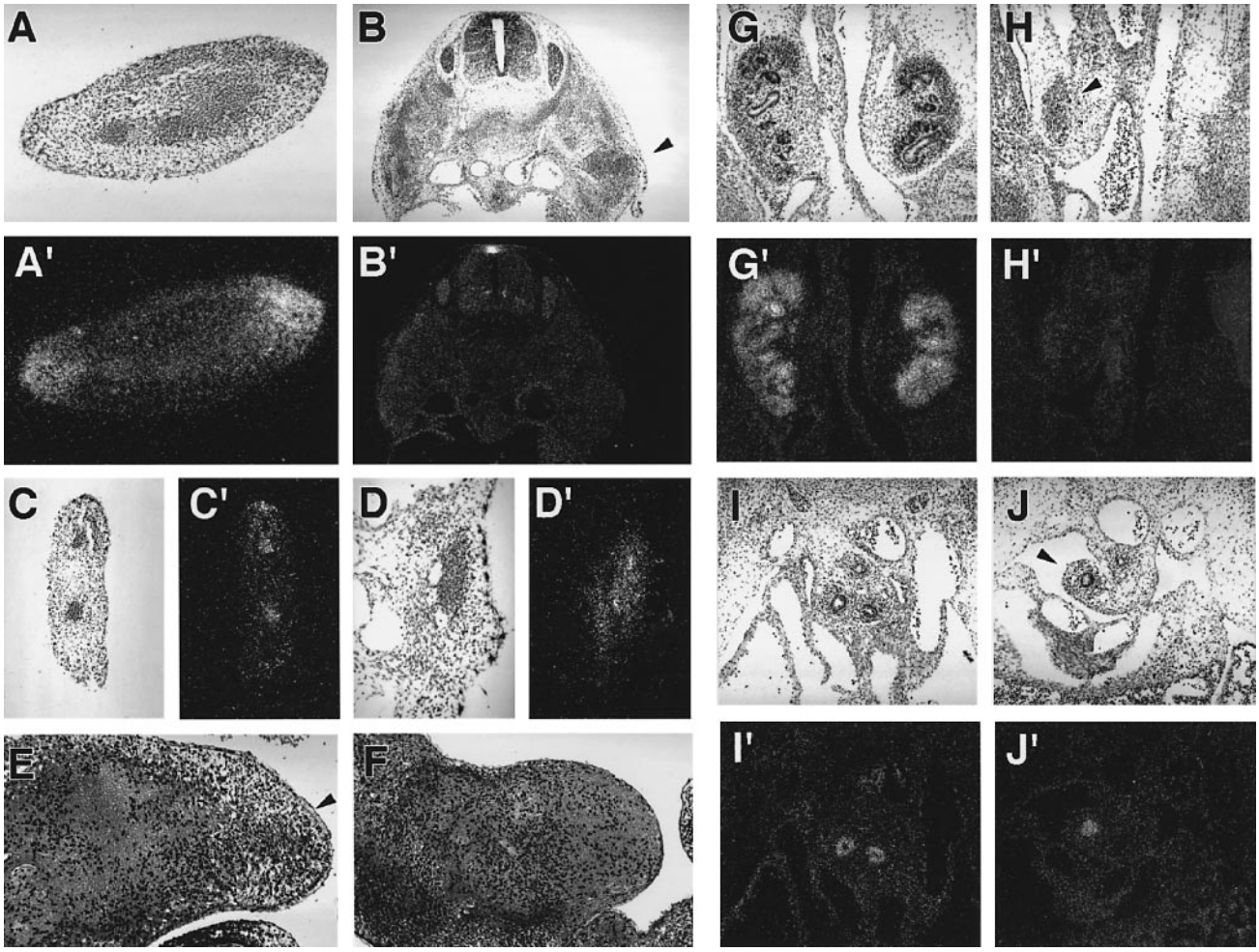

Fig. 6. Diagnostic molecular markers demonstrate disruption of induction and outgrowth in embryos expressing the soluble FGFR2 chimera. Analysis of transcripts by *in situ* hybridization (A–D and G–J), or of BrdU incorporation by immunohistochemistry (E and F). Shown are wild-type (A, C, E, G and I) and transgenic (B, D, F, H and J) E12.5 embryos. Darkfield pictures are indicated by primed letters. *In situ* riboprobes: *Msx-1* (A and B), *Msx-2* (C and D), *Pax-2* (G and H) and *TTF-1* (I and J). (**A–D**) Note that the residual transgenic limb bud (see arrowhead) expresses *Msx-2* but not *Msx-1*. (**E** and **F**) Note that progress zone mesenchyme of the wild-type limb bud (arrowhead) is highly proliferative (BrdU incorporation detected as black nuclei), while the transgenic bud shows no obvious growth. (**G** and **H**) Note that *Pax-2* expression is absent from the rudimentary metanephric transgenic blastema (arrowhead). (**I** and **J**) Note *TTF-1* expression in a single transgenic lung bud (arrowhead). Original magnification: (A, C and E–J) 100×; (B) 50×; (D) 200×.

effective? We propose that cells secreting these soluble dimeric DNRs create an extracellular trap in which a specific subset of FGFs would be 'caught' and effectively inactivated before they could interact with native membrane-bound FGFRs. Once created, such a trap should be maintained by the propensity of FGFR domains to bind to heparan sulfate proteoglycans on the cell surface and in the surrounding extracellular matrix (Johnson and Williams, 1993; Kan *et al*., 1993; Pantoliano *et al*., 1994; Brickman *et al*., 1995). In contrast, dnFGFR-Tm must either compete directly at the cell surface for ligand with relatively abundant wild-type FGFRs, or inactivate native receptors through heterodimerization. Either way, very high levels of the transmembrane form, estimated to be at least 10-fold greater than the wild-type receptor (Ueno *et al*., 1992), would be required to be effective. The secreted chimera may also be more effective because it can disrupt FGFR signaling in neighboring cells not directly expressing the transgene, especially at the mesenchymal–epithelial interface. In this regard, our data suggest that alternatively engineered soluble DNRs could be used to broaden the effective range of transgenes expressed via currently available transcriptional promoters.

It is likely that the ability of this dnFGFR2-HFc to interfere simultaneously with multiple FGFs, including aFGF, FGF3, FGF7 and the recently described FGF10 (Yamasaki *et al*., 1996), and potentially with signaling in all FGFR isoforms, is critical to the realization of many of the resulting transgenic phenotypes. Preliminary analysis of embryos expressing a related chimeric DNR containing the D2 Ig-like domain (D2FGFR-HFc), which preferentially binds to aFGF (Cheon *et al*., 1994), revealed none of the phenotypes associated with dnFGFR-HFc expression (not shown). Moreover, the mild consequences of targeted inactivation of some FGFs suggest that other members can substitute for their loss of function (Mansour *et al*., 1993; Hébert *et al*., 1994; Guo *et al*., 1996). These results, together with the shared recognition and redundant specificity inherent in FGFR signaling, support the notion that different FGFs co-expressed in the same micro-environment possess overlapping and interrelated embryonic functions, as exemplified in the developing limb.

The soluble dnFGFR-HFc appears to act *in vivo* with appropriate specificity. Based on embryonic expression patterns and organ culture experiments, several FGFs that can bind to this FGFR2b isoform have been implicated in development of many tissues demonstrating phenotypic anomalies. Expression of a similar transmembrane dominant-negative FGFR targeted by an SPC gene promoter also inhibited lung formation (Peters *et al*., 1994). Moreover, some tissues that are known targets of the MT promoter, such as the gastrointestinal tract, are overtly unaffected by the dnFGFR-HFc, indicating that this DNR is not indiscriminately deleterious. The possibility that expression of the mouse HFc domain somehow causes the observed phenotypes independently of the FGFR domain can be discounted because transgenic mice expressing either the related D2FGFR-HFc or PDGFR-HFc (J.Jakubczak, G.Merlino and W.LaRochelle, unpublished results) demonstrate none of these embryonic anomalies.

FGFR signaling plays a critical role in embryonic induction and pattern formation

The soluble dnFGFR-HFc can completely inhibit the formation of structures dependent on mesenchymal–epithelial communication for proper development. This strongly suggests that FGFs are required for reciprocal inductive interactions that evoke epithelial budding, mesenchymal–epithelial conversion and proliferation, and establish cell fate during early organogenesis. Here we present for the first time genetic evidence that FGFR signaling critically regulates such interactions in the developing kidney, which expresses FGFR1 in condensing and cortical mesenchyme, and FGFR2b in the ureteric bud, branches and subsequent epithelial structures (Orr-Urtreger *et al*., 1991, 1993; Peters *et al*., 1992, 1993). In transgenic mid-gestation embryos, the metanephric blastema was present, but lacked ureteric bud-derived epithelial structures, much like embryos harboring null mutations in *WT-1* or *GDNF*/c-*ret* (reviewed by Kreidberg, 1996; Massagué, 1996). Moreover, the mesenchymal tissue failed to express Pax-2, a paired box transcription factor thought to help regulate mesenchymal condensation and mesenchymal–epithelial conversion during renal development (Eccles *et al*., 1995; Torres *et al*., 1995; Dehbi *et al*., 1996). These results suggest that FGFR signaling in renal mesenchymal tissue operates upstream of *Pax-2*, and is required for ureteric bud initiation and outgrowth. Unilateral kidney development was observed occasionally in transgenic embryos, always on the right side, perhaps due to differential temporal/spatial transgene expression. However, it was always very small and rudimentary with morphologically abnormal epithelial structures. The inability to elaborate a normal kidney once it was initiated suggests that FGFR signaling is also necessary for an additional stage of renal development, such as mesenchymal condensation and/or nephron differentiation.

In the lung, where FGFR2 is expressed in bud epithelium and FGFR1 in the surrounding mesenchyme (Orr-Urtreger *et al*., 1991, 1993; Peters *et al*., 1992), FGFs are also apparently required in more than one stage of development. Most transgenic embryos initiated one or two lung buds and formed primary bronchi, again dominant on the right side, but typically they demonstrated extremely limited outgrowth and no branching morphogenesis. This result is reminiscent of experiments performed on cultured mouse foregut endoderm, which showed that non-specific mesoderm can induce supernumerary lung buds, but only bronchial mesoderm can induce subsequent branching (Spooner and Wessells, 1970; Wessells, 1970), raising the possibility that FGFR signaling may explain mesoderm specificity. Peters *et al*. (1994) showed that ectopic expression of a transmembrane dominant-negative FGFR2b mutant targeted by the SPC promoter inhibited bronchial branching, but not bud outgrowth. Phenotypic differences in embryos generated using these two approaches are probably due to differences in temporal/spatial transgene expression, or in the activity of the two forms of DNR. It has also been shown that mutations in the *Drosophila* FGFR homolog Breathless cause striking defects in tracheal development (Klambt *et al*., 1992). Together, these results indicate that FGFs, of which FGF7 and FGF10 appear to be excellent candidates (Mason *et al*., 1994; Simonet *et al*., 1995; Nguyen *et al*., 1996; Post *et al*., 1996; Yamasaki *et al*., 1996; Bellusci *et al*., 1997), act at multiple stages of lung development, including early bud formation and branching morphogenesis.

Elegant experiments have suggested that FGF4, FGF8 and FGF10 participate interactively in the initiation of limb bud formation and in the establishment and continued maintenance of the AER, which also expresses FGF4 and FGF8 (Niswander *et al*., 1993, 1994; Cohn *et al*., 1995; Crossley *et al*., 1996; Ohuchi *et al*., 1997). Here we show for the first time that the consequences of AER removal, first demonstrated by Saunders (1948) in the chick, in which AER excision at different times resulted in termination of limb development at progressively more distal levels, appear to be recreated genetically through interference with FGFR signaling. Different E18.5 transgenic embryos displayed varying levels of truncation of forelimb and hindlimb skeletal elements, presumably dependent on the time at which transgene expression reached a critical threshold. Since the soluble DNR is a form of FGFR2b, capable of binding FGF10 (A.Blunt and D.Ornitz, personal communication), our data strongly support the findings of Ohuchi *et al*. (1997), which suggest that FGF10 is involved both in early stages of limb bud initiation and in later maintenance of a functional AER.

Soluble FGFR mutant disturbs programmed differentiation in embryonic skin

Expression and experimental studies suggest that normal development of the skin involves FGFR signaling (Orr-Urtreger *et al*., 1991, 1993; Peters *et al*., 1992; Guo *et al*., 1993; Werner *et al*., 1993, 1994; Mason *et al*., 1994). FGFR1 and FGFR2c transcripts are restricted to the dermis, while FGFR2b expression, detected in ectoderm as early as E10.5, localizes to the basal compartment of the epidermis. Expression of FGFRs in the skin is closely associated with that of aFGF, bFGF, FGF5 and FGF7 (Hébert *et al*., 1990; Mason *et al*., 1994). Here we show that disruption of FGFR signaling alters programed differentiation in the skin. This is evident from the reduction in the transgenic epidermis of apoptotic cells, as well as by the presence of the keratin marker K6, usually associated with hair follicles and wound healing or hyperproliferative disorders (Moll *et al*., 1982; Weiss

et al., 1984; Stoler *et al*., 1988). Concomitant reduction in epidermal proliferation indicates that K6 induction is not strictly a result of hyperproliferation, as observed by others (Sellheyer *et al*., 1993, and references therein). Targeting of a membrane-anchored dominant-negative FGFR2b to basal keratinocytes caused decreased proliferation in the epidermis, indirectly accompanied by dermal thickening (Werner *et al*., 1994). In contrast, our secreted DNR, which can influence directly the dermis as well as the epidermis, inhibited growth in both compartments. Our results confirm that FGFR signaling helps to regulate proliferation, differentiation and specification of the epidermis and its mesenchymal components.

Dominant-negative FGFR mice as models for human skeletal disorders

Recently, mutations have been identified in three of the four FGFRs in a number of human skeletal disorders (reviewed by Muenke and Schell, 1995; Mulvihill, 1995). These syndromes are developmental in nature, characterized by craniofacial anomalies, hand and foot malformations, and/or dwarfism. Several lines of evidence suggest that at least some of these disorders are caused by gain-of-function mutations. Biochemical studies have shown that mutant FGFR2 and FGFR3, associated with Crouzon syndrome and achondroplasia, respectively, are constitutively activated (reviewed by Webster and Donoghue, 1997). FGFR mutations in these syndromes are always of the missense type and never fully inactivating nonsense mutations. Furthermore, although the human disorders are autosomal dominant, loss of one FGFR1 or FGFR2 allele in mice by gene targeting causes no obvious phenotype (Deng *et al*., 1994; Yamaguchi *et al*., 1994; C.Deng, personal communication). It was therefore of great interest that expression of a dominant-negative FGFR2, representing a loss-of-function mutation, causes skull and limb malformations. One interpretation of these data is that either too much or too little FGFR signaling can induce similar developmental anomalies in certain tissues. This point is well illustrated in both hair follicle and lung development, which are perturbed in genetically engineered mice that either overexpress FGF7, lack FGF7 or have disrupted FGFR2b signaling (Guo *et al*., 1993, 1996; Peters *et al*., 1994; Werner *et al*., 1994; Simonet *et al*., 1995).

A possible role for soluble FGFR isoforms in normal mouse development

Our data demonstrate dramatic developmental consequences of expressing a secreted kinase-deficient FGFR mutant, and thus raise the possibility that such truncated isoforms have an important physiological role in normal mouse development. Several reports have described the presence of native soluble FGFR isoforms in mammalian cells (reviewed by Givol and Yayon, 1992). Moreover, it has been shown that a secreted FGFR1 variant with ligand-binding activity structurally related to our soluble DNR is produced in newborn and adult mouse tissues, and in human cell lines (Duan *et al*., 1992; Werner *et al*., 1992). We have used RT–PCR analysis and DNA sequencing to determine that this functional soluble isoform is normally expressed during organogenesis in mid-gestation mouse embryos (not shown). In an environment where membrane-bound FGFRs are relatively abundant and ligands more limiting, such soluble isoforms could act as powerful dominant-negative agents to shield specific cells periodically from FGF stimulation. This might be particularly important in developmental situations requiring alternating cellular growth stimulation and quiescence, as in reciprocal inductive interactions between mesenchyme and epithelium. Moreover, such a mechanism would be especially effective in regulating large signaling families in which ligand and receptor members share overlapping recognition and redundant specificity, because a soluble isoform could simultaneously inactivate multiple ligand types specific for a particular receptor.

Demonstration of the essential nature of FGFR signaling in the development of multiple organs and other developing structures described in this study required the implementation of a novel loss-of-function approach utilizing a secreted soluble dominant-negative mutant uniquely suited to disrupt the highly complex FGF/FGFR superfamily. By modifying spatial and temporal patterns of transgene expression using tissue-specific promoters and an inducible expression system, respectively, and by employing additional soluble DNRs derived from other FGFR isoforms with overlapping and non-overlapping ligand specificities, this approach can now be used effectively to dissect the mechanisms by which the FGF superfamily regulates organogenesis at the cellular and molecular level.

Materials and methods

Expression vectors

The dominant-negative receptor constructs contained the extracellular domain of FGFR2b, cloned as described (Miki *et al*., 1991). The membrane-bound form (dnFGFR-Tm) was generated by PCR with primer 5′-(675)-ATATTGGATCCACCATGGTCAGCTGGGGGCGC-3′ and reverse primer 5′-(1526)-ATATTGGATCCTCAGCTGCTGAAGTCTGGCTTCTT-3′. The soluble chimera (dnFGFR-HFc) was constructed as described (Cheon *et al*., 1994). For *in vitro* studies, dnFGFR-Tm and dnFGFR-HFc forms were cloned into *Bam*HI and *Bgl*II sites, respectively, of MMTneo vector containing the mouse MT promoter, ampicillin and neomycin resistance genes (Cheon *et al*., 1994).

Transgenic animals

MT-dnFGFR-Tm mice and MT-FGFR-HFc embryos were generated by micro-injection into FVB/N zygotes, as described (Jhappan *et al*., 1990). The expression of each transgene was driven by the mouse MT promoter flanked by the MT 5′ and 3′ LCRs, which confer both copy number-dependent and position-independent expression (Palmiter *et al*., 1993). Transgenic mice and embryos were identified by PCR of genomic DNA from tail and yolk sacs, respectively, using a promoter-specific primer 5′-ACTTCAACGTCCTGAGTA-3′ and reverse primer 5′-ATATTGGATCCTCAGCTGCTGAAGTCTGGCTTCTT-3′ (dnFGFR-Tm) or 5′-TTTCTTGTCCACCTTGGTGCT-3′ (dnFGFR-HFc). PCR was done for 30 cycles (94°C, 40 s; 55°C, 40 s; 72°C, 1 min).

RNA analysis

Gene expression of developmental markers was analyzed by *in situ* hybridization using ^{33}P-labeled antisense riboprobes, including *Pax-2* (0.46 kbp), provided by G.Dressler; c-*met* (3.7 kbp), provided by C.Birchmeier; *TTF-1* (1.2 kbp), provided by S.Kimura; *Msx-1* (0.8 kbp) and *Msx-2* (1.0 kbp), provided by R.Maxson, and the mouse HFc (0.8 kbp) on embryos fixed in 4% paraformaldehyde as described (Fox and Cottler-Fox, 1993). Sections were exposed for 2 weeks. Total RNA was extracted from mouse embryos and tissues with TRIzol Reagent (Gibco-BRL). cDNA was prepared from 4 μg of total RNA using the Superscript Preamplification System for First Strand Synthesis (Gibco-BRL).

Skeletal preparation

For skeletal analysis, E18.5 embryos were skinned, eviscerated and then fixed in ethanol, defatted in acetone and stained with 0.015% alcian blue

and 0.005% alizarin red in ethanol with 5% acetic acid for 5 days each. For visualization, stained skeletons were hydrolyzed in 1% KOH overnight, and cleared through graded glycerols.

Immunohistochemistry

Immunohistochemical analysis of embryonal skin was done with monospecific polyclonal antibodies to keratins (Roop *et al*., 1987). Paraffin-embedded sections (5 μm) of skin from E18.5 embryos fixed in 70% ethanol were deparaffinized, rehydrated in phosphate-buffered saline (PBS), and endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methyl alcohol. Tissue sections were blocked with normal goat serum, then incubated overnight at 4°C with affinity-purified rabbit antiserum specific for keratins K14, K1, K6 or anti-mouse IgG (Fc) (Pierce), followed by incubation with biotinylated goat anti-rabbit IgG and diaminobenzidine substrate (Kirkegaard & Perry Laboratories). Apoptotic cells were detected using the ApopTag kit, as described by the manufacturer (Oncor). Labeling indices were determined by immunohistochemical detection of incorporated bromodeoxyuridine (DAKO) as described by the manufacturer and elsewhere (Sharp *et al*., 1995). In embryonic skin, cell proliferation was assessed by quantifying mitotic figures in a minimum of six cross-sections and expressed as number of mitotic figures per mm skin.

Transfection and protein purification

MMTneo-dnFGFR-Tm and MMTneo-dnFGFR-HFc constructs were electroporated into Balb/MK cells, and stable G418-resistant lines established. Stable NIH 3T3 MMTneo-DNR-HFc transfectants were established as described (Cheon *et al*., 1994). A *Xenopus* FGF3 cDNA expression vector was obtained from Dr Clive Dickson and transfected into COS-1 cells with 40 μg of carrier calf thymus DNA by calcium phosphate. Secreted FGF3 was recovered and purified as described (Kiefer *et al*., 1993). Conditioned medium was collected from NIH 3T3 MMTneo-dnFGFR-HFc transfectants, and purified dnFGFR-HFc was prepared using anti-mouse IgG1 (heavy-chain-specific) agarose columns (Sigma).

Protein analysis

Immunoprecipitation and immunoblot analysis of phosphorylated FGFR1 were done essentially as described (LaRochelle *et al*., 1993). NIH 3T3 were starved overnight in Dulbecco's modified Eagle's medium (DMEM) and treated for 5 min with 10 ng/ml of ligand pre-incubated with 1.4 or 5.6 μg of dnFGFR-HFc. Cells were solubilized in radioimmunoprecipitation assay (RIPA) buffer (LaRochelle *et al*., 1993) including 0.1% sodium deoxycholate and 0.1% SDS. Equivalent amounts of lysates were incubated with either anti-phosphotyrosine mAb (PY, 1:1000; Upstate Biotechnology) or anti-Flg (FGFR1, 1:1000; Santa Cruz Biotechnology), immunocomplexes were adsorbed to GammaBind G agarose (Pharmacia Biotech), fractionated on 8% SDS–PAGE under reducing conditions and transferred to Immobilon P membranes. Immunoblotting was done with either PY (1:1000) or aFGFR1 (1:1000) overnight, followed by anti-mouse or anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (Pierce), and visualized by chemiluminescence (Amersham). For the analysis of transgene protein expression, frozen E12.5 embryos were homogenized and sonicated in RIPA buffer, and equivalent amounts of protein were fractionated on SDS–PAGE under reducing conditions and transferred as described. The blot was incubated with rabbit anti-mouse IgG (Fc), and visualized with secondary anti-rabbit IgG conjugated to HRP and enhanced chemiluminescence.

Mitogenic assays

aFGF, bFGF and FGF4 were obtained from R&D Systems, and FGF7 from Biosource International. Thymidine incorporation into Balb/MK MMTneo-dnFGFR-Tm and MMTneo-dnFGFR-HFc transfectants and NIH 3T3 cells was performed as described (Rubin *et al*., 1989). Briefly, cells were plated in 96-well plates pre-coated with fibronectin. To assay DNA synthesis, quiescent Balb/MK transfectants were incubated for 16 h with increasing concentrations of aFGF or KGF, and with [^{3}H]thymidine for an additional 5 h before harvesting. Similarly, NIH 3T3 cells were starved in DMEM containing 1 μg/ml heparin, then treated with 1 ng/ml of each FGF pre-incubated with increasing concentrations of dnFGFR-HFc, typically for 15 min at 4°C, before addition to cells. [^{3}H]Thymidine uptake was measured by liquid scintillation counting. Triplicates of each sample were measured and their averages determined.

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References

Amaya,E., Musci,T.J. and Kirschner,M.W. (1991) Expression of a dominant-negative mutant of the FGF receptor disrupts mesoderm formation in *Xenopus* embryos. *Cell*, **66**, 257–270.

Bellusci,S., Grindley,J., Emoto,H., Itoh,N. and Hogan,B.L. (1997) Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. *Development*, **124**, 4867–4878.

Bottaro,D.P., Rubin,J.S., Ron,D., Finch,P.W., Florio,C. and Aaronson, S.A. (1990) Characterization of the receptor for keratinocyte growth factor. Evidence for multiple fibroblast growth factor receptors. *J. Biol. Chem.*, **265**, 12767–12770.

Brickman,Y.G., Ford,M.D., Small,D.H., Bartlett,P.F. and Nurcombe,V. (1995) Heparan sulfates mediate the binding of basic fibroblast growth factor to a specific receptor on neuronal precursor cells. *J. Biol. Chem.*, **270**, 24941–24948.

Cheon,H., LaRochelle,W.J., Bottaro,D.P., Burgess,W.H. and Aaronson, S.A. (1994) High-affinity binding sites for related fibroblast growth factor ligands reside within different receptor immunoglobulin-like domains. *Proc. Natl Acad. Sci. USA*, **91**, 989–993.

Ciruna,B.G., Schwartz,L., Harpal,K., Yamaguchi,T.P. and Rossant,J. (1997) Chimeric analysis of fibroblast growth factor receptor 1 (FGFR1) function: a role for FGFR1 in morphogenetic movement through the primitive streak. *Development*, **124**, 2829–2841.

Cohn,M.J., Izpisúa-Belmonte,J.C., Abud,H., Heath,J.K. and Tickle,C. (1995) Fibroblast growth factors induce additional limb development from the flank of chick embryos. *Cell*, **80**, 739–746.

Colvin,J.S., Bohne,B.A., Harding,G.W., McEwen,D.G. and Ornitz,D.M. (1996) Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. *Nature Genet.*, **12**, 390–397.

Crossley,P.H. and Martin,G.R. (1995) The mouse FGF8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development*, **121**, 439–451.

Crossley,P.H., Monowada,G., MacArthur,C.A. and Martin,G.R. (1996) Roles for FGF8 in the induction, initiation, and maintenance of chick limb development. *Cell*, **84**, 127–136.

Dehbi,M., Ghahremani,M., Lechner,M., Dressler,G. and Pelletier,J. (1996) The paired-box transcription factor, Pax2, positively modulates expression of the Wilms' tumor suppressor gene (*WT1*). *Oncogene*, **13**, 447–453.

Deng,C., Wynshaw-Boris,A., Shen,M.M., Daugherty,C., Ornitz,D.M. and Leder,P. (1994) Murine FGFR1 is required for early postimplantation growth and axial organization. *Genes Dev.*, **8**, 3045–3057.

Deng,C., Wynshaw-Boris,A., Zhou,F., Kuo,A. and Leder,P. (1996) Fibroblast growth factor receptor 3 is a negative regulator of bone growth. *Cell*, **84**, 911–921.

Deng,C., Bedford,M., Li,C., Xu,X., Yang,Y., Dunmore,J. and Leder,P. (1997) Fibroblast growth factor receptor 1 (FGFR1) is essential for normal neural tube and limb development. *Dev. Biol.*, **185**, 42–54.

Duan,D.-S.R., Werner,S. and Williams,L.T. (1992) A naturally occurring secreted form of fibroblast growth factor (FGF) receptor 1 binds basic FGF in preference over acidic FGF. *J. Biol. Chem.*, **267**, 16076–16080.

Eccles,M.R., Yun,K., Reeve,A.E. and Fidler,A.E. (1995) Comparative *in situ* hybridization analysis of PAX2, PAX8, and WT1 gene transcription in human fetal kidney and Wilms' tumors. *Am. J. Pathol.*, **146**, 40–45.

Fallon,J.F., Lopez,A., Ros,M.A., Savage,M.P., Olwin,B.B. and Simandl,B.K. (1994) FGF-2: apical ectodermal ridge growth signal for chick limb development. *Science*, **264**, 104–107.

Feldman,B., Poueymirou,W., Papaioannou,V.E., DeChiara,T.M. and Goldfarb,M. (1995) Requirement of FGF4 for postimplantation mouse development. *Science*, **267**, 246–249.

Floss,T., Hans-Henning,A. and Braun,T. (1997) A role for FGF-6 in skeletal muscle regeneration. *Genes Dev.*, **11**, 2040–2051.

Fox,C.H. and Cottler-Fox,M. (1993) *In situ* hybridization for the detection of HIV RNA in cells and tissues. In Coligan,J. (ed.), *Current Protocols in Immunology.* Wiley Press, New York, NY, pp. 12.8.1–12.9.20.

Givol,D. and Yayon,A. (1992) Complexity of FGF receptors: genetic basis for structural diversity and functional specificity. *FASEB J.*, **6**, 3362–3369.

Guo,L., Yu,Q. and Fuchs,E. (1993) Targeting expression of keratinocyte growth factor to keratinocytes elicits striking changes in epithelial differentiation in transgenic mice. *EMBO J.*, **12**, 973–986.

Guo,L., Degenstein,L. and Fuchs,E. (1996) Keratinocyte growth factor is required for hair development but not for wound healing. *Genes Dev.*, **10**, 165–175.

Hébert,J.M., Basilico,C., Goldfarb,M., Haub,O. and Martin,G.R. (1990) Isolation of cDNAs encoding four mouse FGF family members and characterization of their expression patterns during embryogenesis. *Dev. Biol.*, **138**, 454–463.

Hébert,J.M., Rosenquist,T., Götz,J. and Martin,G.R. (1994) FGF5 as a regulator of the hair growth cycle: evidence from targeted and spontaneous mutations. *Cell*, **78**, 1017–1025.

Jackson,D., Bresnick,J., Rosewell,I., Crafton,T., Poulsom,R., Stamp,G. and Dickson,C. (1997) Fibroblast growth factor receptor signalling has a role in lobuloalveolar development of the mammary gland. *J. Cell Sci.*, **110**, 1261–1268.

Jhappan,C., Stahle,C., Harkins,R.N., Fausto,F., Smith,G.H. and Merlino,G.T. (1990) TGFα overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. *Cell*, **61**, 1137–1146.

Johnson,D.E. and Williams,L.T. (1993) Structural and functional diversity in the FGF receptor multigene family. *Adv. Cancer Res.*, **60**, 1–41.

Kan,M., Wang,F., Xu,J., Crabb,J.W., Hou,J. and McKeehan,W.L. (1993) An essential heparin-binding domain in the fibroblast growth factor receptor kinase. *Science*, **259**, 1918–1921.

Kelly,S.E., Bachurski,C.J., Burhans,M.S. and Glasser,S.W. (1996) Transcription of the lung-specific surfactant protein C gene is mediated by thyroid transcription factor 1. *J. Biol. Chem.*, **271**, 6881–6888.

Kiefer,P., Mathieu,M., Close,M.J., Peters,G. and Dickson,C. (1993) FGF3 from *Xenopus laevis*. *EMBO J.*, **12**, 4159–4168.

Kimura,S., Hara,Y., Pineau,T., Fernandez-Salguero,P., Fox,C.H., Ward,J.M. and Gonzalez,F.J. (1996) The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. *Genes Dev.*, **10**, 60–69.

Klambt,C., Glazer,L. and Shilo,B.Z. (1992) breathless, a *Drosophila* FGF receptor homolog, is essential for migration of tracheal and specific midline glial cells. *Genes Dev.*, **6**, 1668–1678.

Kreidberg,J.A. (1996) Gene targeting in kidney development. *Med. Pediatr. Oncol.*, **27**, 445–452.

LaRochelle,W.J., Jensen,R.A., Heidaran,M.A., May-Siroff,M., Wang, L.M., Aaronson,S.A. and Pierce,J.H. (1993) Inhibition of platelet-derived growth factor autocrine growth stimulation by a monoclonal antibody to the human alpha platelet growth factor receptor. *Cell Growth Differ.*, **4**, 547–553.

Mansour,S.L., Goddard,J.M. and Capecchi,M.R. (1993) Mice homozygous for a targeted disruption of the proto-oncogene *int-2* have developmental defects in the tail and inner ear. *Development*, **117**, 13–28.

Mason,I.J. (1994) The ins and outs of fibroblast growth factors. *Cell*, **78**, 547–552.

Mason,I.J., Fuller-Pace,F., Smith,R. and Dickson,C. (1994) FGF7 (keratinocyte growth factor) expression during mouse development suggests roles in myogenesis, forebrain regionalisation and epithelial–mesenchymal interactions. *Mech. Dev.*, **45**, 15–30.

Massagué,J. (1996) Crossing receptor boundaries. *Nature*, **382**, 29–30.

Miki,T., Fleming,T.P., Bottaro,D.P., Rubin,J.S., Ron,D. and Aaronson, S.A. (1991) Expression cDNA cloning of the KGF receptor by creation of a transforming autocrine loop. *Science*, **251**, 72–75.

Miki,T., Bottaro,D.P., Fleming,T.P., Smith,C.L., Burgess,W.H., Chan, A.M. and Aaronson,S.A. (1992) Determination of ligand-binding specificity by alternative splicing: two distinct growth factor receptors encoded by a single gene. *Proc. Natl Acad. Sci. USA*, **89**, 246–250.

Minoo,P., Hamdan,H., Bu,D., Warburton,D., Stepanik,P. and deLemos,R. (1995) TTF1 regulates lung epithelial morphogenesis. *Dev. Biol.*, **172**, 694–698.

Moll,R., Franke,W.W., Schiller,D.L., Geiger,B. and Krepler,R. (1982) The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors, and cultured cells. *Cell*, **31**, 11–24.

Muenke,M. and Schell,U. (1995) Fibroblast growth factor receptor mutations in human skeletal disorders. *Trends Genet.*, **11**, 308–313.

Mulvihill,J.J. (1995) Craniofacial syndromes: no such thing as a single gene disease. *Nature Genet.*, **9**, 101–103.

Neubüser,A., Peters,H., Balling,R. and Martin,G.R. (1997) Antagonistic interactions between FGF and BMP signaling pathways: a mechanism for positioning the sites of tooth formation. *Cell*, **90**, 247–255.

Nguyen,H.Q., Danilenko,D.M., Bucay,N., DeRose,M.L., Van,G.Y., Thomason,A. and Simonet,W.S. (1996) Expression of keratinocyte growth factor in embryonic liver of transgenic mice causes changes in epithelial growth differentiation resulting in polycystic kidneys and other organ malformations. *Oncogene*, **12**, 2109–2119.

Niswander,L. and Martin,G.R. (1992) Fgf4 expression during gastrulation, myogenesis, limb and tooth development in the mouse. *Development*, **114**, 755–768.

Niswander,L. and Martin,G.R. (1993) FGF4 and BMP2 have opposite effects on limb growth. *Nature*, **361**, 68–71.

Niswander,L., Tickle,C., Vogel,A., Booth,I. and Martin,G.R. (1993) FGF4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell*, **75**, 579–587.

Nogawa,H. and Ito,T. (1995) Branching morphogenesis of embryonic mouse lung epithelium in mesenchyme-free culture. *Development*, **121**, 1015–1022.

Ornitz,D.M., Xu,J., Colvin,J.S., McEwen,D.G., MacArthur,C.A., Coulier,F., Gao,G. and Goldfarb,M. (1996) Receptor specificity of the fibroblast growth factor family. *J. Biol. Chem.*, **271**, 15292–15297.

Orr-Urtreger,A., Givol,D., Yayon,A., Yarden,Y. and Lonai,P. (1991) Developmental expression of two murine fibroblast growth factor receptors, flg and bek. *Development*, **113**, 1419–1434.

Orr-Urtreger,A., Bedford,M.T., Burakova,T., Arman,E., Zimmer,Y., Yayon,A., Givol,D. and Lonai,P. (1993) Developmental localization of the splicing alternatives of fibroblast growth factor receptor-2 (FGFR2). *Dev. Biol.*, **158**, 475–486.

Ohuchi,H. *et al.* (1997) The mesenchymal growth factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor. *Development*, **124**, 2235–2244.

Palmiter,R.D., Sandgren,E.P., Koeller,D.M. and Brinster,R.L. (1993) Distal regulatory elements from the mouse metallothionein locus stimulate gene expression in transgenic mice. *Mol. Cell. Biol.*, **13**, 5266–5275.

Peratoni,A.O., Dove,L.F. and Karavanova,I. (1995) Basic fibroblast growth factor can mediate the early inductive events in renal development. *Proc. Natl Acad. Sci. USA*, **92**, 4696–4700.

Peters,K.G., Werner,S., Chen,G. and Williams,L.T. (1992) Two FGF receptor genes are differentially expressed in epithelial and mesenchymal tissues during limb formation and organogenesis in the mouse. *Development*, **114**, 233–243.

Peters,K.G., Ornitz,D.M., Werner,S. and Williams,L.T. (1993) Unique expression pattern of the FGF receptor 3 gene during mouse organogenesis. *Dev. Biol.*, **155**, 423–440.

Peters,K., Werner,S., Liao,X., Wert,S., Whitsett,J. and Williams,L. (1994) Targeted expression of a dominant-negative FGF receptor blocks branching morphogenesis and epithelial differentiation of the mouse lung. *EMBO J.*, **13**, 3296–3301.

Post,M., Souza,P., Liu,J., Tseu,I., Wang,J., Kuliszewski,M. and Tanswell,A.K. (1996) Keratinocyte growth factor and its receptor are involved in regulating early lung branching. *Development*, **122**, 3107–3115.

Przylepa,K.A. *et al.* (1996) Fibroblast growth factor receptor 2 mutations in Beare–Stevenson cutis gyrata syndrome. *Nature Genet.*, **13**, 492–494.

Represa,J., Leon,Y., Miner,C. and Giraldez,F. (1991) The *int-2* proto-oncogene is responsible for induction of the inner ear. *Nature*, **353**, 561–563.

Robinson,M.L., MacMillan-Crow,L.A., Thompson,J.A. and Overbeek, P.A. (1995) Expression of a truncated FGF receptor results in defective lens development in transgenic mice. *Development*, **121**, 3959–3967.

Roop,D.R., Huitfeldt,H., Kilkenny,A. and Yuspa,S.H. (1987) Regulated expression of differentiation-associated keratins in cultured epidermal cells detected by monospecific antibodies to unique peptides of mouse epidermal keratins. *Differentiation*, **35**, 143–150.

Rubin,J.S., Osada,H., Finch,P.W., Taylor,W.G., Rudikoff,S. and Aaronson,S.A. (1989) Purification and characterization of a newly identified growth factor specific for epithelial cells. *Proc. Natl Acad. Sci. USA*, **86**, 802–806.

Saunders,J.W. (1948) The proximo-distal sequence of origin of the parts of the chick wing and the role of the ectoderm. *J. Exp. Zool.*, **108**, 363–403.

Sellheyer,K., Bickenbach,J.R., Rothnagel,J.A., Bundman,D., Longley, M.A., Krieg,T., Roche,N.S., Roberts,A.B. and Roop,D.R. (1993) Inhibition of skin development by overexpression of transforming growth factor β1 in the epidermis of transgenic mice. *Proc. Natl Acad. Sci. USA*, **90**, 5237–5241.

Sharp,R., Babyatsky,M.W., Takagi,H., Tägerud,S., Wang,T.C., Bockman, D.E., Brand,S.J. and Merlino,G. (1995) Transforming growth factor α disrupts the normal program of cellular differentiation in the gastric mucosa of transgenic mice. *Development*, **121**, 149–161.

Simonet,W.S., DeRose,M.L., Bucay,N., Nguyen,H.Q., Wert,S.E., Zhou,L., Ulich,T.R., Thomason,A., Danilenko,D.M. and Whitsett,J.A. (1995) Pulmonary malformation in transgenic mice expressing human keratinocyte growth factor in the lung. *Proc. Natl Acad. Sci. USA*, **92**, 12461–12465.

Spooner,B.S. and Wessells,N.K. (1970) Mammalian lung development: interactions in primordium formation and bronchial morphogenesis. *J. Exp. Zool.*, **175**, 445–454.

Stark,K.L., McMahon,J.A. and McMahon,A.P. (1991) FGFR4, a new member of the fibroblast growth factor receptor family, expressed in the definitive endoderm and skeletal muscle lineages of the mouse. *Development*, **113**, 641–674.

Stoler,A., Kopan,R., Duvic,M. and Fuchs,E. (1988) Use of monospecific antisera and cRNA probes to localize the major changes in keratin expression during normal and abnormal epidermal differentiation. *J. Cell Biol.*, **107**, 427–446.

Takayama,H., LaRochelle,W.J., Anver,M., Bockman,D.E. and Merlino,G. (1996) Scatter factor/hepatocyte growth factor as a regulator of skeletal muscle and neural crest development. *Proc. Natl Acad. Sci. USA*, **93**, 5866–5871.

Torres,M., Gómez-Pardo,E., Dressler,G.R. and Gruss,P. (1995) Pax2 controls multiple steps of urogenital development. *Development*, **121**, 4057–4065.

Ueno,H., Gunn,M., Dell,K., Tseng,A. and Williams,L.T. (1992) A truncated form of fibroblast growth factor receptor 1 inhibits signal transduction by multiple types of fibroblast growth factor receptor. *J. Biol. Chem.*, **267**, 1470–1476.

Wang,Y. and Sassoon,D. (1995) Ectoderm–mesenchyme and mesenchyme–mesenchyme interactions regulate Msx1 expression and cellular differentiation in the murine limb bud. *Dev. Biol.*, **168**, 374–382.

Webster,M.K. and Donoghue,D.J. (1997) FGFR activation in skeletal disorders: too much of a good thing. *Trends Genet.*, **13**, 178–182.

Weiss,R.A., Eichner,R. and Sun,T.T. (1984) Monoclonal antibody analysis of keratin expression in epidermal diseases: a 48- and 56-kdalton keratin as molecular markers for hyperproliferative keratinocytes. *J. Cell Biol.*, **98**, 1397–1406.

Werner,S., Duan,D.R., de Vries,C., Peters,K.G., Johnson,D.E. and Williams,L.T. (1992) Differential splicing in the extracellular region of fibroblast growth factor receptor 1 generates receptor variants with different ligand binding specificities. *Mol. Cell. Biol.*, **12**, 82–88.

Werner,S., Weinberg,W., Liao,X., Peters,K.G., Blessing,M., Yuspa,S.H., Weiner,R.L. and Williams,L.T. (1993) Targeted expression of a dominant-negative FGF receptor mutant in the epidermis of transgenic mice reveals a role of FGF in keratinocyte organization and differentiation. *EMBO J.*, **12**, 2635–2643.

Werner,S., Smola,H., Liao,X., Longaker,M.T., Krieg,T., Hofschneider, P.H. and Williams,L.T. (1994) The function of KGF in morphogenesis of epithelium and reepithelialization of wounds. *Science*, **266**, 819–822.

Wessells,N.K. (1970) Mammalian lung development: interactions in formation and morphogenesis of tracheal buds. *J. Exp. Zool.*, **175**, 455–466.

Wilkinson,D.G., Bhatt,S. and McMahon,A.P. (1989) Expression pattern of the FGF-related protooncogene *int-2* suggests multiple roles in fetal development. *Development*, **105**, 131–136.

Yamaguchi,T.P., Harpal,K., Henkemeyer,M. and Rossant,J. (1994) Fgfr-1 is required for embryonic growth and mesodermal patterning during mouse gastrulation. *Genes Dev.*, **8**, 3032–3044.

Yamasaki,M., Miyake,A., Tagashira,S. and Itoh,N. (1996) Structure and expression of the rat mRNA encoding a novel member of the fibroblast growth factor family. *J. Biol. Chem.*, **271**, 15918–15921.

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