Fibroblast Growth Factor Enriches the Embryonic Liver Cultures for Hepatic Progenitors

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Fibroblast growth factors (FGFs) play an important role in hepatic induction during development. The aim of our study was to investigate the effect of exogenous FGFs on *ex vivo* liver development. We begin our analysis by examining FGF signaling during early mouse liver development. Phospho-FGF receptor (Tyr653/654) was detected in embryonic day 10 (E10) to E12 livers only. Next, E10 livers were cultured in the presence of FGF1, FGF4, or FGF8 for 72 hours and examined for histology, proliferation, apoptosis, and differentiation. FGFs especially FGF8 promoted sheet-like architecture, cell proliferation, and survival as compared to the control. All FGFs induced a striking increase in the number of c-kit and α -fetoprotein-positive progenitors, without altering albumin staining. However these progenitors were CK-19-positive (biliary and bipotential progenitor marker) only in the presence of FGF1 or FGF4 and not FGF8. FGFs also induced β -catenin, a stem cell renewal factor in these cultures. In conclusion, the presence of activated FGFR indicates a physiological role of FGF during early liver development. FGF1 and FGF4 enrich the embryonic liver cultures for bipotential hepatic progenitors. FGF8 promotes such enrichment and induces a one-step differentiation toward a unipotential hepatocyte progenitor. Thus, FGFs might be useful for enrichment and propagation of developmental hepatic progenitors. (*Am J Pathol* 2004, 164:2229–2240)

Understanding the process of liver development is essential to unravel the molecular aspects of liver cancer. Comprehending this complex process would also be useful to identify and anticipate roles of various factors that might be of value in hepatic tissue engineering and regenerative medicine applications. Liver development is a complex process that begins at 7 to 8 somites stage of development in mice.^{1–3} The initial mechanism of induction involves intricate physical and molecular events that initiate the commitment of gut endoderm to form liver.

Although such interactions with the cardiac mesenchyme as an inducer of liver development have been known for quite some time, the mechanistic molecular details are beginning to be understood.^{4,5} Many studies have now revealed the importance of factors such as fibroblast growth factors (FGFs) and bone morphogenic protein-4 (BMP-4) in the liver specification.^{6,7} After such commitment, the resident cells of the primitive liver bud undergo balanced events including proliferation, apoptosis, and differentiation to eventually constitute a functioning organ.

Liver bud at embryonic day (E9.5) to E10 stage is constituted predominantly by the hepatic progenitors that are precursors of differentiated cells at later stages of development. Several signaling pathways such as the Wnt/ β -catenin pathway, jagged/notch pathway, and sonic hedgehog pathway have been shown to play a role in maintenance and expansion of stem cells elsewhere.⁸ Of particular importance is the Wnt/ β -catenin pathway because of its involvement in liver growth, regeneration, and cancer as well as its role in epithelial-mesenchymal transitions.^{9–11} We have previously reported high levels of β -catenin protein expression in liver at embryonic day 10 (E10) through E14 of liver development. Using β -catenin anti-sense studies or the Wnt-3A-conditioned media in embryonic liver culture model, we have elucidated the role of this pathway in cell proliferation, apoptosis, and differentiation during early liver development.^{12,13} Embryonic liver cultures have also been used for exploring the effect of exogenous growth factors and to investigate function of novel genes by *in vitro* knockout studies.^{14–16}

The present study focuses on the effect of exogenous FGF1, FGF4, and FGF8 on the developing liver using the *ex vivo* embryonic liver cultures. FGFs are a large family of polypeptide growth factors that function by binding to the known ectodomains of the FGF receptors (FGFRs).^{17,18} This induces receptor dimerization and receptor tyrosine kinase activation that in turn provokes the activation of the ERK pathway via SHP2 and Ras to finally mediate the biological effects. FGFR tyrosine kinase activation entails successful phosphorylation at many tyrosine residues

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especially at 653/654.¹⁹ Once phosphorylated, several events such as nuclear translocation of FGFR and activation of target gene expression have been reported.^{20–22} The current study examines normal FGF activity during early liver development to explore its physiological relevance during this stage. This is followed by a comprehensive analysis of the impact of exogenous FGFs on hepatic progenitors during early *ex vivo* hepatic development including their effect on cell proliferation, apoptosis, and differentiation. This study thus informs of the expected results from the use of such factors in hepatic tissue engineering or cell culture applications.

Materials and Methods

Animals

Pregnant ICR mice were purchased from Charles River (Wilmington, MA). All experiments were performed in strict accordance with the National Institutes of Health guidelines as per the University of Pittsburgh Institutional Animal Care and Use Committee approved protocol.

Embryo Isolation, Embryonic Liver Protein Extraction, and Western Blot

Whole embryos were isolated from E10, E11, E12, and E14 timed-pregnant ICR mice for paraffin embedding. Multiple livers from E12, E14, E16, E17, and adult livers were isolated and pooled together for protein extraction using RIPA buffer as discussed elsewhere.¹¹ Protein content was measured by bicinchoninic acid and 50 μ g of protein for each stage was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis using minigel apparatus (Bio-Rad, Hercules, CA). Western blot analysis using standard protocol was performed for Phospho-FGFR-tyrosine 653/654 antibody (Cell Signaling, Beverly, MA) and equal loading was confirmed by β -actin (Chemicon, Temecula, CA).¹¹

Embryonic Liver Isolation and *ex Vivo* Cultures

The embryonic livers from embryos of E9.5 to E10 pregnant mice were isolated by surgical microdissection using atraumatic microsurgical instruments and a stereomicroscope (Olympus SZX12) as described previously.^{13–16} Dissected livers were placed on a methylcellulose filter paper positioned on a metal screen in six-well plates containing media and different growth factors. The cultures were maintained in an incubator at 37°C in the presence of 5% CO₂ for 72 hours.

Culture Media

All cultures were performed in triplicates and the results shown are representative of such studies. The embryonic livers were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Life Technologies, Inc.,

Grand Island, NY) supplemented with nonessential amino acids (Life Technologies, Inc.) and 10% fetal calf serum and in the presence of gentamicin (Life Technologies, Inc.). In addition, FGF1 (10 ng/ml), FGF4 (25 ng/ml), or FGF8 (25 ng/ml) (R&D Systems, Minneapolis, MN) were added to three liver cultures each. These were cultured for 72 hours and the media replaced every 24 hours with fresh FGFs.

Tissue Processing

The whole embryos from E10, E11, E12, and E14 and the cultured livers were formaldehyde fixed and paraffin embedded as described elsewhere.¹³ Four- μ m-thick sections were cut using a microtome (Leica Microsystems) and subjected to further histological and immunohistochemical examination.

Histology and Immunohistochemical Characterization

Hematoxylin and eosin staining was performed to evaluate any changes in histology in response to FGF in comparison to the controls (serum only). Immunohistochemistry was performed by an indirect immunoperoxidase procedure for immunohistochemical localization of various proteins has been described previously.¹¹ Primary antibodies used were against β -catenin and α -fetoprotein (α -FP) from Santa Cruz (Santa Cruz, CA), c-kit from Oncogene (Boston, MA), albumin and CK19 from DAKO (Carpinteria, CA). The secondary antibodies were from Chemicon and the signal was detected using the ABC Elite kit (Vector Laboratories, Burlingame, CA). For negative control, the sections were incubated with secondary antibodies only. ApopTag peroxidase kit (Intergen Company, Purchase, NY) was used to detect apoptosis in the embryonic livers cultured under different conditions. Terminal dUTP nick-end labeling (TUNEL)-positive apoptotic nuclei were detected by the presence of positive brown staining. For proliferation assay, immunohistochemistry was performed for Ki-67 (Santa Cruz) that specifically recognizes cells in the S phase of cell cycle. All stained slides were viewed on a Zeiss upright research microscope (Axioskop 40). Digital images were obtained on a Nikon Coolpix 4500 camera. Collages were prepared using Adobe Photoshop 5.0 software.

Quantitative and Statistical Assessment

For quantitative analysis of various immunohistochemical stains, the total cells and positively staining cells for each marker were counted to derive percentages of positively staining cells ($\pm$ SEM) from three representative high-power fields from each of the three livers cultured under one growth condition. Further comparisons to address the effect of growth conditions on each of the markers were made by the multiple column comparisons by one-

Figure 1. Presence of activated FGFR during early liver development indicates role of FGF at this stage of hepatic development. **A:** Section from E10 liver demonstrates a number of phospho-FGFR-Tyr653/654-positive cells (**arrows**) with its nuclear localization. **B:** E11 liver also demonstrates presence of activated FGFR in the nuclei of several resident cells (**arrows**). **C:** Similar localization is maintained in a significant number of positive cells (**arrows**) at E12 stage. **D:** E14 liver is devoid of any phospho-FGFR-Tyr653/654-positive cells (**arrowhead**) indicating its role in previous stages of hepatic development. **E:** Western blot using phospho-FGFR-Tyr653/654 antibody demonstrates its presence in the livers at E12 stage of development only as compared to all later gestational stages (**top**). β -Actin blot (**bottom**) reveals uniform protein loading.

-way analysis of variance using the Instat software 2.01 (GraphPad, San Diego, CA). Statistical significance was judged by the two-tailed *P* value and a *P* value of less than 0.05 was considered statistically significant. This was followed by appropriate posttests and because only four experimental groups were to be compared (<5),

Bonferroni *t*-test (STATsimple 2.0.5; Nidus Technologies), was used to determine the statistically significant differences between these test groups. Again, the differences were labeled as being statistically significant if the *P* value was less than 0.05 or highly significant if the *P* value was less than 0.01.

Figure 2. Characteristic changes in histology; cell proliferation (increase) and apoptosis (decreased) are evident in the embryonic liver cultures in the presence of different FGFs. **A:** Normal embryonic liver culture (in presence of 10% FCS) demonstrates cell arrangement as ducts (**arrowhead**) or sheets (**arrow**). **B:** FGF1 promotes sheet-like arrangement (**arrow**) and the ducts are fewer and single layered (**arrowhead**) as compared to the control cultures. **C:** FGF4 treatment enhances sheet-like arrangement of cells (**arrow**) with fewer single or multi-layered ducts (**arrowhead**) as compared to normal cultures. **D:** FGF8 promotes sheet-like arrangement of cells (**arrow**) in embryonic cultures without any duct formation. Also the morphology of the cells resembles normal hepatocytes at different stages of differentiation and maturation. **E:** Normal embryonic liver culture (in the presence of 10% FCS) demonstrates ~10% apoptotic nuclei (**arrowhead**) in sheet-like and ductal arrangement by TUNEL immunohistochemistry. Most cells in either arrangement are viable (**arrow**). **F:** Some decrease in apoptotic nuclei (**arrowhead**) is observed in the cultures in the presence of FGF1. **G:** FGF4 also ensures similar cell viability (**arrow**) with less than 10% of cells showing apoptosis by TUNEL (**arrowhead**). **H:** In the presence of FGF8, only 5% of all cells showed apoptosis (**arrowhead**) that were interspersed among the predominant viable cells (**arrow**). **I:** Ki-67-positive cells (**arrows**) comprise ~12% of all cells in serum-grown cultures seen interspersed among the negative cells (**arrowhead**). **J:** A twofold increase in the number of Ki-67-positive cells (**arrow**) is observed in the presence of FGF-1 indicating an increase in cell proliferation. **K:** FGF4 also increases Ki-67 positivity although to a lesser extent than FGF1 with ~17% of cells being Ki-67-positive (**arrow**). **L:** In the presence of FGF8, ~30% of cells were Ki-67-positive (**arrow**) while the remaining cells were Ki-67-negative (**arrowhead**), indicating a threefold increase in cell proliferation as compared to the normal cultures. Original magnifications, $\times 40$.

Results

Presence of Activated FGFR in Early Liver Development

We began the analysis by investigating the status of the FGF pathway in early liver development. For this purpose

we used a phospho-specific antibody that recognizes FGFR, which is tyrosine phosphorylated at residues 653 and 654. These sites are shared by all four FGFRs and tyrosine phosphorylation at these sites represents catalytic activity and activation of the FGF pathway.¹⁹ Nuclear localization of FGFR has been associated with FGF acti-

Figure 3. Effect of FGFs on apoptosis and proliferation in embryonic liver cultures. TUNEL (apoptotic nuclei)- or Ki-67 (proliferation)-positive cells were counted to derive percentages of positive cells in three representative high-power fields from sections from three independent embryonic livers cultured in each growth condition. Analysis of variance for TUNEL analysis reveals statistically significant differences between control and FGFs as judged by the two-tailed *P* value of 0.0402. Posttests (Bonferroni *t*-tests) revealed statistically significant differences between the DMEM and FGF8 cultures and FGF4 and FGF8 cultures (*P* < 0.05) only. For Ki-67 staining comparisons, analysis of variance for proliferation assay reveals a statistically significant difference between control and FGFs (*P* = 0.0017). Bonferroni *t*-tests reveal highly significant differences between DMEM and FGF1 cultures and DMEM and FGF8 cultures (*P* < 0.01), and significant differences between DMEM and FGF4 cultures and FGF4 and FGF8 cultures (*P* < 0.05) only. *, *P* < 0.05; **, *P* < 0.01.

vation in many studies.^{23–25} Sections from whole embryos at the E10, E11, E12, and E14 stages were examined for localization of phospho-FGFR. Several cells were positive for activated FGFR at the E10 stage (Figure 1A). FGFR-Tyr 653/654 localized to the nuclei of numerous resident cells indicating active FGF signaling. Similar localization was also maintained at the E11 and E12 stages (Figure 1, B and C). There were very few cells that showed any FGFR staining at the E14 stage (Figure 1D) indicating a significant decrease in FGF activity at this stage of development. To corroborate these findings we used protein extracts from developing livers to examine the levels of activated FGFR. We found appreciable levels of phospho-FGFR at E12 followed by its complete loss at all later stages of hepatic development as shown in a representative Western blot (Figure 1E). This analysis thus confirms an ongoing FGF signaling in the developing livers at the E10 to E12 stage.

FGFs Promote Sheet-Like Arrangement and Inhibit Duct Formation in Embryonic Liver Cultures

In the presence of 10% fetal calf serum, the embryonic liver cultures display cells arranged in sheet-like or ductal structures (Figure 2A). Multilayered ducts that constitute the predominant ductal arrangement occupy ~30% of

the overall section. In the presence of FGF1 a sheet-like arrangement persists but only 10% of the area shows mainly single layered ductal structures (approximately threefold decrease) (Figure 2B). In the presence of FGF4 there was again a predominance of sheet-like arrangement, although 20% of the section showed multilayered or single-layered ducts (approximately twofold decrease) (Figure 2C). The difference in the FGF8 cultures was most pronounced and the cells were observed in sheet-like arrangement only without any evidence of ductal structures (Figure 2D). Morphologically, the cells comprising sheet-like architecture in these conditions resemble hepatocytes at different stages of differentiation and maturation.

Effects of FGFs on Apoptosis and Proliferation in Embryonic Liver Cultures

For this analysis three different embryonic liver cultures under the same growth condition were examined by TUNEL for apoptosis and Ki-67 for cell proliferation. Normal embryonic liver cultures show $12 \pm 3\%$ apoptotic nuclei by TUNEL staining as evident by the brown staining (Figure 2E). There was a consistent decrease in the number of apoptotic nuclei ($8 \pm 1\%$) on inclusion of FGF1 in the cultures (Figure 2F). A minor decrease as compared to the control was evident in the FGF4 cultures in which $10 \pm 1\%$ were TUNEL-positive (Figure 2G). In the presence of FGF8 there was a noteworthy decrease in the number of apoptotic nuclei ($5 \pm 1\%$) as compared to the rest of the conditions (Figure 2H).

Normal embryonic liver cultures in the presence of 10% serum grow more than twice their original size and 95 to 100% of the resident cells demonstrate proliferating cell nuclear antigen positivity.¹³ Ki-67 stain recognizes cells in S phase of the cell cycle and is a more specific indicator of proliferation. In the following analysis we compare the Ki-67 positivity under different growth conditions. Approximately $11 \pm 2\%$ of all cells were Ki-67-positive in the control (Figure 2I). FGF1 grown cultures showed $21 \pm 2\%$ of all cells to be Ki-67-positive (Figure 2J). A similar increase was also apparent ($17 \pm 2\%$) in the presence of FGF4 (Figure 2K). However, a more dramatic increase was evident in the presence of FGF8 in the cultures in which $30 \pm 4\%$ of all cells were Ki-67-positive (Figure 2L).

To examine the statistical significance of the changes observed in the apoptosis and proliferation assay, multiple sections were used for calculating the percentage of positively staining cells followed by appropriate column comparisons as described in the Materials and Methods section. For apoptosis assay, analysis of variance depicts a statistically significant variation among columns with a two-tailed *P* value of 0.0402 (Figure 3). Posttest analysis was then performed by the Bonferroni *t*-tests to examine significance between specific growth conditions. The differences observed in apoptosis between the DMEM and FGF1 cultures or DMEM and FGF4 cultures were statistically insignificant (*P* > 0.05). Also changes were insignificant between the FGF1 and FGF4 cultures

and the FGF1 and FGF8 cultures. However, the Bonferroni *t*-tests revealed statistically significant differences between the DMEM and FGF8 cultures, as well as the FGF4 and FGF8 cultures ($P < 0.05$) signifying an overall cytoprotective effect of FGF8.

Similar analysis was also performed for Ki-67 immunohistochemistry. Analysis of variance revealed a two-tailed *P* value of 0.0017 indicating a significant positive impact of FGFs on cell proliferation (Figure 3). Bonferroni post *t*-tests were again performed to identify experimental groups that showed significant changes in number of Ki-67-positive cells. Highly significant differences were observed in DMEM and FGF1 cultures and DMEM and FGF8 cultures ($P < 0.01$), and a significant difference was observed between DMEM and FGF4 cultures ($P < 0.05$). Although differences between the numbers of Ki-67-positive cells were insignificant between FGF1 and FGF4 cultures or FGF1 and FGF8 groups, a significant difference was observed between the FGF4 and FGF8 cultures ($P < 0.05$). Thus, although all three FGFs promoted cell proliferation, FGF1 and FGF8 had a more noteworthy effect on cell proliferation in the *ex vivo* embryonic liver cultures.

FGFs Enrich the Embryonic Liver Cultures for Undifferentiated Progenitors

c-Kit is known to be expressed in early undifferentiated hepatic progenitors.^{26–29} Normally E10 embryonic liver in mouse is predominantly composed of c-kit-positive cells (~90%), that drops to ~25% at E13.5 to E14 indicating their differentiation (not shown). Approximately $24 \pm 5\%$ of all cells (similar to E13 to E14 embryonic liver) in the normal organ cultures displayed c-kit positivity as shown in a representative section (Figure 4A). In the presence of FGF1, these cultures demonstrated an increase in the total number of c-kit-positive cells to $\sim 76 \pm 7\%$ (Figure 4B). FGF4 also induced a similar increase to $\sim 72 \pm 9\%$ (Figure 4C). FGF8 also enriched the culture for c-kit-positive cells with $\sim 82 \pm 8\%$ of all cells staining positive for this marker (Figure 4D).

Next, we examined the cultures for α -FP that is expressed in hepatic progenitors and immature/fetal hepa-

toocytes.^{30–32} Approximately $56 \pm 6\%$ of all cells in the normal embryonic liver culture are α -FP-positive (Figure 4E). Phenotypically, these α -FP-positive hepatic progenitors are intermediate to large size cells that contain larger nuclei. In the presence of FGF1, there was a noteworthy increase in the number of α -FP-positive cells to $\sim 78 \pm 7\%$ (Figure 4F). FGF4 also promoted a similar increase in the number of α -FP-positive cells to $\sim 80 \pm 7\%$ (Figure 4G). Similarly, FGF8 enhanced α -FP positivity with $\sim 87 \pm 5\%$ of all cells staining positive for this marker (Figure 4H). Most of the resident cells in the FGF8 cultures were positive for α -FP except the smaller hematopoietic cells. A high proportion of the α -FP-positive cells were also positive for c-kit as observed in sequential section staining and one such representative comparison (for organ culture in the presence of FGF8) can be appreciated in Figure 4, D and I. Thus increase in c-kit and α -FP-positive cells in the presence of FGFs indicates an increase in hepatic progenitors within the culture.

These observations were then corroborated for any statistical significance (Figure 5). Overall, column comparison within the c-kit group shows significant variation by analysis of variance (two-tailed *P* value = 0.0005). Bonferroni *t*-tests were next applied to determine statistical differences among various experimental groups. This test revealed highly significant variation in c-kit positivity between DMEM and FGF1 cultures, DMEM and FGF4 cultures, and DMEM and FGF8 cultures ($P > 0.01$). However, no significant differences were observed among any of the FGF cultures ($P < 0.05$). For the α -FP staining, analysis of variance again revealed statistically significant variation with a two-tailed *P* value = 0.0115 (Figure 5). Bonferroni post *t*-tests identified highly significant differences in the number of α -FP-positive cells between DMEM and FGF1 cultures, DMEM and FGF4 cultures, as well as DMEM and FGF8 cultures ($P < 0.05$). On the other hand differences observed in the number of α -FP-positive cells among the FGF1, FGF4, and FGF8 cultures were statistically insignificant ($P > 0.05$). Cumulatively, it seems that FGF1, FGF4, and FGF8, all enhanced c-kit and α -FP positivity in the embryonic liver cultures, thus indicating successful enrichment for hepatic progenitors.

Figure 4. FGF1 and FGF4 enrich the cultures for bipotential hepatic progenitors, whereas FGF8, in addition, also promotes one-step differentiation of such cells into unipotential progenitors. **A:** In normal embryonic liver culture (in the presence of 10% FCS) after 72 hours, ~25% of all cells are c-kit-positive (**arrowhead**). Remaining cells are more mature or differentiated and are c-kit-negative (**arrow**). **B:** In the presence of FGF1 ~75% of the entire organ culture demonstrates c-kit positivity (**arrowhead**). **C:** FGF4 also induced c-kit positivity (**arrowhead**) comparable to FGF1 with a few negative cells (**arrow**). **D:** A representative section of FGF8-grown embryonic livers demonstrates ~90% of cells to be c-kit-positive (**arrowhead**). **E:** α -FP staining shows a group of immature hepatocytes (**arrowhead**) in normal embryonic liver cultures. **F:** In the presence of FGF1, increased numbers of cells were α -FP-positive (**arrowhead**). **G:** Similar increased number of α -FP-positive cells (**arrowhead**) is seen in the presence of FGF4 with a few negatively staining cells (**arrow**). **H:** Strong α -FP positivity (**arrowhead**) is seen in the FGF8-cultured embryonic livers demonstrating a noteworthy enrichment for liver stem cells. The only negative cells seen are smaller hematopoietic cells (**arrow**). **I:** A low-power view of the FGF8 cultures demonstrates numerous α -FP-positive cells (**arrowhead**) similar to c-kit-positive cells observed in a consecutive section (**D**). **J:** Albumin-positive (**arrowhead**) cells displaying hepatocytic (stem cell/immature/mature) phenotype are observed in normal embryonic liver cultures in DMEM with 10% FCS, that are interspersed by negatively staining smaller cells (**arrow**) of the hematopoietic lineage. **K:** Similar albumin-positive cells (**arrowhead**) are observed in the presence of FGF1 with a few negative hematopoietic cells (**arrow**). **L:** FGF4 also induces a similar pattern of albumin staining. **M:** FGF8 also shows a similar phenotype with numerous albumin-positive cells (**arrowhead**) and negatively staining hematopoietic cells. **N:** CK19-positive cells are seen lining the innermost (luminal) layer (**arrowhead**) of multilayered ductal structures in normal cultures (**left**). **Right:** An area of CK19-positive cells outside the ducts in the sheet-like arrangement that represents bipotential progenitors (also c-kit-, α -FP-, and albumin-positive). **O:** Extra ductal cells also stain positive for CK19 (**arrowhead**) in FGF1 cultures indicating their bipotentiality. Again the hematopoietic cells are negative for CK19 (**arrow**). **P:** In the presence of FGF4, a ductal area shows only the innermost layer to be CK19-positive (**arrowhead**) and the cells away from the lumen are CK19-negative (**arrow**). **Right:** CK19-positive cells in sheet arrangement. **Q:** Decreased CK19 staining in sheet arrangement of cells is observed in FGF8 cultures. Fewer cells with less intense staining are observed (**arrowhead**). Many hematopoietic (white **arrowhead**) and nonhematopoietic (**arrow**) cells are CK19-negative. Also note that no ducts form in these cultures. **R:** Another FGF8-grown culture shows decreased CK19 positivity with a few faintly CK19-positive cells (**arrowhead**) among the negatively staining cells (**arrow** and white **arrowhead**). Original magnifications: $\times 10$ (A–D, I); $\times 40$ (E–H, J–R).

Figure 5. Quantitative and statistical analysis of the effect of FGFs on hepatic progenitors and hepatocytes. Percent positive cells for each lineage marker were computed from three high-power fields from three independent embryonic livers cultured per growth condition and compared for statistical significance. Significant variation in c-kit staining between the control and FGFs is observed by analysis of variance as judged by the two-tailed *P* value of 0.0005. Posttests (Bonferroni *t*-tests) identified highly significant differences in c-kit positivity between DMEM and FGF1 cultures, DMEM and FGF4 cultures, as well as DMEM and FGF8 cultures (*P* < 0.01) only. For α -FP staining, analysis of variance again reveals an overall statistically significant variation between the DMEM and FGFs (*P* = 0.0115). Next, Bonferroni *t*-tests showed significant differences in the number of α -FP-positive cells between DMEM and FGF1 cultures and DMEM and FGF4 cultures (*P* < 0.05); and highly significant differences between DMEM and FGF8 cultures (*P* < 0.01). Lastly, albumin-staining analysis revealed insignificant column variation between the DMEM and FGF groups (*P* > 0.05). Bonferroni *t*-tests also failed to detect any significant differences in albumin positivity between any of the experimental groups (*P* > 0.05). *, *P* < 0.05; **, *P* < 0.01.

Effect of FGFs on Hepatocyte and Biliary Differentiation

Our next aim was to investigate any effect of the FGFs on biliary or hepatocyte differentiation. For hepatocytes we used albumin immunohistochemistry and although it is a marker for hepatic stem cells and immature and mature hepatocytes, useful information can be gathered and interpreted from a combined analysis with c-kit and α -FP staining. Normal embryonic liver cultures demonstrate $\sim 77 \pm 6\%$ of albumin-positive cells (Figure 4J). Most cells within the culture are albumin-positive except the smaller hematopoietic cells. A comparable number of albumin-positive cells were found in the FGF1 ($75 \pm 6\%$), FGF4 ($80 \pm 8\%$), and FGF8 ($77 \pm 4\%$)-containing cultures (Figure 4; K, L, and M). Statistical analysis by analysis of variance revealed insignificant differences in the albumin-positive cell populations among these conditions (*P* > 0.05) (Figure 5). Bonferroni post *t*-tests analysis also failed to detect any significant differences in albumin-positive cell percentages the DMEM, FGF1, FGF4, or FGF8 groups (*P* > 0.05). Thus, although albumin analysis alone is inconclusive, when interpreted with the concurrent increase in c-kit and α -FP-positive cell populations, we can infer that FGFs do not alter maturation of

hepatocytes, although they do promote progenitor cell population enrichment in the embryonic liver cultures.

Next, we tested these cultures for CK-19, an established biliary marker.^{33,34} CK-19 also recognizes the bipotential hepatic progenitors.³⁵ Thus the analysis was aimed at examining the effect of FGFs on biliary differentiation and also confirming the changes observed in the hepatic progenitor population addressing their true bipotentiality in response to these factors. In the control cultures, the innermost layers of cells in the ductal arrangement are CK-19-positive (Figure 4N, left). A distinct population of positive cells (25% of section) is also observed in the remaining sheet-like arrangement (Figure 4N, right). Consecutive slide staining showed these cells to be also positive for albumin, α -FP, and c-kit indicating their bipotentiality or a progenitor phenotype (not shown) as has also been reported earlier.¹³ In the presence of FGF1, these cultures displayed an increase in the number of such CK-19-positive cells in the sheet-like arrangement (Figure 4O). These cells reflect dual potentiality owing to their concurrent positivity to c-kit, α -FP, and albumin as shown in the consecutive serial sections (seen in low power in Figure 4B for c-kit and high power in Figure 4, F and K, for α -FP and albumin, respectively). FGF4 cultures also displayed a similar pattern for CK-19 staining with the innermost layer in the duct structures (left panel) and the cells in sheet-like arrangement (right panel) displaying CK-19 positivity (Figure 4P). No duct formation was observed in the FGF8 cultures. The sheet-like arrangement of cells showed a weak to no CK-19 staining (Figure 4, Q and R). However, these cells simultaneously possess other hepatic progenitor markers such as c-kit, α -FP, and albumin suggesting that FGF8 enriches for a unipotential hepatic progenitor phenotype rather a true bipotential progenitor.

FGFs Increase Nuclear Localization of β -Catenin, a Stem Cell Renewal Factor

Our final aim was to identify any changes in β -catenin distribution in response to the FGF treatment in the embryonic liver cultures. Convergence of the FGF and Wnt pathways during development has been reported in several tissues.^{36,37} We have previously demonstrated high levels of β -catenin during early liver development along with its nuclear localization that corresponds to its role in regulating cell proliferation and apoptosis of the resident cells at that stage.¹³ Because, β -catenin is also one of the known factors to be involved in stem cell renewal in other cell types we wanted to establish if FGF treatment was in fact inducing any redistribution of this protein in embryonic liver cultures that might suggest a mechanism of such enrichment and/or one-step differentiation of the hepatic progenitors within these cultures.^{38,39} In the normal embryonic liver cultures in the presence of 10% fetal calf serum (FCS), the cells exhibit mainly cytoplasmic β -catenin in $\sim 22 \pm 1\%$ of resident cells (Figure 6A). In the presence of FGF1, there was an increase in the number of cells displaying cytoplasmic β -catenin positivity ($63 \pm 1\%$) with a few cells also showing its nuclear

Figure 6. FGFs enhance β -catenin immunoreactivity in embryonic liver cultures. **A:** β -Catenin-positive cells (arrow) are seen in the normal embryonic liver cultures. **Inset**, high-power view shows the staining to be mainly cytoplasmic. **B:** An increase in β -catenin staining (arrow) occurs in the presence of FGF1, with a few cells also showing nuclear β -catenin (arrowhead) as seen in the **inset**. **C:** Increased number of β -catenin-positive cells showing that cytoplasmic (arrow), membranous (red arrowhead), and nuclear (arrowhead) localization was evident in the presence of FGF4. Smaller hematopoietic cells (green arrowhead) stained negatively. A high-power view in the **inset** also shows a similar pattern. **D:** A low-power view is included to appreciate a noteworthy increase in β -catenin positivity (arrowhead) that is observed in the presence of FGF8 in the embryonic liver cultures. **E:** Increased β -catenin localization is observed in the cytoplasm (arrow), at the membrane (red arrowhead) and nuclei (arrowhead) of most cells with only a few hematopoietic cells staining negatively (green arrowhead). **Inset** reveals clear nuclear (arrowhead) and membranous (red arrowhead) localization of β -catenin. Original magnifications: $\times 40$ (A–C, E); $\times 4$ (D).

localization (Figure 6B). In FGF4 a similar increase ($68 \pm 2\%$) as well as localization was observed (Figure 6C). In the presence of FGF8, there was a dramatic increase in the total number of β -catenin-positive cells as can be appreciated in the low-power view (Figure 6D). There was a concomitant increase in the number of cells showing nuclear localization of β -catenin (Figure 6E). Interestingly, more than 80% of all cells showed discrete membranous localization as compared to the other conditions in which less than 10% of all cells showed any membranous localization. A quantitative analysis for overall β -catenin positivity was then performed to examine statistical significance of the observed changes. Analysis of variance reveals a highly statistically significant variation among all conditions with a two-tailed P value of less than 0.0001 (Figure 7). Bonferroni post t -tests were next performed for interexperimental group comparisons. Highly significant differences in β -catenin positivity were observed between DMEM and FGF1, DMEM and FGF4, and DMEM and FGF8 cultures ($P < 0.01$). In addition, although Bonferroni t -tests failed to reveal significant difference between the FGF1 and FGF4 experimental groups ($P > 0.05$), highly significant differences were observed in the number of β -catenin-positive cells between the FGF1 and FGF8 cultures as well as FGF4 and FGF8 cultures ($P < 0.01$). These results indicate a significant positive effect of the examined FGFs on β -catenin and might be one of the contributing factors for hepatic progenitor enrichment in these *in vitro* embryonic liver cultures. Also, a noteworthy increase in membranous

Figure 7. FGFs induce β -catenin increase in embryonic liver cultures. Percent positive cells displaying β -catenin immunoreactivity were calculated from three high-power fields from three independent embryonic livers cultured under specific growth conditions and compared for statistical significance. Significant increase in β -catenin positivity was detected by analysis of variance between the DMEM and FGF cultures (two-tailed $P < 0.0001$). Posttests (Bonferroni t -test) identified highly significant differences in the number of β -catenin-positive cells between DMEM and FGF1, DMEM and FGF4, DMEM and FGF8, FGF1 and FGF8, and FGF4 and FGF8 cultures ($P < 0.01$) only. Statistically insignificant difference was observed between FGF1 and FGF4 cultures ($P > 0.05$). **, $P < 0.01$.

localization of β -catenin in FGF8 cultures might be part of a one-step differentiation process in these cultures.

Discussion

FGFs are known to play a role in early development and organogenesis. FGF4 has also been recognized as one of the genes that is one of the identifying characteristics of the embryonic stem cells and is also required in propagating these cells in culture.^{40,41} Specifically FGF1, FGF2, and FGF8 have been shown to play a role in liver development.^{1,2} However FGF knockouts have not been informative and redundant signaling is considered one of the contributing factors. FGF and Wnt pathway interactions have also been defined previously especially in the limb development.⁴²

The present study was aimed at examining the roles of FGFs, specifically FGF1, FGF4, and FGF8 during early liver development. At the E9.5 to E10 stage, the resident cells of the liver are the hepatic progenitors that differentiate into the hepatic or biliary cells later during development. These cells are positive for c-kit (stem cell marker), α -FP, albumin, and CK-19, that on their own are markers for immature hepatocytes, mature hepatocytes, and biliary epithelial cells, respectively. How liver development proceeds from this point and how these cells commit to either cell type is unclear, especially at the level of molecular cues that further the process of differentiation. *Ex vivo* embryonic liver cultures allow us to manipulate and examine effects of various growth factors by studying their effect on cell proliferation, apoptosis, histology, tissue organization, and cell differentiation and to specifically address these from the perspective of their effect on hepatic progenitors because these cells comprise most of the primitive liver at this stage of liver development. Once cultured in standard conditions in the presence of 10% serum for 72 hours, the livers resemble the E13 to E14 stage of normal liver development.

Our initial aim was to examine the presence of FGF during the early stages of liver development. Immunohistochemistry for activated FGFR on the E10 to E12 livers revealed its presence in several resident cells. We also demonstrate the presence of activated FGFR in the nuclei of several resident cells especially at E10 that was maintained until E12 stage followed by a drastic decrease that was also confirmed by Western blot analysis. Nuclear localization of activated FGFR has been an indicator of active FGF signaling.^{23–25} Thus our analysis provides evidence that the FGF pathway is active during early liver development in the postinductive and morphogenic stages of hepatic development suggesting a physiological role of this pathway during development. It has been shown that FGFs have also been shown to promote self-renewal of stem cells in pluripotent stem cells such as embryonic stem cells and also in multipotent stem cells such as neural progenitors.^{43,44} Our results showing activated FGFR during early liver development might suggest a role of this pathway in hepatic progenitor renewal along similar lines.

The effect of FGF1 and FGF4 were overall similar on the observed tissue histology. They promoted the cell arrangement in sheet-like structures and did promote ductal differentiation to the same extent as 10% serum. In the presence of FGF8, no duct formation was observed and the cells arranged in sheets only. The apoptosis data reveals only marginal effect of FGF1 and FGF4. In contrast FGF8 significantly promoted cell survival in the embryonic liver culture. This observation suggests that the decrease in the ductal structures (in the presence of FGF1/4) or their complete absence (FGF8) was not because of any increase in cell death of the ductal cells and may be directly augmenting the sheet-like architecture. Cell proliferation of resident cells increased significantly in the presence of any of the tested FGFs indicating an overall morphogenetic effect on the cells in embryonic liver cultures. The balance between these two cellular events of apoptosis and proliferation is crucial for physiological growth in multiple organs and tissues including the hepatic growth during early liver development.⁴⁵ Moreover aberrations in their balance result in pathologies ranging from hyperplasia to neoplasia.⁴⁶ Our data suggests that FGF signaling is one such pathway that is dictating a balance of these events as they affect apoptosis (especially FGF8) as well as promote resident cell proliferation in the *in vitro* organ cultures, suggesting an overall hepatotrophic effect.

Another important observation was a dramatic increase in the number of hepatic progenitors in the culture containing FGF1 or FGF4 as evident by the expansion of the progenitor cells. Whereas the total number of such cells after 72 hours in cultures is ~25% in a normal embryonic liver culture, in the presence of FGF1 or FGF4 this number was consistently increased to more than 70%. The progenitor status can be concluded by a significant cell population being positive for c-kit, α -FP, albumin, and CK19 as seen by consecutive section staining for various markers. This demonstrates the capability of these factors to enrich the organ cultures for such hepatic progenitors by promoting their proliferation, decreasing apoptosis, and at the same time maintaining their dedifferentiated state. This leads us to believe that FGFs may play an important role in hepatic progenitor renewal. This observation is also strengthened by an accompanying increase in the β -catenin in the cytoplasm and nuclei of the resident cells in response to FGF1 and FGF4 that could be one of the contributing mechanisms for hepatic progenitor enrichment and expansion. These findings again suggest that the FGF pathway is acting at the postinductive stage of hepatic development and is playing a role in maintenance and propagation of hepatic progenitors during morphogenesis as well.¹

FGF8 treatment induced a more dramatic phenotype. Eighty to ninety percent of the entire organ culture was constituted by c-kit-, α -FP-, and albumin-positive hepatic progenitors. However, these cells lacked CK-19 positivity precluding them from being true bipotential progenitors. Also, failure of formation of any ductal structures is also supporting this observation. This suggests that FGF8 does induce progenitor enrichment, but in addition, also induces a one step-differentiation of these progenitors

toward hepatocytic lineage. Thus FGF8 enriches the culture for unipotential hepatocytic progenitors by promoting cell proliferation and survival. We again observe a significantly higher β -catenin staining and its nuclear localization in response to FGF8. Also, a substantially higher number of resident cells demonstrates membranous localization of β -catenin that might be indicative of a more differentiated cell type, reflecting a contributory mechanism for the one-step hepatocytic differentiation of the bipotential hepatic progenitors. At the same time, the nuclear and cytoplasmic β -catenin might be more crucial in regulating cell proliferation, apoptosis, and eventually in maintenance and enrichment for the c-kit and α -FP-positive progenitor cells. These observations pertaining to the effect of FGF8 on the enrichment and differentiation are crucial because the molecular mechanisms that dictate bifurcation of the differentiation of the bipotential hepatic precursors are primarily obscure. Although, more studies will be required to positively address this role, we feel that FGF8 might be one of the crucial factors assisting the decision-making in the *ex vivo* embryonic livers.

These results from the use of FGFs on *ex vivo* liver development can be applied to hepatic stem cell biology and tissue culture applications. Hepatic tissue engineering for cell therapy or bioreactor applications is being investigated in liver failure for short-term or long-term relief because of an obvious paucity of donor organs for orthotopic liver transplantation. However this area faces several challenges including the absence of a suitable cell source.^{47–49} Use of a hepatic progenitor cell population is an attractive alternative because these cells possess not only a higher plasticity and survival but also possess an ability to act as a source of their own kind. Having such cells in a hepatic bioreactor or for cell therapy applications will thus provide a constant source of differentiated and dedifferentiated cells under appropriate growth factor conditions. The positive impact of FGF1, FGF4, and FGF8 on hepatic progenitor pool expansion might find application in cell therapies, hepatic tissue engineering, and artificial liver devices.

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