FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 43, No. 4, 2005
pp. 203-208

Review article

Basic fibroblast growth factor and its receptors in human embryonic stem cells

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Abstract: Human embryonic stem cells (hESCs) are pluripotent stem cells with long-lasting capacity to self-renew and differentiate into various cell types of endodermal, ectodermal or mesodermal origin. Unlike mouse ESCs (mESCs), which can be maintained in an undifferentiated state simply by adding leukemia inhibitory factor (LIF) into the culture medium, hESCs are notorious for the sustained willingness to differentiate and not yet clearly defined signaling pathways that are crucial for their "stemness". Presently, our knowledge involves only limited number of growth factor signaling pathways that appear to be biologically relevant for stem cell functions *in vitro*. These include BMP, TGFβ, Wnt, and FGF signaling pathway. The purpose of this review is to summarize recent data on the expression of FGFs and their receptors in hESCs, and critically evaluate the potential effects of FGF signals for their undifferentiated growth and/or differentiation in context with our current understanding of FGF/FGFR biology.

Key words: Basic FGF - Human embryonic stem cells - Self-renewal - Differentiation

FGF family and FGFR signaling pathway

Fibroblast growth factors (FGFs) constitute a large family of signaling polypeptides that are expressed in various cell types from early embryos to adults. Since the first discovery of FGF in 1974 [14], 22 distinct members of the FGF family have been described [reviewed in 22]. FGF family members range from 16 to 34 kDa and share 13-71% amino acid identity. Across vertebrates, FGFs are highly conserved and promiscuously bind to four high-affinity transmembrane receptor tyrosine kinases, denoted as FGFR1, 2, 3, and 4. In addition to high-affinity FGFRs, FGFs interact also with extracellular matrix-derived or membrane-bound heparan sulfate proteoglycans or their fragments that either potentiate or inhibit FGF activity [11, 16]. To date, four known members of the signaling FGFR family have a similar structure that is comprised of hydrophobic leader sequence, two or three immunoglobulin-like domains, acidic box, short transmembrane region, and two tyrosine kinase domains. Common hallmark of FGFR family is that the receptor diversity is controlled by alternative splicing, resulting in the generation of multiple splice variants (Fig. 1). These splice variants of FGFRs exhibit differential ligand-binding characteristics and are expressed in differentiation- and tissue-specific manner [reviewed in 5].

During embryonic development, FGFs function in multiple biological processes including cell proliferation, differentiation, and migration [2]. In mammalian embryos, members of FGF family are expressed in many cell types, but the timing of expression varies. In addition, several FGFs are present exclusively in embryonic tissues, whereas others, including basic FGF (bFGF or FGF-2), are expressed in embryonic and adult tissues. In the adult organism, FGFs represent important homeostatic factors and play a role in response to injury and tissue repair [6]. Importantly, aberrant expression of

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Lecture presented at the Third Annual Meeting of the European Stem Cell Therapeutics Excellence Centre, October 6-9, 2005, Cracow, Poland

some FGFs may lead to malignant transformation and may contribute to the pathophysiology of cancer, *e.g.* by regulating apoptosis or neovascularization [21, 33]. As mentioned above, an important feature of FGF biology is tissue-specific alternative splicing and expression of the receptors that ensure differential action of FGFs during development. In this regard, FGFR1 plays an essential role in early development, mainly in mesoderm patterning and cell migration during gastrulation, and it is also involved in cell differentiation at several levels during early stages of organogenesis. Conversely, FGFR2 is expressed and functions later during organogenesis, and FGFR3 is involved in differentiation of oligodendrocytes and astrocytes in central nervous system, and also regulates bone formation. FGFR4 is highly expressed in the definitive endoderm and embryonic muscle plate [5].

The activation of FGFRs involves two different ligands, FGFs and heparan sulfate proteoglycans or their side chains. Cooperation between both types of ligands is essential as the binding of FGFs or heparan sulfate proteoglycans alone is insufficient for FGFR dimerization and initiation of downstream signaling. The signal cascade downstream of FGFRs involves tyrosine phosphorylation of the docking protein FRS2 followed by recruitment of several Grb2 molecules. Activated Grb2 molecules bound to FRS2 recruit the nucleotide exchange factor SOS. The formation of FRS2-Grb2-SOS complexes results in activation of the Ras-Raf-MAPK signaling pathway followed by cell response. Parallel, phosphorylated FGFRs activate also PLCγ-PKC pathway and Grb2 molecule recruits the docking protein Gab1 that activates PI3K-Akt cell survival pathway. Therefore, FGF signaling through FGFRs is mediated primarily by assembly of a multidocking protein complex and involves several layers of control [28]. The complexity of FGF signaling via FGFRs is schematized in Figure 2.

Expression of FGF and FGFR in hESCs

It is widely accepted that hESCs should be maintained on feeder layers of mouse embryonic fibroblasts or other suitable feeder cells. Also culture medium contains bFGF, in order to sustain self-renewal of hESCs and their capacity to differentiate (Fig. 3). Although it is likely that exogenous bFGF supports growth of undifferentiated hESCs [1, 34], the mechanism of bFGF action is not yet clearly established.

The first step to understanding the action of members of FGF family, including bFGF, is to determine the expression of individual components of FGF signaling pathway. As one of the most powerful tools of modern biology, large scale transcriptional profiling of undifferentiated and differentiating hESCs provides the opportunity (1) to identify genes that are involved in the mechanisms responsible for pluripotency or early differentiation events, and (2) to develop unique molecular signatures of independently-derived hESC lines. Using cDNA microarray analysis, several groups have detected elevated levels of key components of the FGF signaling pathway in undifferentiated hESCs compared with their differentiated counterparts. These include bFGF, FGF-11, and FGF-13, as well as FGFR1, FGFR2, FGFR3, and FGFR4 [9, 13, 24, 27, 29]. More recently, transcriptomes of undifferentiated hESCs and hESC-derived embryoid bodies (EBs) were compared by massively parallel signature sequencing (MPSS) that permits more complete mapping than conventional cDNA microarrays [32]. MPSS clearly shows that hESCs express substantial levels of mRNAs coding bFGF and its three high-affinity receptors, FGFR1, FGFR3, and FGFR4. In addition, hESCs express mRNA for docking protein FRS2 that represents a major target of activated FGFRs (see Figure 2). Interestingly, FGF4 that is highly expressed and functional in mESCs, is absent in hESCs.

In concert with these bulk analyses of the transcriptome, we recently determined using Western blot analysis that undifferentiated as well as differentiating hESCs are abundant in several molecular-mass isoforms of FGF-2, including those that are exportable (18 kDa low molecular-mass isoform) or localize into the nucleus (22, 22.5, and 24 kDa high molecular-mass isoforms). Interestingly, using quantitative RT-PCR we also revealed that the expression of FGFRs in undifferentiated cells follows a specific pattern, with FGFR1 being the most abundant receptor and other receptors showing lower expression in the order: FGFR3> FGFR4>FGFR2. When hESCs are induced to differentiate, expression of all four FGFRs remarkably increases; with FGFR1 being elevated about six-fold, FGFR2 about two-fold, and FGFR3 and FGFR4 about seven-fold [10]. Together, several groups including our own have clearly demonstrated that hESCs are well equipped to accept and transmit FGF signals. Taken together, it became obvious for the first time that, thanks to the expression of multiple exportable and nuclear forms of FGF, FGF signaling pathway in hESCs may function in an autocrine manner, and that the capacity to accept FGF signals may increase when cells undergo differentiation.

Effects of bFGF on growth of undifferentiated hESCs

Since their first derivation in 1998 [30], hESCs are routinely cultured on feeder layers of mouse or human fibroblasts in medium that is supplemented with bFGF at the concentration of 4-5 ng/ml. Due to expected clinical requirements, many laboratories have begun culturing hESCs in a feeder-free system. In this case they increased the concentration of bFGF to 8 ng/ml [4].

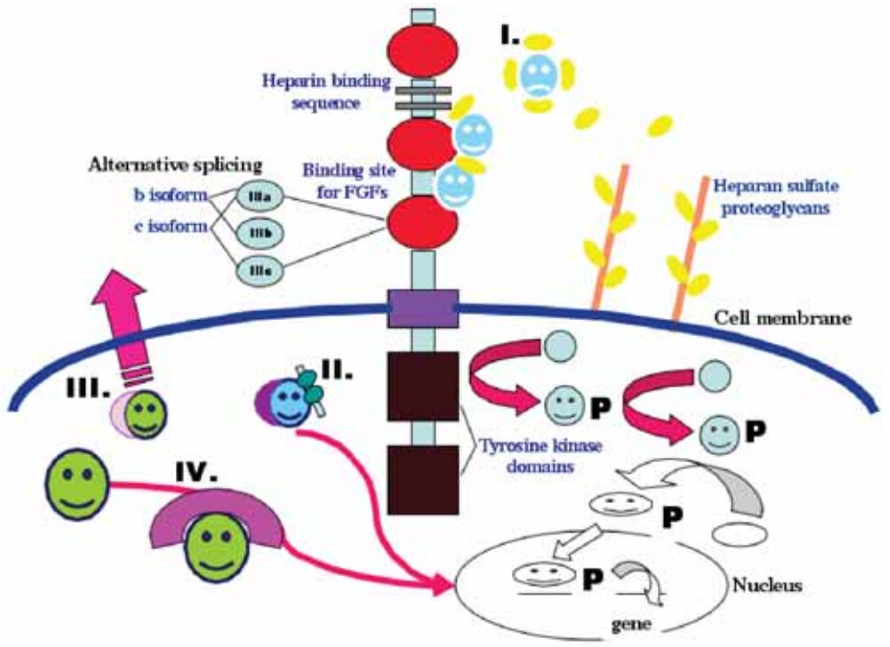

Fig. 1. Structural features of the FGFR and action of bFGF. I. Exogenous bFGF binds to its high-affinity receptors and triggers "classical" transduction pathway that consists of recruitment, assembly, and phosphorylation/activation (P) of downstream signaling proteins. In addition, FGF action could be enhanced or inhibited by heparan sulfate proteoglycans or their fragments. II. Low molecular-mass isoform of bFGF binds to its high-affinity receptors, principally FGFR1, and the resulting complex is internalized and translocated to the nucleus. Nuclear low molecular-mass bFGF may function by activating genetic programs related to cell growth, differentiation, and adaptive responses to culture conditions. III. Endogenously produced low molecular-mass bFGF lack an obvious signal sequence and could be released only in complex with other molecules (*e.g.* HSP 27) by exocytotic mechanism that is independent of the endoplasmatic reticulum-Golgi pathway. Then exported bFGF acts in an autocrine or paracrine manner. IV. Endogenously produced high molecular-mass bFGF associates with bFGF-Interacting Factor (FIF) and is directly transported to the nucleus where mediates the pro-survival and anti-apoptotic activities.

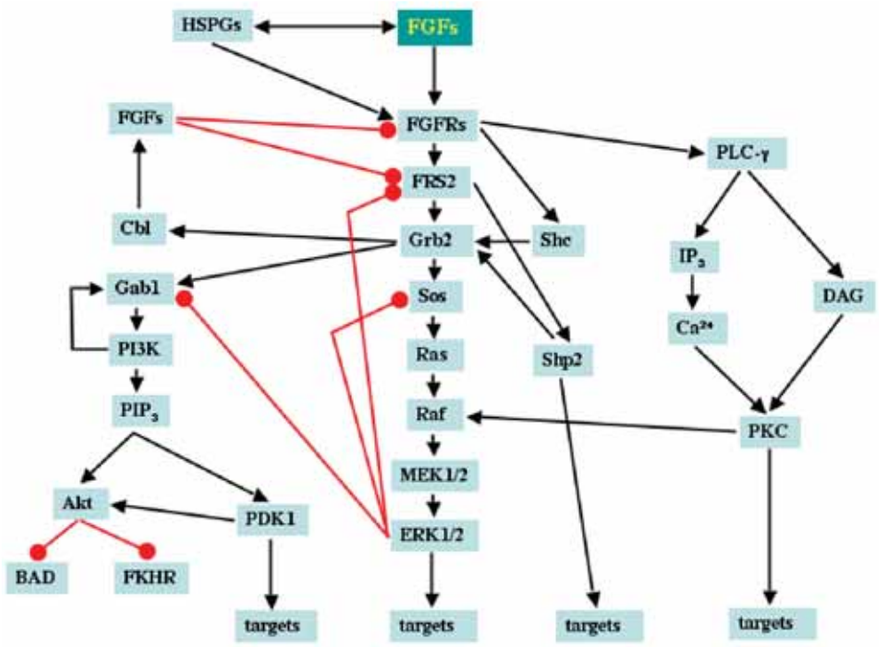

Fig. 2. Signaling pathways activated by FGFs. Stimulatory and inhibitory stimuli are depicted in black and red, respectively. The abbreviations for all elements of canonical FGFR intracellular signaling pathway are accessible at http://stke.sciencemag.org/cgi/cm/stkecm;CMP_15049. Adapted from Schlessinger, 2004.

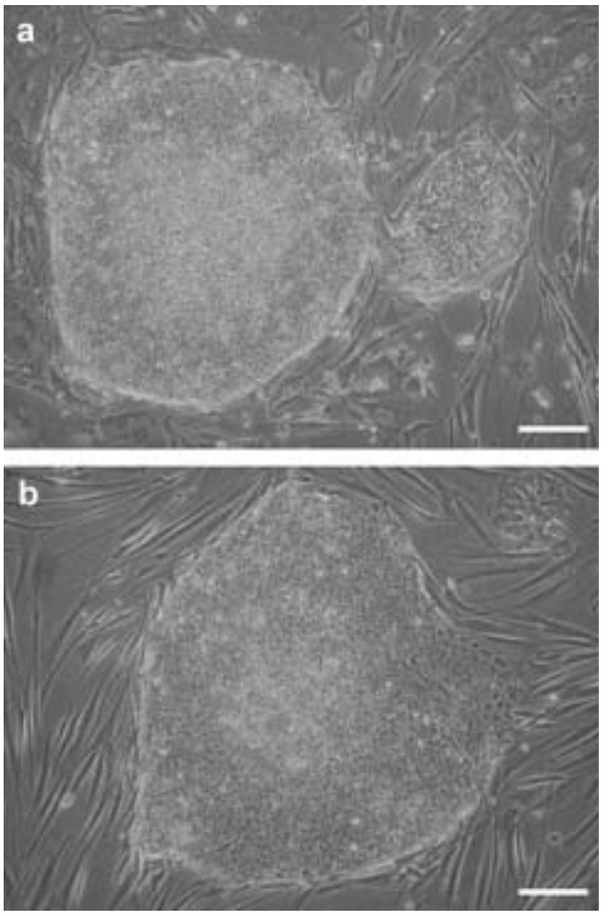

Fig. 3. Morphology of hESCs. HESCs cultured on feeder layers of mouse embryonic fibroblasts (A) and human foreskin fibroblasts (B). Bars = 50 μm.

Obvious interpretation of the effects on hESCs is that bFGF supports growth of undifferentiated cells and increases cloning efficiency. Although such promoting effect of exogenous bFGF on growth of undifferentiated hESCs has been reported by several groups [1, 4, 18, 26, 34], the exact signaling mechanism remains unknown. It becomes apparent that hESCs , similarly to other types of *in vitro* expanded stem cells, may employ intracrine or autocrine signaling pathways to retain their "stemness" in addition to exogenous growth factor signals. On the other hand, certain autonomous signaling pathways may also be activated when hESCs are induced to differentiate by various differentiation stimuli. Importantly, it is of note that hESCs represent a greatly *in vitro* expanded cell population that is only reminiscent to inner cell mass-derived primitive ectoderm in the embryo. Therefore, at least some signaling pathways in hESCs might not have relevance *in vivo* and should be studied only in the context with future clinical applications.

Recently, Xu *et al.* [36] and Wang *et al.* [31] have shown that noggin combined with bFGF at extremely high concentration (40 ng/ml) supports the undifferentiated proliferation of hESCs in the absence of fibroblast feeder cells. As hESCs cultured in fibroblast-unconditioned medium show high levels of bone morphogenetic protein (BMP) signaling activity compared to hESCs maintained in fibroblast-conditioned medium, this effect was explained in a such way that BMP antagonist noggin synergizes with bFGF to repress trophoblast-inducing BMP signaling and thus sustains undifferentiated growth of hESCs. It has been reported that also bFGF alone is sufficient to maintain hESC self-renewal in the absence of feeder cells [35].

Notably, the effect of bFGF on undifferentiated growth of hESCs is apparent only at very high molar concentration (40 ng/ml represents 2.4 nM) that may be irrelevant to the binding capacity of the FGFRs expressed in hESCs. For instance, it has been reported that human leukemia K562 cells that are known for their high expression of FGFRs and high FGF binding activity have about 1600 high-affinity binding sites per cell [27]. Also in bFGF-high responsive aortic endothelial cells the expression of FGFRs is at the same order of magnitude, with about 5000 FGFR sites per cell [23]. Moreover, as mentioned earlier, the binding of bFGF to hESCs might be significantly modulated by membrane-bound or soluble low-affinity binding sites that can either potentiate or inhibit bFGF biological activity [11, 16]. However, currently there is no information on the expression and action of low-affinity FGF receptors in hESCs. This very important issue regarding the capacity of undifferentiated hESCs to accept bFGF signals remains to be addressed.

Although several groups have suggested effect of exogenous bFGF on the maintenance of proliferating hESCs in the undifferentiated state, proliferation rate itself was found not to be significantly affected. In fact, some other differences in hESCs cultures depending on the concentration of exogenous bFGF were observed by us and by other groups. These differences were mainly manifested by the growth of smaller cells and ability of cells to spread on the feeder layer of mouse fibroblasts. Interestingly, in our hands the outgrowth of hESC colonies in medium containing 10 or 20 ng/ml bFGF was reduced compared to the outgrowth in medium without or with only 5 ng/ml bFGF. This observation is in agreement with studies on various cell types indicating that bFGF has a potential to modulate cell attachment and spreading. This may happen either by induction of cell adhesion molecules [7, 12, 15] or *via* complexing FGFR1 expressed on target cells with feeder cell-associated heparan sulfates [25].

Our recent insights into the mechanisms of FGF/FGFR signaling in hESCs

It is apparent that bFGF added to culture media may stimulate hESCs only *via* their high-affinity receptors. In contrast, bFGF produced endogenously by hESCs may function in two ways depending on the presence or absence of the nuclear localization sequence: high molecular-mass isoforms can be directly targeted to the nucleus and operate independently of cell surface receptors in an intracrine manner; whereas the low molecular-mass isoform may be exported from the cells and act *via* FGFRs as an autocrine or paracrine factor.

We have shown that stimulation of hESCs by bFGF at the concentration that is routinely used for undifferentiated hESC culture leads to tyrosine phosphorylation of various proteins and to activation of extracellular signal-regulated kinases, ERK1/2, in particular. However, we also noted that undifferentiated hESCs maintained for several days in bFGF-free medium possess an unexpectedly high basal level of phosphorylation of ERK1/2, which contrasts with the undetectable ERK1/2 phosphorylation typically demonstrated by mESCs. We hypothesized that such constitutive activation of ERK1/2 could be caused, at least in part, by unusually high expression of exportable and nuclear isoforms of bFGF in hESCs conferring a reduced dependency of ERK activity on extracellular signals.

To test whether autocrine FGF signaling is important for growth of undifferentiated hESCs, we treated hESCs cells with the pharmacological inhibitor of FGF receptor tyrosine kinases, SU5402, which specifically interacts with intracellular catalytic domain of FGFRs [20]. After 2 days of continuous exposure to SU5402, cells in the centers of some colonies acquired differentiated morphology. Upon further culture in the presence of SU5402, such morphology, accompanied by a loss of markers of undifferentiated cells Oct-4 and SSEA-4, developed in all hESC colonies. Concomitantly, decreased phosphorylation of mitogen-activated protein kinase kinase (MEK1/2) and its substrate ERK1/2 were both observed in cells that were maintained in medium with the inhibitor. Similar effect was observed in hESCs irrespectively of the presence or absence of recombinant bFGF. Importantly, the occurrence of differentiated cells was accompanied by up-regulation of cyclin-dependent kinase inhibitor p27, a common event that characterizes differentiation of cells of early embryonic origin [3], and by a slower proliferation. Moreover, differentiation process was manifested by up-regulation of TROMA-1, a marker for primitive endoderm, and of nestin, a marker for developing neuroepithelium and for epithelial precursors in the embryonic pancreas.

As mentioned above, hESCs contain significant amount of nuclear high molecular-mass isoforms of bFGF. The nuclear accumulation of bFGF is linked with post-translational methylation, which is important for protein-protein and protein-RNA interactions [17]. Moreover, some data also suggest that nuclear localization of bFGF accounts for the regulation of genes that are involved in the pro-survival phenotype of cells [8]. If this is true also for hESCs remains to be investigated.

We conclude that undifferentiated hESCs possess functional FGF signaling pathway that can basically operate in two different ways: (a) autocrine via low molecular-mass bFGF and FGFRs and (b) intracrine through translocation of high molecular-mass bFGF into the nucleus. We propose that autocrine FGF signaling pathway is unconditionally vital for proliferation of hESCs in the undifferentiated state.

Acknowledgements: This work was supported by the Grant Agency of the Czech Republic (305/05/0434), Ministry of Education, Youth, and Sports (1M0021620803), and Academy of Sciences of the Czech Republic (AV0Z50390512).

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Received: June 30, 2005
Accepted: July 1, 2005