Physiological Functions and Underlying Mechanisms of Fibroblast Growth Factor (FGF) Family Members: Recent Findings and Implications for Their Pharmacological Application

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1. INTRODUCTION

The fibroblast growth factor (FGF) family is a group of multifunctional signaling molecules that act very differently from what their name implies. In mammals, the FGF family is comprised of 22 structurally related proteins that have a wide variety of functions contributing to organogenesis, tissue remodeling, nervous system control, angiogenesis and regulation of metabolism. Although structurally related, FGFs exhibit diverse modes of action, mechanisms of secretion and ultimate biological consequences. The proteins have therefore been further grouped into several subfamilies, each sharing both genetic and functional similarity. These are the FGF1 subfamily (FGF1 and FGF2), FGF4 subfamily (FGF4, FGF5, FGF6), FGF7 subfamily (FGF7, FGF10 and FGF22), FGF8 subfamily (FGF8, FGF17 and FGF18), FGF9 subfamily (FGF9, FGF16 and FGF20), FGF19 subfamily (FGF19, FGF21 and FGF23), and FGF homologous factor (FGF) subfamily (FGF11 (FHF3), FGF12 (FHF1), FGF13 (FHF2), and FGF14 (FHF4)).

Standing out among these is the FHF subfamily, which, unlike the others, it is comprised of intracellular proteins acting on intracellular targets without activating a cell surface, transmembrane receptor tyrosine kinase. Thus although FHFs appear to be important for the regulation of neural cells and various stem cells, they will not be discussed further in this review article. FGF members belonging to the FGF1, FGF4, FGF7 and FGF8 subfamilies exert their activities mainly via paracrine and/or autocrine modes of action by activating one or more cell surface receptor tyrosine kinases. These proteins regulate the growth, differentiation, survival and other functions of nearby cells. For receptor activation by these so-called canonical FGFs, the presence of heparan sulfate/heparin together with the FGF ligand and the FGF receptor (FGFR) is necessary. By contrast, the FGF19 subfamily (also called endocrine FGF or eFGF) act as hormones in an endocrine mode to regulate various physiological functions in distant cells. FGF19 subfamily members also transduce their physiological signal through FGFRs and require the presence of a co-receptor, in this case one of the Klotho family of proteins (i.e., αKlotho or βKlotho).

This review summarizes our recent studies of the FGF system from mechanistic, physiological, and application-oriented viewpoints.

2. MAKING PRE-EXISTING RECEPTORS SPECIFIC TO NEWCOMERS: RECRUITMENT OF CO-RECEPTORS AND GLYCOSAMINOGLYCANS FOR SPECIFIC AND SENSITIVE SIGNALING OF ENDOCRINE FGFs

It is thought that at some point during evolution in animal kingdom, the hormonal FGFs (i.e., FGF19, FGF21 and FGF23 in humans) emerged as regulators of metabolism. By then, there existed FGFRs encoded by four genes whose translation products have been classified into seven major protein forms, including FGFR1c (FGFR1IIIC), FGFR1b (FGFR1IIIb), FGFR2c (FGFR2IIIc), FGFR2b (FGFR2IIIb), FGFR3c (FGFR3IIIC), FGFR3b (FGFR3IIIb) and FGFR4. These receptors, which are abundantly and widely expressed, had been used as cognate receptors for pre-existing FGFs, including members of the FGF1, FGF4, FGF7 and FGF9 subfamilies. We hypothesize that because the FGF19 subfamily evolved to act as endocrine factors that require specific and sensitive recognition by target cells distant from their expressing, Klotho family proteins were recruited to make up a specific recognition system while using the pre-existing FGFRs for signal transduction.

Fgf21 was originally identified as a new FGF family gene in 2000 using homology-based polymerase chain reaction (PCR). Its biological significance remained unknown until it was found that FGF21 increased expression of glucose transporter (GLUT)-1 mRNA and stimulated glucose incorporation...
in adipocytes, indicating the new FGF to be a glucose regulator.\(^5\) Pharmacological administration of FGF21 to diabetic rodents revealed that it could mediate a reduction in blood glucose and triglycerides to near normal levels. Moreover, transgenic mice overexpressing FGF21 were resistant to diet-induced obesity. FGF21 was also shown to regulate ketogenesis and lipolysis.\(^6,7\) The signaling pathway via which FGF21 acts remained unclear, however.

We confirmed that FGF21 increases the expression of GLUT1 mRNA in 3T3-L1 adipocytes but not in undifferentiated 3T3-L1 fibroblasts. However, contrary to our expectation that a difference in FGFR levels underlies the difference in the sensitivity of 3T3-L1 adipocytes and fibroblasts to FGF21, 3T3-L1 fibroblasts expressed both FGFR1 and FGFR2 mRNA, and the levels of both gradually declined as the cells differentiated into adipocytes. These data suggest that the expression level of FGFR1 or FGFR2 cannot, by itself, account for the unresponsiveness of 3T3-L1 fibroblasts to FGF21.

Because the senescence-related molecule Klotho (also called \(\alpha Klotho\)) had been reported to function as a co-receptor for FGF23,\(^8,9\) we then assessed expression of Klotho mRNA in 3T3-L1 cells, but failed to detect it at any time before or during the adipogenic differentiation. The absence of Klotho from the differentiated 3T3-L1 adipocytes indicated that another molecule likely functions as a co-receptor for FGF21 in 3T3-L1 cells. We therefore examined the mRNA expression of another member of the same protein family, \(\beta Klotho\), which exhibits 41.2% amino acid identity to Klotho.\(^10\) We found that \(\beta Klotho\) mRNA was undetectable in undifferentiated 3T3-L1 fibroblasts, but its expression dramatically increased 6–8 d after the induction of adipogenic differentiation. Then using a BaF3 cell proliferation assay system, we tested whether \(\beta Klotho\) could serve as a co-receptor for FGF21. The BaF3 cell system was initially established to determine the receptor specificity of canonical FGF signaling. BaF3 cells are a pro-B lymphoma line that does not endogenously express detectable levels of any of the components suggested for FGF21 recognition/signaling, but does harbor the intracellular mediators necessary to transduce a mitogenic signal from FGFRs. We prepared BaF3 transfectants stably expressing one of the four human (h) tyrosine kinase FGFR subtypes (hFGFR1c, hFGFR2c, hFGFR3c or hFGFR4) with or without \(\beta Klotho\).

In addition, the intracellular kinase domains for all these constructs were swapped with that of hFGFR1c to enable direct comparison of the receptor activation induced by ligand binding to the respective FGFR extracellular domains. We found that FGF21 stimulated proliferation of BaF3 transfectants expressing FGFR1c only when the cells co-expressed \(\beta Klotho\). FGF21 activity was detected even in the absence of heparin, a crucial co-factor necessary for most FGFs to activate their cognate receptors, though heparin did enhance FGF21 activity. These results suggest that \(\beta Klotho\) functions as a co-receptor for FGF21 in BaF3 cells expressing FGFR1c.

We next determined which FGFRs, in addition to FGFR1c, would transduce the FGF21 signal in cooperation with \(\beta Klotho\). Among the BaF3 transfectants, only those expressing FGFR3c or FGFR1c plus \(\beta Klotho\) proliferated in response to stimulation with FGF21, and this mitogenic activity was increased approximately 2-fold by the presence of heparin. We also found that FGF21 induced phosphorylation of two downstream mediators of the FGF signal, FRα2α and mitogen-activated protein kinase (MAPK) (Erk1/2), in FGFR1c/\(\beta Klotho\)/BaF3 and FGFR3c/\(\beta Klotho\)/BaF3 cells, but not in FGFR4/\(\beta Klotho\)/BaF3 cells.

Earlier studies with FGF23 revealed that the Klotho-FGFR1c complex constitutes the functional FGF23 receptor.\(^5,9\) Using our BaF3 system, we confirmed that FGF23 induced proliferation of only Klotho/FGFR1c-expressing cells. Conversely, \(\beta Klotho/FGFR1c\)-expressing cells, but not Klotho/FGFR1c-expressing cells, proliferated in response to FGF23 stimulation. Thus \(\beta Klotho\) and Klotho appear to selectively endow FGFR1c with sensitivity to FGF21 and FGF23, respectively (Fig. 1).

To confirm our interpretation that \(\beta Klotho\) acts as an FGFR co-receptor, we evaluated the activation of FRα2α and MAPK, in \(\beta Klotho\)-transfected, undifferentiated 3T3-L1 fibroblasts. Phosphorylation of FRα2α and MAPK was detected in cells exposed to FGF21; moreover, the phosphorylation was inhibited by a specific FGFR tyrosine kinase inhibitor, which confirmed that FGF21 signaling was via FGF21 activation. Furthermore, 3T3-L1 fibroblasts expressing \(\beta Klotho\) alone were able to express GLUT1 mRNA in response to FGF21. Our finding was soon confirmed by another research group using different (L6 myoblast) transfectants overexpressing \(\beta Klotho\).

![Fig. 1. Combination of a FGFR Subtype and Klotho Protein Determines Specificity for Endocrine FGFs, as Shown Using a BaF Transfectants System](image-url)
Relevant to its endocrine nature, we found that FGF21 does not bind to heparin in physiological saline. This feature may enable high levels of FGF21 to be sustained in the circulation under pathophysiological conditions. Similarly, FGF23 shows only weak affinity for heparin, which means that once released into the circulation from their expressing cells both FGF21 and FGF23 will have access to distant organs, where they could act in an endocrine fashion. In that context, we would expect that the crucial requirement for βKlotho as a coreceptor for FGF21 or Klotho for FGF23 is a key determinant of their respective target tissues when those tissues express FGFR subtypes that respond to both factors (e.g., FGFR1c) (Fig. 1).

Given that pharmacologically administered FGF21 stimulates glucose uptake by adipocytes and lowers serum glucose and triglyceride levels, FGF21 and βKlotho may be useful targets for development of therapeutic agents for the treatment of diabetes. This line of research and development are being conducted at many pharmaceutical entities around the world.

FGF19 is another member of endocrine FGF subfamily. Secreted from intestine, human FGF19 controls bile acid synthesis in the liver. Its expression in intestine and secretion into the circulation is induced by bile acid, and after reaching the liver via the portal vein, it suppresses Cyp7A1, a key liver enzyme involved in bile acid biosynthesis, thereby closing a feedback loop regulating bile acid homeostasis. FGF19 also reportedly contributes to the regulation of blood glucose levels, along with other metabolic regulators, including FGF21 and insulin. Earlier studies suggested that FGF19 at 10–100 nm concentrations signals through FGF4 in the presence of βKlotho, but its activity and receptor specificity at physiological concentrations (pM levels) remained unclear.

To precisely investigate the molecular mechanism by which FGF19 induces cellular signaling, we again used a modified BaF3 cell system. We initially examined the ability of FGF19 at nanomolar levels, the concentration range used in earlier studies, to induce DNA synthesis. We found that nanomolar FGF19 was able to induce DNA synthesis only when hFGFR4 was co-expressed with βKlotho (Fig. 1). Moreover, although the combination of hFGFR4 and βKlotho was sufficient to elicit a partial response to FGF19, the response was enhanced by heparan sulfate (HS) or chondroitin sulfate (CS)-B (Fig. 2). The three other receptors tested, hFGFR1c, hFGFR2c and hFGFR3c, were not activated by FGF19, even when co-expressed with βKlotho.

In contrast to HS and CS, heparin enabled FGF19 (at 0.46 nm and higher) to signal through hFGFR1c, hFGFR2c and hFGFR3c when co-expressed with βKlotho. In addition, heparin enabled FGF19 (at 0.46 nm and higher) to signal via hFGFR4 in the absence of βKlotho. Such βKlotho-independent signaling was not observed in the presence of HS or CS. Our findings are consistent with those of earlier studies by others, which examined the activity of FGF19 at concentrations ranging from 10 to 100 nm. However, FGF19 concentrations in the human circulation are reportedly in the subnanomolar range, most likely around 30 pm, which is 3–4 orders of magnitude lower than the concentrations tested previously.

We found that activation of hFGFR4 using FGF19 at concentrations ranging from 3 to 500 pm was strongly dependent on the presence of sGAGs and on the co-expression of βKlotho. In the presence of HS or heparin, 500 pm FGF19 alone did not activate hFGFR4, but the receptor was maximally activated when co-expressed with βKlotho. With this system, evoked DNA synthesis could be detected at FGF19 concentrations as low as 3 pm in the presence of heparin, HS, CS-B or CS-E; By contrast, CS-A, CS-C and CS-D were not sufficient to mediate the effect. Because liver is the FGF19 target organ, we prepared sGAGs from liver and found that heparin and the hepatic sGAGs were equally potent enhancers of picomolar FGF19 signaling via hFGFR4 co-expressed with βKlotho.

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**Fig. 2. Schematic Diagram of the Signaling Complex for Canonical and Endocrine FGFs**

Left: for canonical FGFs (i.e., FGF1, FGF4, FGF7, FGF8, FGF9 subfamily members), a FGF ligand specifically binds to a FGFR of the corresponding subtype in the presence of heparin (HP) or heparan sulfate (HS), which induces activation of the receptor dimer, thereby mediating signal transduction within the cells. Right: for endocrine FGFs (eFGFs) (i.e., FGF19, FGF21 and FGF23), βKlotho or αKlotho is recruited to each ligand-receptor pair to form the active signaling complex mediating signal transduction. The importance of HP/HS varies depending on the eFGF.
Like FGF21, FGF19 shows very low affinity for heparin\(^{11,23}\), which is presumably necessary for its endocrine mode of action. In addition, the requirement for βKlotho as a co-receptor and the strong positive regulation of FGF19/hFGFR4 signaling by HS/CS establishes a highly specific and sensitive recognition system.

In contrast to the effects of picomolar FGF19, the results obtained with nanomolar FGF19 in the presence of heparin indicate that the high concentration severely reduced the target specificity of FGF19. In that context, it is intriguing that FGF19 expression is elevated in the cancerous tissues of some patients with lung or colon cancer,\(^{24}\) and some cancers also show upregulation of FGFR4.\(^{24}\) Whether FGF19 signaling via hFGFR4 in the presence of endogenous heparin/sGAGs contributes to the pathology of these diseases remains unknown, but our findings shed new light on the mechanisms underlying the physiological and pathological activities of FGF19.

3. REGULATING THE QUIESCENT STATE OF STEM CELLS: FGF18 REGULATES THE RESTING PHASE OF THE HAIR GROWTH CYCLE\(^{25,26}\)

Hair shafts repeatedly elongate and then shed from the same mini-organs called hair follicles throughout, or near so, during the life span of animals with hair. The hair growth cycle can thus be regarded as the cyclic morphogenesis of hair follicles. Hair follicles are appendages of the skin and are composed of multiple cell types derived from both epithelial and mesodermal lineages. Each hair growth cycle is composed of three phases: anagen (the growth or morphogenesis phase), catagen (the regression or apoptosis phase) and telogen (the rest or quiescent phase) in that order. Studies by ourselves and others indicate that products of Fgf5 gene are important regulators of catagen induction at the end of anagen.\(^{27,28}\) (Fig. 3). We showed that FGF5 inhibits activation of dermal papilla cells\(^{29}\) and that the level of FGF5 mRNA expression is much higher at anagen VI (the last stage of anagen) than in telogen, which is suggestive of its ability to induce catagen.\(^{25}\)

During our study of FGF5 and the hair cycle, we noted that many other FGFs are also expressed at high levels in skin, hair follicles and other skin appendages.\(^{25,30}\) Therefore, to gain insight into the involvement of FGFs in hair follicle regulation, we quantified the mRNA expression of all 22 FGF family members and the four FGFRs in adult mouse full-thickness skin at various stages of the hair growth cycle. We found that expression of several FGFs varied throughout the hair growth cycle: mRNA expression of FGF18 and FGF13 peaked at telogen, FGF7 and FGF10 at anagen V, and FGF5 and FGF22 at anagen V.\(^{25}\)

Because telogen is the resting phase of the hair growth cycle, during which hair follicle (epidermal) stem cells are quiescent, we were particularly interested in the experimental result showing that FGF18 expression is at its highest during this phase. We therefore further investigated the possible association between strong FGF18 expression and the telogen phase of the hair cycle.\(^{26}\) C3H/HeN mice develop coat hairs approximately one week after birth as the result of hair follicle morphogenesis.\(^{31}\) We found that FGF18 mRNA was expressed only weakly during hair follicle morphogenesis, but the FGF18 mRNA was strongly expressed once the follicles entered morphogenesis catagen and telogen. The expression then declined in the subsequent anagen of the first hair cycle, and increased again in the following catagen and telogen. We were able to induce synchronized anagen by plucking the club hairs, which elicited an immediate decline in FGF18 mRNA. Levels of FGF18 mRNA then remained low throughout anagen but increased during catagen and telogen. Expression of FGF18 mRNA peaked on day 92 and was sustained at a high level for as long as telogen continued in the mice (we observed for up to 188 d). Thus strong FGF18 transcription is strictly associated with catagen and the entire telogen phase.

We also showed that FGF18 protein is associated with cells in the telogen bulge region, a hair stem cell niche, though some epidermal basal layer cells also weakly express FGF18. The FGF18-positive cells are also positive for keratin 15 or CD34, two proteins preferentially expressed in the bulge stem cells,\(^{32}\) which confirms that FGF18 is expressed within the hair stem cell niche.

The cognate receptors for FGF18 are FGFR3c and FGFR4.\(^{33}\) We found that high levels of FGFR3 mRNA are expressed in both telogen and anagen skin.\(^{25}\) Immunostaining for FGFR3 revealed that it is strongly expressed in telogen bulge cells, outer root sheath cells, basal-layer epidermal cells and dermal papilla cells.\(^{26}\) Within bulge cells, FGFR3 was primarily detected in the nuclear/perinuclear region and, to a lesser degree, in the cytosol, which we confirmed using two validated antibodies and two different visualization methods: immunohistochemistry\(^{26}\) and immunofluorescence confocal microscopy.\(^{26}\) Within basal layer cells and outer root sheath cells, FGFR3 is detected primarily in the cytosol and, to a lesser degree, in the nuclear/perinuclear region.\(^{26}\) Although the biological significance of the nuclear/perinuclear localization of FGFR3 is not well understood, it has been suggested that the receptor’s nuclear localization plays a role in its signaling.

To gain insight into the physiological function of FGF18 in hair follicles, we performed a gene knockout study. Because conventional Fgf18 knockout was lethal,\(^{34}\) we generated Fgf18 conditional knockout (cKO) mice in which Cre recombinase was used to selectively knock out the Fgf18 allele containing exon 3, resulting in depletion of the mature FGF18 protein exclusively in keratin 5-positive cells.\(^{26}\) The Fgf18 cKO mice

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**Fig. 3. Proposed Model of FGF Signaling in the Regulation of the Hair Growth Cycle**

In wild-type mouse hair follicles, FGF18 signaling maintains the telogen phase and prevents transition to the anagen phase. FGF5 signaling is strong in the last stage of anagen and induces transition to the catagen phase. FGF7, FGF10 and probably FGF1 and FGF2 promote anagen.\(^{29,30}\)
were healthy and fertile. Assessed based on the presence of the exon 3-coded sequence, the levels of FGF18 mRNA in the telogen skin in these mice were less than 2% of the levels in wild-type mice. We also confirmed that mature FGF18 protein is diminished in the hair bulbs of Fgf18 cKO mice.

When the mice were 32 d old, the dorsal hair follicles of both the Fgf18 cKO and wild-type mice were in the first physiological anagen (blackish skin). At 37 d the neck regions of both mice entered telogen (pinkish skin), and at 40 d all of the dorsal follicles were in telogen. In the Fgf18 cKO mice, the next anagen quickly started on day 47 (bluish skin). This change in the hair cycle phase was verified by histochimical examination of skin sections from individual mice. By contrast, telogen continued for a longer period in the heterozygous cKO littermates, and for a much longer period in the wild-type mice.

The dorsal hair growth waves seen in Fgf18 cKO mice smoothly progressed posteriorly from the neck region, making it possible to describe the general trend of the hair cycle phase transition. We analyzed multiple Fgf18 cKO mice and characterized the transition of the hair cycle phase (anagen or telogen for simplicity) of the dorsal hair follicles histologically (for mice younger than 30 d old) or based on the skin color, as well as the weekly hair growth in the region located about 1 cm posterior to the ear-to-ear line. The summarized results (Fig. 4) indicate that whereas the morphogenesis and the first hair cycle in the Fgf18 cKO mice took place at approximately the same time as in wild-type mice, each telogen thereafter lasted only a week or so, making the average duration of each hair cycle, including telogen, only about 3 weeks in the Fgf18 cKO mice. The finding that telogen is longer in the heterozygous littermates than in homozygous cKO mice confirms that the loss of mature FGF18 was responsible for the telogen shortening, and suggests the effect may be dose-dependent.

In wild type mice, the telogen following the first hair cycle generally lasted 3–5 weeks or more, and the onset time and location of the next anagen varied among individual mice and depended on the hair cycle domains within the dorsal skin. As a result, the second natural hair cycle was rarely complete before the age of 90 d (Fig. 4). In addition, hair follicle growth during anagen was strongly suppressed by local delivery of FGF18 in wild-type mice. All these results demonstrate that epithelial FGF18 signaling and its reduction in the milieu of hair stem cells are crucial for the maintenance of the resting and growth phases, respectively (Fig. 3).

Notably, in older Fgf18 cKO mice, after a rapid succession of hair cycles, follicles in the same phase were aligned so that they formed a stripe, and the number of stripes increased with the mouse’s age. We found that the duration of each hair cycle in these aged mice was 3 weeks in the anterior region, but was about 4 weeks in the posterior region. This difference in hair cycle duration may account for the compacted distance between the stripes in aged Fgf18 cKO mice.

It was recently reported that FGF18 and bone morphogenetic protein (BMP)-6 are expressed in isolated telogen bulge cells, and that other BMPs are expressed during early telogen in interfollicular dermal cells. This suggests BMPs are able to slow keratinocyte growth without inducing terminal differentiation, and it has been hypothesized that both FGF18 and BMP6 place bulge stem cells in a quiescent state. The phenotype of our Fgf18 cKO mice not only supports this hypothesis but also demonstrates that FGF18 imposes a strong natural inhibitory signal on hair follicle stem cells in vivo. BMPs are reportedly essential for hair follicle morphogenesis and hair shaft differentiation, while the absence of FGF18 does not result in abnormal hair follicle morphology. Thus the FGF18 and BMP signals control telogen and hair growth in temporally and spatially different ways, though the proteins share several characteristics as inhibitory factors.

FGF18 acts to maintain telogen and inhibit anagen, whereas FGF7 and FGF10 act to promote anagen (Fig. 3). This likely reflects, at least in part, their different receptors. FGF18 activates FGFR3c and FGFR4, while both FGF7 and FGF10 activate FGFR2b, an epithelium-specific FGFR subtype. Signaling via FGFR2b mediates epithelial cell proliferation, while signaling via FGFR3c and FGFR4 induces a complex phenotype that is sometimes inhibitory. For example, FGFR3 signaling suppresses chondrocyte proliferation and hypertrophic differentiation during late embryonic and postnatal skeletal growth. This probably explains why the biological effects of FGF18 and FGF7/FGF10 differ so significantly.

4. APPLICATION OF FGFs FOR THERAPEUTIC PURPOSES: A STABLE FGF CHIMERIC PROTEIN SUITABLE FOR RADIODISPROTECTION

Because FGFs exert strong biological effects in numerous pathophysiological contexts, they are thought to be promising agents for various pharmaceutical applications. However, the instability of their wild-type forms generally makes their direct use as drugs problematical. In particular, the structural instability of wild-type FGF1 and its dependence on exogenous heparin for optimal activity markedly diminishes its potential utility as a therapeutic agent. FGF2 is more stable.
than FGF1, but it does not activate all FGFR subtypes. We evaluated a FGF1:FGF2 chimeric protein we created previously and designated FGFC (FGF-C1211)\(^{43}\) for its receptor affinity, absolute heparin-dependence, stability and potential clinical applicability. The structure of FGFC is illustrated in Fig. 5. The short sequence (shown in black) was derived from FGF2, while the remainder of the molecule was from FGF1a, a fully active form of FGF1 purified from brain. FGFC and FGF1 exhibit approximately the same binding affinity for heparin-Sepharose.\(^{42}\)

FGF1 is known to activate all seven FGFR subtypes, while FGF2 shows somewhat greater receptor specificity, activating only FGFR1c, FGFR2c, FGFR3c, FGFR4 and, to a lesser degree, FGFR1b.\(^{43}\) Using a BaF3 cell-based assay system overexpressing each FGFR subtype, we found that, like FGF1, FGFC activates the seven FGFR subtypes in the presence of heparin. Furthermore, the biological activity of FGFC is equal to or slightly superior to that of FGF1. By contrast, FGF2 was less able to induce FGFR2b/BaF3 cell proliferation, as was described previously.\(^{44}\) Thus, although FGFC is composed of partial sequences from both FGF1 and FGF2,\(^{44}\) its receptor specificity resembles that of FGF1.\(^{42}\) FGFC also stimulated keratinocyte proliferation much more strongly than FGF2, as would be expected from its ability to activate FGFR2b.\(^{42}\)

Importantly, FGFC showed greater structural stability than wild-type FGF1 or FGF2.\(^{42}\) Polypeptide growth factors generally lose activity during storage, which is in part attributable to degradation of the active three-dimensional fold of the protein. We therefore compared the time-dependent changes in the emission spectra of FGFC and FGF1.\(^{56-58}\) Upon unfolding, 353-nm emission from the single tryptophan\(^{46}\) residue (Trp-107 of FGF1, Trp-122 of FGFC) is seen. When FGF1 and FGFC were incubated at 25°C in phosphate buffer containing 0.7 M guanidinium chloride, FGF1 showed a marked increase in emission at 353 nm, whereas FGFC showed a much smaller 353-nm signal, indicating that a greater proportion of FGF1 than FGFC molecules had unfolded, exposing their tryptophan. When these proteins were incubated first at 70°C and then at 25°C, FGF1 and FGFC showed similar spectra with a distinct peak at 353 nm, indicating that both had unfolded.

We also found that FGFC showed greater resistance to trypsin digestion than either parent protein.\(^{42}\) Upon application of a polypeptide growth factor to a wound or other tissue damage, it is susceptible to degradation by proteases expressed in both the tissue and the infiltrating inflammatory cells. To examine their ability to serve as an exogenously applied therapeutic agent, we compared the susceptibility of FGFC, FGF1 and FGF2 to trypsin digestion in vitro. FGFC exhibited greater resistance to trypsinization than FGF1 or FGF2. Whereas 69% of FGFC remained intact after digestion with 0.01% trypsin, only 36% of FGF2 and no FGF1 remained intact.

We then compared the stability of FGFC during storage at 37°C in solution. The FGFs were incubated for various periods at 37°C in phosphate buffered saline (PBS) containing 10% bovine serum albumin (BSA), after which their remaining biological activity was assayed. In addition, the amount of each FGF remaining in the samples was determined. Whereas the activity of wild-type FGF1 rapidly declined, with only 1% of the initial activity remaining after 6 h, the activity of FGFC remained nearly unchanged for 6 h. FGFC was also more stable than FGF2.\(^{42}\) Loss of the soluble form of active polypeptide growth factors during storage can also occur as a result of adsorption onto the surface of the storage vessel and/or as a result of precipitation of an aggregate. We found that 54% of the original concentration of FGFC was retained after storage for 48 h, whereas only 17% of FGF1 remained in solution at the same time.\(^{42}\)

FGF2 and FGF7 (or KGF, keratinocyte growth factor) are currently approved by the Pharmaceuticals and Medical Devices Agency and Food and Drug Administration in Japan and the United States, respectively, and have been used in clinical settings for several years, the former for treatment of skin ulcers and the latter for treatment of mucositis.\(^{51}\) However, FGF2 is not active with FGFR2b, and FGF7 is active only with FGFR2b, a receptor subtype specific for epithelial cells. In many cases tissue healing after an injury involves proliferation of cells of both mesenchymal and epithelial lineage, so an ability to stimulate epithelial cells is important for a therapeutic agent.\(^{52}\) From the viewpoint of clinical application, it is noteworthy that FGFC activates all target cells previously shown to be activated by FGF2 or FGF7. This ability of FGFC to activate all FGFR subtypes, including FGFR2b, is a great advantage over FGF2 and FGF7. We also found that FGFC has a much greater ability to stimulate proliferation of MK2 keratinocytes in the absence of heparin than FGF1, and that FGF2 has comparatively little ability to stimulate MK2 cell proliferation.\(^{42}\)

The advantages of using FGFC as a therapeutic agent may become most apparent in life-threatening situations.\(^{42,43}\) Among the potential clinical applications of FGFs, protection against radiation-induced damage is of particular interest.\(^{53}\) To date, the abilities of FGF1,\(^{53,54}\) FGF2,\(^{55,56}\) FGF7,\(^{57}\) FGF10\(^{57}\) and FGF20\(^{58}\) to protect various organs against radiation-induced damage have been evaluated. FGF7 was approved for use in the prevention and treatment of oral mucositis in cancer patients receiving radiochemotherapy. We previously showed that among the wild-type FGFs examined (FGF1, FGF7 and FGF10), FGF1 exerts the strongest protective effect against radiation-induced injury of small intestine crypts, the intestinal epithelial stem cell niche.\(^{59,60}\) Strong gamma or X-ray radiation causes death of the irradiated animals within days, mainly due to failure of the regeneration of the intestinal epithelial cells. Using this assay system, we assessed the protective effects of FGFC and found that when 10 μg of FGFC or FGF1 were intraperitoneally administered to BALB/c mice 24 h before administration of 10 Gy of whole body γ-irradiation, the crypt survival after 3.5 d was significantly greater than in control mice receiving saline. Furthermore, the protective effect of FGFC was most apparent when administered 24 h before irradiation. Therefore, our experiments suggest that FGFC may have potential for the treatment of radiation-induced injury when administered before irradiation.
Fig. 6. FGFC Improves Survival among Mice Exposed to High-Dose Irradiation

Simple saline or saline containing 30 µg of FGFC were intraperitoneally administered to BALB/c mice 24 h before 8 Gy total body γ-irradiation. Each group was composed of eight mice, and the numbers of surviving mice per group was determined every day after irradiation.

Acknowledgments This article reviews our recent publications, as cited in the text. A few of our unpublished results are also described. I would like to thank Drs. Masashi Suzuki and Masahiro Asada at the National Institute of Advanced Industrial Science and Technology (AIST) and Fumiko Nakayama at the National Institute of Radiological Sciences (NIRS), Japan, for major contributions to the results described in this review.

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