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Original Article

Targeted disruption of fibroblast growth factor receptor-1 blocks maturation of visceral endoderm and cavitation in mouse embryoid bodies

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ABSTRACT The cellular response to fibroblast growth factors (FGFs) is mediated by receptor tyrosine kinases (FGFR-1 – 4) whose patterns of expression are spatially and temporally restricted during embryogenesis. These receptors have differential ligand binding capacities and are coupled to diverse signalling pathways. In the present study, we have characterized the ability of FGFR-1-deficient mouse embryonic stem (ES) cells to bind FGF-2 and to proliferate in the absence or presence of exogenous FGF-2. Under the same conditions, we also analysed the differentiation of FGFR-1-deficient ES cells into three dimensional, post-implantation, embryonic tissues, known as embryoid bodies (EBs). We show that the targeted disruption of *FGFR-1* leads to a reduced binding of FGF-2 which has no significant effect on the proliferation of undifferentiated ES cells. In addition, lack of functional FGFR-1 in differentiating EBs leads to a reduced expression of the endoderm marker gene α-*fetoprotein (AFP).* This deregulation of the *AFP* gene correlates with defects in the formation of the visceral endoderm, proper differentiation of the ectoderm and thus the organization of the columnar epithelium, and a block of cavitation. Although the addition of exogenous FGF-2 further reduced the expression of *AFP* mRNA in differentiating mutant EBs, corresponding morphological changes were not observed. Our results indicate that FGFR-1 may play a vital role in endoderm formation.

KEY WORDS: *fibroblast growth factor receptor 1, fibroblast growth factor 2, embryonic stem cell, embryoid body, differentiation*

Introduction

FGFs constitute an extensive family of signalling molecules with variable functions in development and in adult physiology and pathology (Burdsal *et al.,* 1998; Boilly *et al.,* 2000). In the developing mouse embryo, FGF signals are thought to be transmitted via all four members of the FGFR family, of which FGFR-1 and -2 function from the earliest stages of development (Deng *et al.,* 1994; Yamaguchi *et al.,* 1994; Arman *et al.,* 1998), whereas FGFR-3 and -4 become more important later during definitive organogenesis (Colvin *et al.,* 1996; Deng *et al.,* 1996; Weinstein *et al.,* 1998). Each member of the FGFR family interacts with a particular FGF with a characteristic affinity, and the combination of ligand binding specificity together with diverse signal transduction cascades, defines a precise cellular response. Several FGFs have been suggested to play important roles during early embryogenesis. This has been shown particularly for FGF-4 mRNA which is expressed as early as in the 1-cell stage embryo (Rappolee *et al.,* 1994). FGF-2 is also known to be involved in early mouse embryogenesis, since it has been found to be expressed in the primitive ectoderm and therefore in ES cells, and was also detected in pregnant mouse uterus (Jirmanova *et al.,* 1999). During early embryogenesis, FGF-2 co-regulates the patterning of mesodermal and neural cell lineages (Bursdal *et al.,* 1998; Ciccolini, 2001). Also FGF-3, -5, -8, and –17 were detected in prestreak- and streak-stage embryos (Wilkinson *et al.,* 1988; Hébert *et al.,* 1991; Crossley and Martin 1995; Maruoka *et al.,* 1998). FGF-2 has been reported to signal strongly via FGFR-1 and less intensely via FGFR-2 (Ornitz *et al.,* 1996). As both of these FGF-2 cognate receptors were found to be expressed in mouse blastocysts (Campbell *et al.,* 1992), their crucial role in the determination of the fate of inner cell mass (ICM)-derived ES cells is strongly suspected.

In keeping with this idea, several reports describing targeted disruption of *FGFR-1*, *-2*, and *FGF-2* genes have shown their

Abbreviations used in this paper: AFP, alpha-fetoprotein; cc, cystic cavity; cd, cell debris; ce, columnar epithelium; DBA, *Dolichos biflorus;* EB, embryoid body; en, endothelium cells; ES, embryonic stem cell; fgf, fibroblast growth factor; me, mesoderm cells; sc, stem cell; ve, visceral endoderm.

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critical impact on normal development. FGFR-1-deficient mice die prior to, or during gastrulation, due to aberrant patterning of the primitive streak and other axial structures (Deng *et al.,* 1994; Yamaguchi *et al.,* 1994). Similarly, targeted disruption of *FGFR-2* leads to abortion of the egg cylinder and dominant negative homozygotes die early after implantation of blastocysts (Arman *et al.,* 1998). In contrast, FGF-2-deficient mice are viable, but display severe defects in the architecture of the cerebral cortex. These defects are probably not caused by an abnormal growth of neuronal progenitors, since it has been shown that these cells are properly formed and proliferate normally. Rather, defects of the cerebral cortex seem to stem from aberrant migration and retarded differentiation of neuronal precursors (Dono *et al.,* 1998; Ortega *et al.,* 1998).

In the present report, we have examined the molecular and morphogenetic consequences of the absence of FGFR-1 in ES cells in the presence/absence of FGF-2, during a time window corresponding to the developmental period early after implantation. To this end, we used conventional, adherent cultures of ES cells, which permit a precise evaluation of cell growth. Since this model is not optimal for the study of differentiation, we employed suspension cultures of EBs, which are considered to be a good model of early post-implantation differentiation *in vivo*. We found that the inactivation of FGFR-1 led to a reduction in the binding of FGF-2 to undifferentiated ES cells but surprisingly did not significantly alter ES cell proliferation in response to FGF-2. In addition, the absence of FGFR-1 was found to be associated with the aberrant differentiation of ES cell-derived EBs into visceral endoderm and columnar epithelium, and a block of cavitation. Our results indicate that these early changes may account for the abnormal mesoderm induction and organization of mesodermal derivatives in FGFR-1-deficient embryos as described by Deng *et al.* (1994) and Yamaguchi *et al.* (1994).

Results

Expression of FGFRs in Undifferentiated FGFR-1-Deficient and Wild-Type ES Cells and Differentiating EBs

Over-expression of FGFR-2, truncated in the tyrosine kinase domains, completely inhibits FGF signalling via all FGFRs and blocks differentiation of ES cells into cystic EBs (Chen *et al.,* 2000). In our model system we employed a targeted disruption of only FGFR-1 to study its role in the proliferation and differentiation of ES cells. We first compared the expression of individual *FGFRs* in FGFR-1-deficient and wild-type ES cells and EBs in order to evaluate the possible altered expression of other FGFR isotypes as a compensatory measure for the loss of FGFR-1.

In undifferentiated wild-type ES cells, weak mRNA signals for *FGFR-1*, *FGFR-2* and *FGFR-4* were detected, whereas *FGFR-3* was not detectable. The same expression pattern was found in undifferentiated mutant ES cells, with the exception of *FGFR-1*. During differentiation of wild-type ES cell-derived EBs, *FGFR-1* and *FGFR-2* were strongly up regulated by day 6 and their expression remained invariable up to day 11 of culture. Similarly, mutant EBs strongly up-regulated *FGFR-2* expression by day 6, which was maintained at high levels up to day 11 of differentiation. The expression of *FGFR-4* also increased, although to a more limited extent, at day 6 and remained constant to day 11 of differentiation in both types of EBs. Different expression kinetics were observed for *FGFR-3*. In both wild-type and mutant EBs, expression of *FGFR-3* gradually increased, reaching a maximum by day 11 of differentiation. The expression of any of the *FGFRs* was not affected by treatment with exogenous FGF-2. The results of this analysis are shown in Fig. 1.

Ligand Binding Properties of FGFR-1-Deficient and Wild-Type ES Cells

FGF-2, like other members of the FGF family, binds to, and signals via, high-affinity transmembrane FGFRs. In addition, exogenous FGF-2 interacts with heparan sulfate proteoglycans, which are non-signalling low-affinity binding sites that further modulate the cell response. During early development of the mouse embryo, such a role is played by abundantly expressed embryonic cell-specific proteoglycans, called embryoglycans, which under certain conditions may affect the *in vitro* growth of ICM-derived ES cells (Dvorak *et al.,* 1998; Jirmanova *et al.,* 1999). Thus, it appeared to be important to ascertain the relative low- and high-affinity FGF-2 binding capacities of undifferentiated FGFR-1-deficient ES cells compared to wild-type cells.

In our experiments, we used a standard technique to distinguish low- and high-affinity binding by means of a step-wise releasing of bound ^{125}I-FGF-2 with 0.8 M and 2 M salt concentrations. Wild-type and FGFR-1-deficient ES cells were found to bind negligible amounts of FGF-2 to their low-affinity binding sites, as the radioactivity released by 0.8 M NaCl from cells incubated with ^{125}I-FGF-2 in the presence of a 100-fold molar excess of unlabeled FGF-2 was roughly equal to the radioactivity obtained from cells incubated only with ^{125}I-FGF-2 (Fig. 2A). This finding may be due to the low binding constant and the extremely rapid association and dissociation of FGF-2 from the low-affinity surface binding sites which makes this assay difficult to accomplish. In contrast, wild-type ES cells were

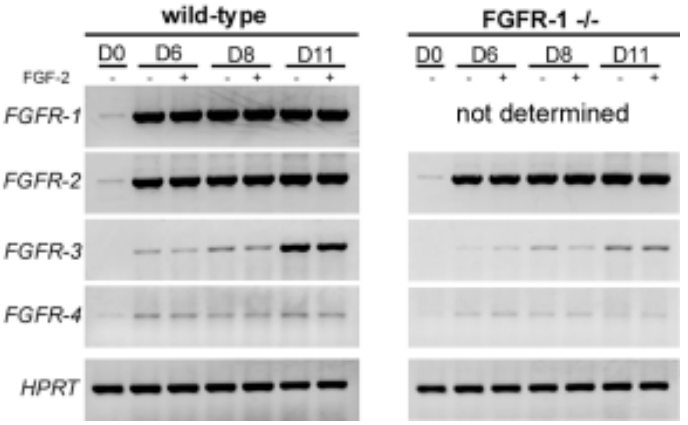
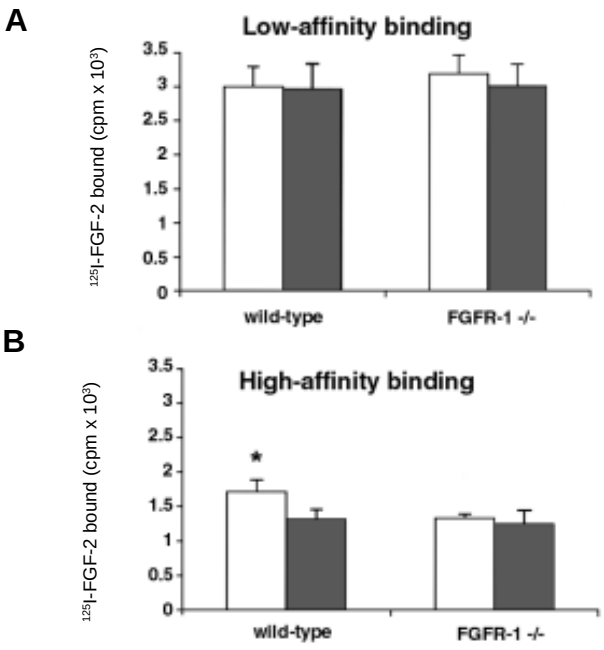


Fig. 1. Expression of *FGFRs* in undifferentiated ES cells (D0) and in differentiating embryoid bodies (D6, D8, D11). FGFR *expression was examined by RT-PCR with primers described in Table 1. Undifferentiated wild-type ES cells weakly express* FGFR-1, -2 *and* -4. *The same expression pattern, except for* FGFR-1, *was found in undifferentiated* FGFR-1*-deficient ES cells. Notably,* FGFR-3 *mRNA was not detected in either undifferentiated wild-type or* FGFR-1*-deficient ES cells. At the onset of EB differentiation, the levels of* FGFR-1 *and* FGFR-2 *mRNA (wild-type EBs) and* FGFR-2 *mRNA (mutant EBs) dramatically increase and remain elevated until day 11 of culture. However, the expression dynamics of* FGFR-3 *mRNA in both groups of EBs was quite distinct, showing up-regulation towards the end of the differentiation period.* HPRT *transcripts were examined as a control for the integrity and quantity of the RNA used in this analysis. Data representative of three different experiments are shown.*



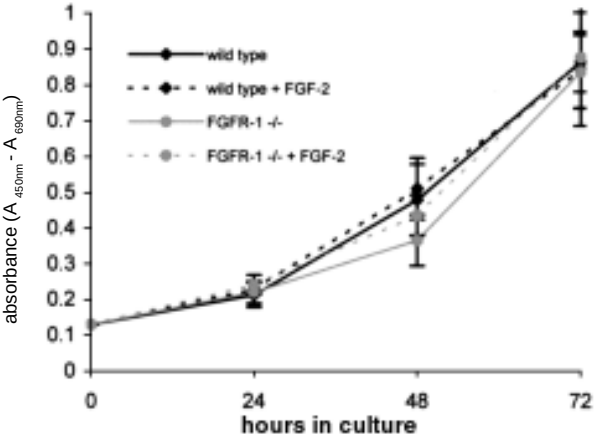


Fig. 2. (Left) Binding of ^{125}I-FGF-2 to undifferentiated ES cells. *Binding of ^{125}I-FGF-2 to low-affinity* **(A)** *and high-affinity* **(B)** *receptors was carried out in the absence (open bars) or the presence (solid bars) of a 100-fold excess of unlabeled FGF-2. Specific binding was estimated as the value of ^{125}I-FGF-2 binding (open bars) minus the value of ^{125}I-FGF-2 binding in the presence of unlabeled ligand (solid bars). Each value represents the mean of eight independent measurements in one of two independent experiments, which showed very similar tendency. Only high-affinity binding of ^{125}I-FGF-2 in wild-type ES cells was statistically significant. This binding was not observed in the corresponding mutant cells. Standard deviations are indicated. (*, $p < 0.05$* versus *binding in the presence of unlabeled FGF-2).*

Fig. 3. (Right) Proliferation of ES cells. *The basal growth of both ES cell lineages, as measured by their metabolism of the WST-1 substrate was very similar at 24 h, slightly retarded in mutant cells at 48 h, and again roughly equal at 72 h of culture. Similarly, the addition of 10 ng/ml of exogenous FGF-2 weakly stimulated the growth of both wild-type and* FGFR-1*-deficient ES cells at 48 h of culture but had no effects at 72 h of culture. Note that the observed differences were not statistically significant. Cultures of wild-type as well as* FGFR-1*-deficient ES cells were still subconfluent and both ES cell lineages grew optimally even after 72 h of culture. Data are presented as the mean of at least ten measurements in one of two independent experiments, which showed very similar tendency. Standard deviations are indicated.*

capable of high-affinity binding of ^{125}I-FGF-2, but the absence of FGFR-1 led to the abolition of this high affinity binding (Fig. 2B).

It should be noted, however, that the above data must be considered only as relative, because of several limitations of the assay, including the potential sensitivity of fragile ES cells to low and high salt washes. Despite this limitation, our data verify that FGFR-1-deficient ES cells are remarkably distinct from wild-type cells in terms of high-affinity receptor binding.

TABLE 1

OLIGONUCLEOTIDE PRIMERS USED FOR GENE EXPRESSION ANALYSIS

Gene	Primer sequence	Annealing temp. (°C)	Cycles (bp)	Size	Reference
FGFR-1	5' -TGCCACCTGGAGCATCATAATG - 3' 5' -TCCGATAGAGTTACCCGCCAAG - 3'	56	33	415	-
FGFR-2	5' -CCTATGACATTAACCGTGTCCC - 3' 5' -AAACACAGAATCGTCCCCTG - 3'	58	33	635	Jirmanova *et al.*, 1999
FGFR-3	5' -GATGCTGAAAGATGATGCGACTG - 3' 5' -GTGGGTGTAGACTCGGTCAAAAAG - 3'	58	30	496	-
FGFR-4	5' -GCTGAAAGACAATGCCTCCGAC - 3' 5' -GCACTTCCGAGACTCCAGATACTG - 3'	64	30	313	-
Pax-6	5' -TGCCCTTCCATCTTTGCTTG - 3' 5' -TCTGCCCGTTCAACATCCTTAG - 3'	54	33	178	-
Brachyury	5' - GAGAGAGAGCGAGCCTCCAAAC - 3' 5' -GCTGTGACTGCCTACCAGAATG - 3'	56	29	230	Rohwedel *et al.*, 1998
GATA-4	5' -GAAAACGGAAGCCCAAGAACC - 3' 5' -TGCTGTGCCCATAGTGAGATGAC - 3'	54	32	186	-
AFP	5' -ATGTATGCCCCAGCCATTCTGTCC - 3' 5' -GAGATAAGCCTTCAGGTTTGACGC - 3'	54	29	466	-
HPRT	5' -CTTGCTGGTGAAAAGGACCTCTC - 3' 5' -CAAATCAAAAGTCTGGGGACGC - 3'	56	24	350	-

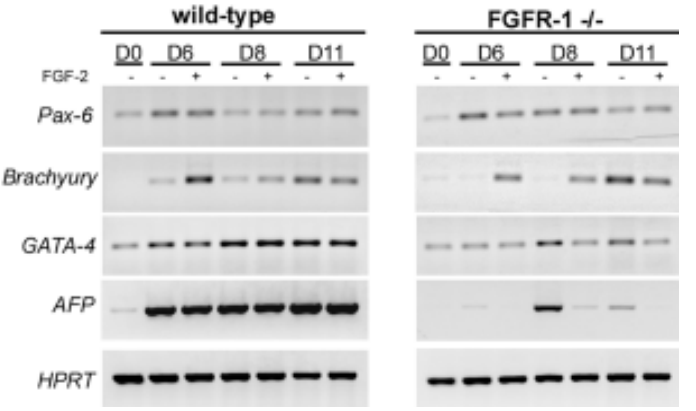


Fig. 4. Expression of marker genes in undifferentiated ES cells (D0) and differentiating EBs (D6, D8, D11). *Upon culturing wild-type and mutant EBs, the expression level of the neuroectoderm marker gene* Pax-6 *and the early endoderm gene* GATA-4 *remained unchanged from day 6 to day 11, regardless of FGF-2 treatment. Notably, mRNA for the mesoderm marker gene* Brachyury *was upregulated in both groups of EBs in response to FGF-2 at days 6 and 8, but not at day 11 of differentiation. In contrast to the comparable behaviour of marker genes for the neuroectoderm, early endoderm and mesoderm during this time course, remarkable differences in the expression of the late endoderm marker gene* AFP *were observed in wild-type and mutant EBs and also following FGF-2 incubation. At all intervals (D6, 8, 11), lowered levels of* AFP *mRNA were further down-regulated after FGF-2 treatment in mutant EBs, compared to the constantly high expression levels observed in wild-type EBs. The expression of* HPRT *gene was used as a loading control. The results are representative of three independent experiments.*

The Absence of FGFR-1 does not constitute a Significant Disadvantage for the Growth of ES Cells

Deng *et al.* (1994) have shown that FGFR-1-deficient blastocysts from the J1 mouse strain display a remarkable outgrowth retardation when cultured for an extended period of time (5-6 days). Thus, before morphological analyses of ES cell-derived EBs, we assayed the proliferation of undifferentiated ES cells, either in the absence or presence of exogenous FGF-2, to evaluate if a growth imbalance between wild-type and mutant ES cells may account for the observed outgrowth defects.

FGFR-1-deficient ES cells were found to proliferate, in the presence or absence of exogenous FGF-2, in a manner similar to that observed in wild-type ES cells (Fig. 3). Thus, despite differences between wild-type and mutant ES cells in their ligand binding, the growth characteristics of both ES cell lineages remained largely unaffected.

The Absence of FGFR-1 affects the Expression of AFP mRNA in ES Cell-Derived Embryoid Bodies

A previous study has shown that the targeted disruption of *FGFR-1* does not abrogate the early formation of ES cell aggregates and EBs (Deng *et al.*, 1994). To address the potential molecular consequences evoked by the absence of FGFR-1 after this period of time, we used standard conditions which permit ES cell aggregation and RT-PCR to analyse the transcription of *Pax-6* (neuroectoderm marker gene), *Brachyury* (mesoderm marker gene), *GATA-4* and *AFP* (endoderm marker genes) for several days following formation of EBs. Additionally, we examined if a saturating

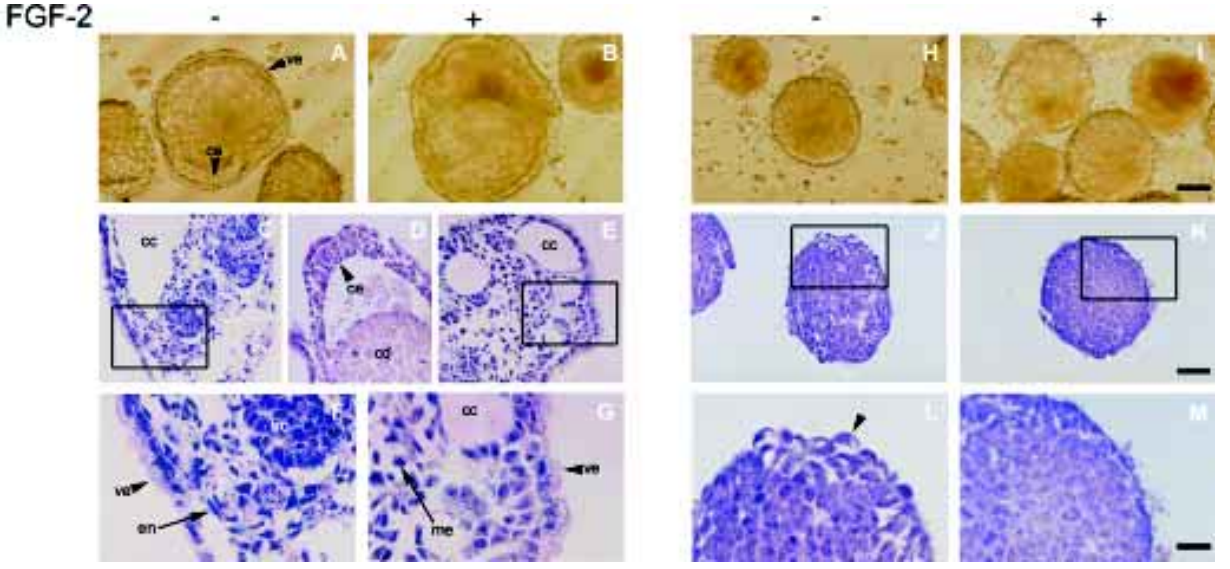


Fig. 5. Morphology of wild-type (left panel) and FGFR-1-deficient (right panel) EBs. *Wild-type EBs cultured in either the absence* **(A,C,D,F)** *or presence* **(B,E,G)** *of exogenous FGF-2 formed outer layers of visceral endoderm (ve) and columnar epithelium (ce) covering a cystic cavity (cc) or, in some cases, cavities. The process of cavitation likely occurs by massive cell death from the centre of the EBs, as demonstrated by the presence of cell debris (cd) stained with eosin. Higher magnification of such EBs (bordered areas and* **(F,G)***) shows apically oriented vacuoles in visceral endoderm cells (ve), some endothelial cells (en), mesenchymal cells (me) and localised clumps of stem cells (sc) with a characteristic undifferentiated morphology. In contrast to wild-type EBs, FGFR-1-deficient EBs* **(H,I,J,K,L,M)** *were smaller in size, showed no sign of visceral endoderm and cavitation, and displayed a characteristic rounded shape. As in wild-type EBs, this morphology appeared to be unaltered by FGF-2 treatment* **(I,K,M).** *Higher magnification views of mutant EBs (bordered areas and* **(L,M)***) reveal a partially organised peripheral layer of cells which lack the typical features of visceral endoderm; however, some vacuolisation was observed (arrowhead). Scale bars: A,B,C,D,E,H,I,J,K, 50 μm; F,G,L,M, 20 μm.*

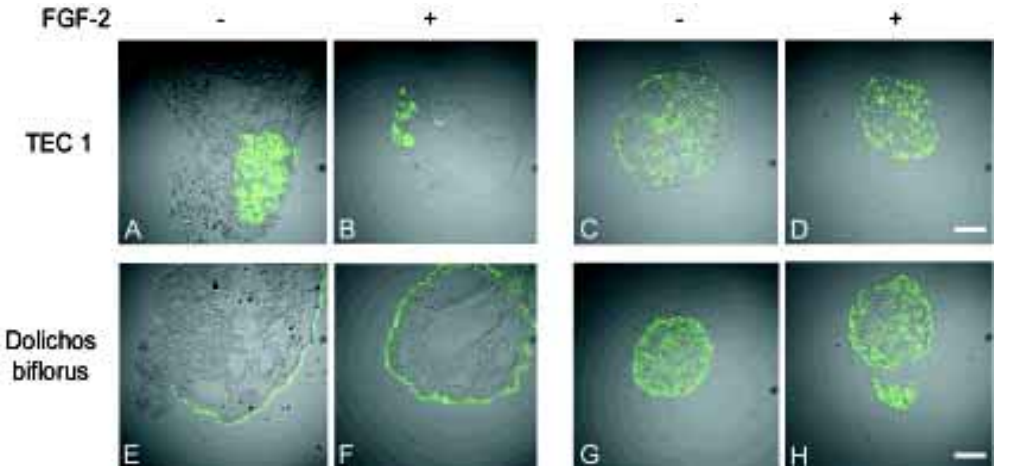


Fig. 6. Expression pattern of TEC 1 and *Dolichos biflorus* agglutinin binding sites in wild-type (left panel) and FGFR-1-deficient (right panel) EBs. *Immunostaining of serial sections with the anti-TEC 1 antibody revealed the presence of localised clumps of undifferentiated cells in wild-type EBs* **(A,B).** *The incidence of these clumps was quite low in both untreated and FGF-2-treated wild-type EBs. In mutant EBs, however, TEC 1-positive cells were observed throughout the sections, again irrespective of FGF-2 treatment* **(C,D)**. Dolichos biflorus *agglutinin bound to wild-type EBs, giving rise to an exclusively peripheral staining of mature visceral endoderm* **(E,F)**. *In mutant EBs, this lectin recognises the majority of cells spread throughout the sections with more intense staining at the periphery* **(G,H)**. *The pattern of lectin binding in both types of EB was apparently unaltered by incubation with FGF-2. These staining patterns again confirm that mutant EBs are less differentiated and still contain cells with an undifferentiated phenotype, as* Dolichos biflorus *binding sites could be detected in the embryonic ectoderm and its early derivatives as well as in visceral endoderm. Scale bars, 50 μm.*

concentration of exogenous FGF-2 (10 ng/ml) modifies the expression of these genes in wild-type or FGFR-1-deficient EBs. As illustrated in Fig. 4, the expression of both *Pax-6* and *GATA-4* was essentially the same in wild-type and mutant ES cell derived EBs over the observed time course, and in response to the presence of FGF-2. In contrast, the expression of *Brachyury* was increased by exogenous FGF-2 in both types of ES cell-derived EBs at day 6 and 8 of differentiation, as assessed by the relative quantification of three independent experiments using control *HPRT* (hypoxanthine phosphoribosyltransferase) mRNA. However, FGF-2 failed to modify the expression of *Brachyury* at day 11 of culture.

The *AFP* gene was found to be robustly expressed in wild-type EBs at all time points examined and its level of expression was unaltered by the presence of FGF-2 (Fig. 4). In striking contrast, *AFP* expression in mutant EBs maintained without exogenous FGF-2 was significantly reduced early after formation (Day 6), but increased at Day 8 and again slightly decreased by Day 11, which corresponds to the end of the process of endoderm formation and cavitation in control wild-type EBs (Day 11). Moreover, the levels of expression of the *AFP* gene were reduced in response to FGF-2 treatment at all intervals, qualitatively imitating the same time-dependent expression pattern as observed in the absence of FGF-2.

Together, these data demonstrate that the lack of FGFR-1 results in a lowered expression of the late endoderm marker gene *AFP*, and that this effect could be further enhanced by the presence of exogenous FGF-2.

The Absence of FGFR-1 impedes the Differentiation of the Visceral Endoderm and Columnar Epithelium and prevents the Cavitation of Embryoid Bodies

The data described above showed that the expression pattern of the endoderm-specific gene *AFP* in FGFR-1-deficient EBs significantly differs from that observed in wild-type controls. This raises the possibility that FGFR-1 is required for the proper differentiation of the endoderm. To determine whether endoderm-specific molecular changes are accompanied by changes in the phenotype of FGFR-1-deficient EBs, we performed microscopic examination at the level of whole EBs and morphological and immunochemical analyses of semi-thin sections of fixed EBs. We first tested if the absence of FGFR-1 interferes, under our experimental conditions, with the initial steps in the process of EB formation. In agreement with previously published data (Deng *et al.,* 1994), we determined that both mutant and wild-type ES cells first give rise to very similar aggregates and then to simple EBs three days after transferring them to suspension culture. However, we found marked differences between wild-type and mutant EBs later during differentiation. Somewhat surprisingly, we also found that the gross morphology of differentiating wild-type and mutant EBs was not influenced by the addition of exogenous FGF-2. Specifically, we showed that during 3-7 days following EB formation, wild-type EBs (Fig. 5; left panel) formed cystic structures with a well-defined outer layer of endoderm. The process of cavitation was preceded by apparent signs of massive cell death, which in the majority of wild-type EBs, was completed by day 10 from the start of differentiation in suspension culture (Table 2). In such cystic EBs, in certain cases with several smaller cavities, we found using higher magnification typical vacuolated visceral endoderm cells at the periphery, an underlying layer of ectoderm-derived columnar epithelium, endothelial and organized mesenchymal cells toward the centre of the EBs and rare compacted clumps of undifferentiated stem cells.

Compared to wild-type EBs, the same analysis of mutant EBs revealed a very different phenotype (Fig. 5; right panel). In general, these EBs exhibited several clearly distinguishable abnormalities:

TABLE 2

PROCESS OF CAVITATION IN WILD-TYPE AND FGFR-1-DEFICIENT EBs* AS SEEN ON DAY 10 OF CULTURE WITHOUT OR WITH FGF-2

	wild-type	wild-type	FGFR-1 -/- + FGF-2	FGFR-1 -/- + FGF-2
well developed cavity	94 %	91 %	6 %	8 %
signs of cavitation	2 %	2 %	11 %	15 %
no cavity	4 %	7 %	83 %	77 %
total EBs analysed	63	214	52	80

* Note that FGFR-1-deficient EBs were maintained in culture up to day 14 with no progress in the process of cavitation (data not shown).

a smaller size, the absence of a defined visceral endoderm layer and columnar epithelium, the absence of cavities, and no signs of cell death. Higher power magnification showed a partially organized layer of epithelial-like cells which, although lacking features of a typical visceral endoderm, were still slightly vacuolated.

In a series of parallel analyses of serial sections through 10 day-old EBs, we also determined the expression of TEC 1 (the anti-TEC 1 antibody recognizes the same epitope as the anti-SSEA 1 antibody), a marker of undifferentiated stem cells (Fig. 6 A-D). Consistent with our morphological observations, we observed that wild-type EBs express TEC 1-positive cells which were located in rare clumps of well-bordered cells and had the morphological features of stem cells (e.g. large nuclei). The quantity of such clumps of TEC 1-positive cells appeared to be unaltered by FGF-2 treatment. In contrast, FGFR-1-deficient EBs showed TEC 1-positive cells spread throughout the entire section, indicating a higher proportion of undifferentiated or less differentiated cells. This finding indicates that mutant EBs are much less differentiated compared to their wild-type counterparts. As in wild-type cells, the quantity of these TEC 1-positive cells did not appear to be modified by exogenous FGF-2 treatment.

We next examined the ability of the lectin *Dolichos biflorus* to bind to the surface of visceral endoderm cells to document the presence of this cell type in wild-type EBs and to assess the phenotype of peripheral epithelial-like cells in FGFR-1-deficient EBs (Fig. 6 E-H). *Dolichos biflorus* binding sites were observed exclusively at the periphery of both FGF-2-nontreated and treated wild-type EBs. In contrast, FGFR-1-deficient EBs expressed *Dolichos biflorus* binding sites throughout the entire section. Nonetheless, the staining intensity was higher at the periphery and decreased towards the centre. This expression pattern was uniform and again irrespective of FGF-2 treatment and may be due to the fact that undifferentiated embryonic ectoderm and its earliest derivatives also weakly express *Dolichos biflorus* agglutinin binding sites (Fan *et al,.* 1998). Importantly, these significant differences between wild-type and FGFR-1-deficient EBs, persisting from day 6 to day 10 of culture, suggest that defects in the formation of the visceral endoderm and columnar epithelium and the block of cavitation observed in mutant EBs are not caused simply by a delay in differentiation. Furthermore, the timing of the morphogenetic changes in wild-type EBs is roughly coincident with normal embryonic events, while mutant EBs remain non-cavitating and begin to degenerate after day 14 of culture (data not shown). It is of special note that either wild-type or mutant EBs maintained from the start of suspension culture with FGF-2 did not present obvious differences, either in the morphology of specific cellular compartments or in the dynamics of their appearance, compared to those EBs differentiated without exogenous FGF-2. This suggests that FGF-2 signals alone may be insufficient to induce observable changes in EB morphology.

Discussion

FGFs and their receptors, FGFRs, show a high level of redundancy in their mutual interactions (Green *et al.,* 1996). In addition, the formation of ligand-receptor complexes results in the activation of specific intracellular targets that may initiate different signalling pathways (Wang *et al.,* 1994). Moreover, the biological response of a particular cell type to exogenous stimuli *in vivo* likely results from the concerted activities of several growth factors and is further regulated by membrane heparan sulfate proteoglycans (Yayon *et al.,* 1991) and intercellular communication within a given tissue. Thus, an evaluation of the specific impact of an individual FGF or FGFR during particular stages of development is intrinsically limited, due to the high degree of redundancy and synergy which characterizes the FGF-FGFR signalling system.

Bearing in mind this limitation, we have used targeted disruption of the *FGFR-1* gene to interfere with FGF signalling in adherent cultures of undifferentiated ES cells and in ES cell-derived differentiating EBs. Using this simple system we have demonstrated that in undifferentiated ES cells, FGFR-1 plays a dominant role in receptor-ligand interactions. However, we found that the lack of functional FGFR-1 does not significantly affect the proliferation of ES cells. Rather, it functions to regulate the differentiation of ES cell-derived EBs. Specifically, we present evidence that the absence of FGFR-1 results in a reduced expression of the late endoderm-specific gene *AFP.* Correspondingly, lack of FGFR-1 suppresses the formation of the visceral endoderm and the specification of outer layers of the primitive ectoderm into the columnar epithelium, and thus leads to a block of cavitation.

In the mouse, genes encoding all four FGFRs are known to be expressed during pre- and/or early post-implantation development (Campbell *et al.,* 1992; Rappolee *et al.,* 1994). However, targeted disruption of either *FGFR-3* (Colvin *et al.,* 1996; Deng *et al.,* 1996) or simultaneous inactivation of *FGFR-3* and *-4* (Weinstein *et al.,* 1998) suggested that these two receptors operate rather at later stages of embryogenesis and fetal development. On the other hand, FGFR-1 and –2 are essential for pre-gastrulation development, as the corresponding homozygous mutant embryos die early after implantation (Deng *et al.,* 1994; Yamaguchi *et al.,* 1994; Arman *et al.,* 1998). Since both these receptors are also expressed in undifferentiated ES cells (Mummery *et al.,* 1993; Fig. 1 of this paper), the inactivation of one of them, can be expected to significantly affect the ligand-binding capacity of the cells. Thus, in our assay high-affinity binding of FGF-2 was abolished in FGFR-1-deficient ES cells.

As mentioned above, FGFR-1-deficient embryos are morphologically normal prior to the developmental period of implantation (Deng *et al.,* 1994; Yamaguchi *et al.,* 1994). However, after 5-6 days of *in vitro* culture, FGFR-1-deficient blastocyst outgrowths show some retardation in size compared to the corresponding wild-type outgrowths (Deng *et al.,* 1994). This growth restriction could be interpreted as being indicative of a role of FGFR-1 in stimulating embryonic mitogenesis. However, using an alternative experimental approach with FGFR-1-deficient ES cells, we have shown that wild-type and mutant ES cells exhibit very similar growth characteristics, indicating that this receptor-ligand system does not mediate the proliferation of undifferentiated ES cells. In keeping with this idea, undifferentiated FGF-4-deficient ES cells proliferate in the same way as wild-type cells (Wilder *et al.,* 1997), whereas FGF-4-deficient embryos die early after implantation (Feldman *et al.,* 1995). Such a discrepancy may be due to an inequality in the requirements for extracellular signals between entire ICM within the blastocyst and isolated ES cells. In this regard, FGF-4 (Rappolee *et al.,* 1994; Wilder *et al.,* 1997) but also FGF-2 (Jirmanova *et al.,* 1999) produced by the undifferentiated cells can acts as a paracrine growth factors for their differentiated progeny. Also generally lower expression of FGFRs, which is typical of undifferentiated ES cells, may also explain the absence of a significant effect of exogenous FGF-2 on cell proliferation. The fact that undifferentiated embryonal carcinoma (EC) cells bind FGFs to their high-affinity receptors

without increasing their degree of mitogenesis (Rizzino *et al.,* 1988) supports this explanation.

It has been repeatedly proven that ES cell-derived EBs contain cells of ectodermal, mesodermal and endodermal origins (e.g. Doetschman *et al.,* 1985). We used a panel of marker genes and morphological and immunochemical analyses to determine how the differentiation of FGFR-1-deficient EBs correlates with the suppressed ligand binding activity of FGFR-1-deficient ES cells in adherent cultures. We found that the expression of the neuroectoderm marker gene *Pax-6* (Walther and Gruss, 1991) was unchanged and indistinguishable from that observed in control wild-type EBs over the 6-day course of RT-PCR analysis. Consistently, both FGFR-1-deficient and wild-type EBs contain cells having the morphology, and expressing the cell markers, of ectodermal and/or undifferentiated stem cells. These results suggest that early ectodermal derivatives within EBs are normally formed in the absence of FGFR-1.

Embryoid bodies derived from normal ES cells form a mesoderm and express the early marker of the mesodermal lineage, *Brachyury*. *Brachyury* is required *in vivo* for the formation of the posterior mesoderm and notochord (Herrmann *et al.,* 1990). In this work we demonstrated that expression of the *Brachyury* gene in mutant and wild-type EBs can be enhanced by FGF-2 treatment from day 6 to 8 of differentiation. A similar FGF-2-mediated increase in expression of *Brachyury* has been demonstrated previously (Bursdal *et al.,* 1998). When *Brachyury* expression was then analysed at day 11 of differentiation (at this time point wild-type EBs already show a mesenchyme-like morphology while mutant EBs still display characteristics of less differentiated ectodermal cells), the level of *Brachyury* mRNA in both types of EBs was not affected by exogenous FGF-2. These data suggest that in the case of *Brachyury,* other FGFRs, primarily FGFR-2, can compensate for the absence of FGFR-1, particularly during early phases of differentiation.

The combined analyses presented in this work show that FGFR-1-deficient EBs display some initial characteristics of endodermal differentiation. However, by further analyses we have also revealed that mutant EBs do not develop mature visceral endoderm and columnar epithelium. We also found that the organization of mesoderm-derived mesenchyme-like cells in mutant EBs was disrupted, compared to wild-type controls. It is likely that roughly by day 6 of differentiation, the primitive endoderm is formed in both mutant and wild-type EBs. This was indicated by the sustained expression of the early endoderm marker gene *GATA-4* (Arceci *et al.,* 1993) and subsequently by the increased expression of *Dolichos biflorus* binding sites on the periphery of EBs (Sato and Muramatsu 1985; Fan *et al.,* 1998). Then, during the course of differentiation, the primitive endodermal cells begin to change into highly polarized and vacuolated visceral endoderm in wild-type EBs, whereas they remain immature in mutant EBs. This is evident, not only from their typical morphology, but also from the coincident deregulation of *AFP,* which is considered to be a marker of mature visceral endoderm (Dziadek and Adamson, 1978). Notably, a very similar phenotype was observed in EBs over-expressing *FGFR-2* cDNA truncated in its catalytic domain (Chen *et al.,* 2000; Li *et al.,* 2001). However, over-expression of truncated *FGFR-2* cDNA can, in fact, inhibit multiple FGFRs including FGFR-1, due to possible heterodimerisation of receptors (Chen *et al.,* 2000). Thus, this latter finding is not incompatible with our suggestion that EB endodermal differentiation requires FGFR-1 signals to take place in a specifically coordinated manner. Interestingly, in our experimental model we found that *AFP* expression was suppressed after treatment of mutant EBs with FGF-2. A sustained equilibrium between mesodermal and endodermal differentiation fates may account for these observations since exogenous FGF-2 simultaneously increases the expression of *Brachyury* and decreases the expression of *AFP.* This may not be the case in wild-type EBs, in which determination of the fate of embryonic cell lineages and major morphogenetic changes accompanying the process of cavitation occur well before day 8 of differentiation. Remarkably, even when expression of *Brachyury* was enhanced and expression of *AFP* was suppressed in the medium with exogenous FGF-2, EBs displayed no morphological changes and displayed the same phenotype as those maintained without FGF-2. Thus, our observations also indicate that FGF-2 signals alone are not sufficient to induce gross morphogenetic changes in differentiating EBs.

Overall, our results indicate that the absence of FGFR-1 in ES cell-derived EBs disturbs the maturation of the visceral endoderm, which in turn fails to produce factors that instruct the proper differentiation of the inner primitive ectoderm. This may constitute a primary event triggering defects of mesodermal patterning in FGFR-1-deficient mice.

Materials and Methods

ES Cell Lines and Cell Cultures

FGFR-1-deficient ES cells derived from the J1 mouse strain and control, wild-type J1 ES cells containing empty vector were kindly provided by Dr. Chu-Xia Deng (NIH, Bethesda, MD). Cells were routinely maintained in DMEM (PAA Laboratories, Linz, Austria) supplemented with 20% fetal calf serum (PAA Laboratories, ES cell-pretested), 1 x MEM non-essential amino acids (Gibco BRL, Paisley, UK), 100 mM nucleosides (Sigma, St. Louis, MO), 100 units/ml penicillin and 0.1 mg/ml Streptomycin (Gibco BRL), 0.1 mM β-mercapthoethanol (Sigma) and 1000 units/ml leukemia inhibitory factor (LIF, Gibco BRL). For all procedures we used ES cell-pretested fetal calf serum incubated with heparin-agarose beads for 12 h and depleted of beads by centrifugation and filtration (heparin-binding growth factor-depleted serum; HGFD serum). The levels of FGF-2 in this serum were always below detectability, as was determined by an ELISA-based kit with a detection limitation of 1 pg/ml (Quantikine High Sensitivity, R&D Systems, Wiesbaden, Germany). Undifferentiated ES cells were expanded in cell culture dishes with a feeder layer of mitomycin C-treated mouse embryonic fibroblasts. To initiate differentiation, ES cells were depleted of embryonic fibroblasts and transferred into bacteriological dishes, which allow for the formation of EBs. Subsequently, ES cells growing in the three-dimensional EBs were maintained in DMEM without LIF and with or without 10 ng/ml FGF-2 (Sigma) until used for further analyses.

Receptor Binding Assays

To determine low- and high-affinity receptor binding of FGF-2 in mutant and wild-type ES cells, cells were grown without a feeder layer in 24-well culture dishes in complete DMEM supplemented with LIF. Subconfluent cells were then washed twice with cold DMEM, 15 mM HEPES, 0.1 % bovine serum albumin (DMEM/H/B) and incubated for 90 min at 4°C with 1 ng/ml ^{125}I-FGF-2 (Amersham Pharmacia Biotech, Vienna, Austria; specific activity 1440 Ci/mmol) in a total volume of 0.5 ml DMEM/H/B. After incubation, the cells were washed twice with cold DMEM/H/B and treated for 5 min with 0.8 M NaCl in 20 mM HEPES pH 7.4. Using this salt concentration, low-affinity ^{125}I-FGF-2 bound to the cell surface heparan sulfate proteoglycans could be removed. High-affinity ^{125}I-FGF-2 bound to the transmembrane receptors was then determined by 2 M NaCl, 20 mM NaOAc (pH 4) extraction. The salt extracts (at least

8 replicates for each treatment) were counted for gamma emission for 200 s (Gamma Counter Tesla, Pardubice, Czech Republic). Non-specific binding of ^{125}I-FGF-2 was considered to be the amount of released radioactivity obtained in the presence of a 100-fold excess of unlabeled FGF-2, and these values of non-specific binding were subtracted from the counts obtained with ^{125}I-FGF-2 in the absence of unlabeled ligand.

Proliferation Analyses

FGFR-1-deficient and wild-type ES cells were depleted of feeder cells and expanded in complete DMEM without LIF, supplemented with 10% HGFD serum. These cells were seeded into 96-well cell culture plates at an initial density of 1.5 x 10^3 cells per well. Twenty-four h later, various concentrations of FGF-2 were added into the culture medium and the cells were grown for the next 24, 48, or 72 h. Following each period of culture, 10% WST-1, a cell proliferation reagent (Boehringer, Mannheim, Germany), was added to each well for a duration of 3 h. The absorbance of the converted formazan dye, which is a measure of the number of living cells, was then determined by spectrophotometry at 450 nm with a reference wavelength of 690 nm.

RT-PCR

Total RNA was isolated from ES cell-derived EBs using ion exchange columns (RNeasy kit; Qiagen, Valencia, CA), according to the manufacturer's instructions. One µg of RNA was then reverse transcribed using M-MLV reverse transcriptase (Gibco BRL) and cDNA was amplified with AmpliTaq Gold DNA polymerase (Perkin-Elmer, Beaconsfield, UK). Table 1 shows the primer sequences, PCR conditions and the length of the obtained PCR products. To control for possible contamination with genomic DNA, PCR reactions were performed with samples which had not been reverse transcribed. PCR amplification of the *HPRT* gene served as an internal control for the integrity of cDNA in each sample. PCR products were separated on 1.5 % agarose gels and visualized by ethidium bromide staining. The identity of PCR products was verified by restriction enzyme analysis (not shown).

Morphological and Histological Analyses

Whole EBs were examined and photographed using an Olympus IX70 inverted microscope equipped with Hoffman modulation contrast (Olympus C & S, Prague, Czech Republic). For sectioning, EBs were fixed for 2 h in ice-cold 4% formaldehyde, freshly generated from paraformaldehyde, slowly dehydrated in ethanol and embedded in polyester wax (Agar Scientific Ltd., Essex, UK). Five µm thick sections were cut, mounted on slides, dewaxed with ethanol, rehydrated and stained with hematoxylin/eosin. Serial sections were then examined and photographed using an Olympus BX60 microscope. For immunostaining of TEC 1 epitopes and the detection of endoderm-specific *Dolichos biflorus* agglutinin (DBA) binding sites (Sato and Muramatsu, 1985), representative serial sections selected after hematoxylin/eosin staining were dewaxed and rehydrated. The sections were subsequently pretreated with blocking solutions containing either 5% normal goat serum, 1% BSA in PBS for TEC 1 staining, or 0.1% BSA for detection of DBA binding sites. After blocking, the sections were incubated with the mouse monoclonal anti-TEC 1 antibody (IgM isotype) or biotinylated DBA (Vector Laboratories, Burlinghame, CA). This was followed by incubation with goat anti-mouse IgM FITC-conjugated antibody (Sigma) or mouse monoclonal anti-biotin FITC-conjugated antibody (Sigma), respectively. Controls without primary antibody or biotinylated lectin were also included. Analyses were performed using an Olympus Fluoview confocal laser scanning microscope system equipped with Nomarski DIC.

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