HHS Public Access

Author manuscript

Semin Cell Dev Biol. Author manuscript; available in PMC 2017 May 01.

Published in final edited form as:

Semin Cell Dev Biol. 2016 May ; 53: 94–100. doi:10.1016/j.semcdb.2015.10.004.

Mechanisms of FGF gradient formation during embryogenesis

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Abstract

Fibroblast growth factors (FGFs) have long been attributed to influence morphogenesis in embryonic development. Signaling by FGF morphogen encodes positional identity of tissues by creating a concentration gradient over the developing embryo. Various mechanisms that influence the development of such gradient have been elucidated in the recent past. These mechanisms of FGF gradient formation present either as an extracellular control over FGF ligand diffusion or as a subcellular control of FGF propagation and signaling. In this review, we describe our current understanding of FGF as a morphogen, the extracellular control of FGF gradient formation by heparan sulfate proteoglycans (HSPGs) and mechanisms of intracellular regulation of FGF signaling that influence gradient formation.

Keywords

Heparan sulfate proteoglycans; Morphogen; Fibroblast growth factors

1. Introduction

Early embryonic development comprises of several crucial processes such as cell proliferation and differentiation, cell survival, patterning of the embryonic axis, maintenance of cell lineages, cell migration and morphogenesis. These processes are tightly regulated by various factors. A group of signaling molecules – the secreted growth factors such as fibroblast growth factors (FGFs), Wnt pathway factors, Hedgehog family factors (Hhs), transforming growth factors (TGFs), bone morphogenetic proteins (BMPs) play crucial roles in the patterning and morphogenesis of the early developing embryo. The potency and importance of morphogens in early development were demonstrated by a series of elegant transplantation studies by Spemann and Mangold in the early 1900s. More recent studies have discovered that morphogens secreted by cells diffuse extracellularly to their target cells and bind their cell surface receptors to influence intracellular signaling cascades. The extracellular diffusion of morphogens determines a concentration gradient in the developing embryo and is crucial for establishing positional identities of differentiating cells. It is thus pertinent to understand the various mechanisms governing the establishment of a morphogen concentration gradient. Recent discoveries elucidate the extracellular role of HSPGs in

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creating, maintaining and regulating morphogen concentration gradients. Morphogen concentration gradients are also impacted by subcellular processes that control morphogen signal propagation. In this review, we focus specifically on the mechanisms of extracellular control of FGF morphogen gradient formation by HSPGs and also discuss the latest advances in the knowledge of intracellular control on FGF signaling.

2. FGF as a morphogen during embryonic development

FGFs are a family of polypeptide growth factors that have been evolutionarily conserved across metazoan species. A member of the family was first identified in pituitary extracts and described for its role in stimulating cell growth and proliferation of mouse fibroblasts [1]. Since their discovery, FGFs have been implicated in regulating a plethora of cell processes ranging from cell proliferation and differentiation to cell migration, survival and apoptosis (reviewed in [2–4]). Various members of the FGF family are actively involved in regulating crucial developmental events [5,6], including but not restricted to, coordinating cell migration and formation of primitive streak [7–9], mesoderm induction and patterning [9], neural induction [10,11] and endoderm formation and patterning [12,13]. The diversity in function exhibited by the FGF family derives directly from the diversity in FGF ligands and FGF receptors. In the mouse and human genomes, 22 FGF genes have been described and can be phylogenetically classified into distinct subfamilies: intracellular FGF subfamily, hormone-like or endocrine FGF subfamily and canonical or paracrine FGF subfamily which further contains five subfamilies [14–16]. Intracellular FGFs are localized to the cytoplasm and are not secreted. They interact with voltage-gated sodium channels in a manner that is independent of FGF receptors [17]. Hormone-like or endocrine FGFs are able to enter the bloodstream and can hence influence target tissues located farther away from the source of their secretion. Endocrine FGFs employ the *Klotho* gene family of transmembrane proteins as co-receptors to bind and signal *via* FGF receptors [18]. Canonical or paracrine FGFs are secreted and localized in the extracellular matrix (ECM) and the cell surface close to the site of their secretion. They rely primarily on diffusion to influence target tissues and, like endocrine FGFs, signal through FGF receptors. Here, we focus primarily on paracrine FGFs and the mechanisms through which their concentration gradient is created.

The status of FGFs as morphogens has been well established. Pioneering studies in the *Xenopus* embryos have demonstrated that secreted FGFs are diffusible and present a concentration gradient at the target tissue leading to expression of different marker genes at varying concentrations [19–21]. Early studies in mouse embryos have also shown that FGF morphogen released from the rostral midline specifies the rostral identity of the developing neocortex [22,23]. Furthermore, studies on the spinal cord in the developing chick embryo have demonstrated that the positional identity of thoracic and branchial motor neurons is established by an FGF gradient that directly influences Hox-c expression profiles [24–26]. Taken together, FGFs are secreted signaling molecules that diffuse to form a concentration gradient at the target tissue, thereby influencing the specification of cell fates in a dose-dependent manner. FGF therefore complies with the classical definition of a morphogen.

FGF morphogen ligands bind with a high degree of specificity to cell surface FGF receptor (FGFR) tyrosine kinases. In vertebrates, four highly related FGFRs (FGFR1-R4) are capable

of binding FGF ligands with varying degrees of specificity [27,28]. FGF receptors themselves also exist in alternative splicing isoforms. This generates diversity in FGF ligand–receptor binding that translates into a functional diversity [3,29]. FGFRs consist of an extracellular ligand-binding domain, a transmembrane domain and a cytoplasmic tyrosine kinase domain [30]. Dimerization of FGFR by FGF ligand binding leads to tyrosine kinase activation that can further direct three independent signal transduction pathways, Ras/MAPK, PI3k/Akt and PLCγ/Ca^{2+} – that contribute to the functional diversity elicited by FGFs. Binding of FGF to FGFR and downstream activation of FGF signaling is regulated by heparin/heparan sulfate (HS) [5,31–36]. Details on mechanisms of HS modulation of FGF gradient formation and signaling are presented in the following section.

3. Extracellular control of FGF gradient formation and signaling by HSPGs

Heparan sulfate proteoglycans (HSPGs) are glycoproteins consisting of a core protein to which heparan sulfate glycosaminoglycan polymer chains are covalently linked (Fig. 1). HSPGs are typically categorized based on the location of their core protein – transmembrane type HSPGs (syndecans) and glycerophosphatidylinositide (GPI) anchored HSPGs (glypicans) that are confined to the cell surface, and extracellular matrix HSPGs that are directly secreted and localized in the extracellular matrix (ECM) and basal lamina (perlecans, agrin) [37–42]. HSPG core proteins are evolutionarily conserved across species and are capable of binding ligands and matrix components to regulate biological processes of various growth factors [43,44]. HS side-chains exhibit a great degree of structural heterogeneity based on their length, composition and the microenvironment during development [45]. A concerted effort by both HS side chains and the HSPG core protein effectively regulates growth factor diffusion and gradient formation. A review detailing the importance of HS side chains and HSPG core protein interactions with different morphogens is available [41]. While HSPGs interact with a wide plethora of factors, of particular interest to this review are the FGF ligands that bind HSPGs. By binding FGF with moderate affinity, HSPGs serve many purposes such as restricting diffusion of ligands, preventing their enzymatic degradation, functioning as reservoirs for ligands, transporting ligand to neighboring cells, physically approximating ligands to receptors and promoting stability of ligand–receptor complex by acting as co-receptors [37,39]. HSPGs hence play a pivotal role in eliciting FGF signaling.

Biosynthesis of HSPGs begins in the Golgi apparatus wherein a precursor polymer is synthesized, which is then transported to the cell surface for further modifications. For the interested reader, details of HSPG biosynthesis have been extensively described in several reviews [37,38,41,46]. Briefly, the HSPG biosynthesis cascade begins with a xylosylation at specific serine residues on the core protein (Fig. 1). This is followed by the incorporation of repeating units of glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc) residues that constitute the HS side chain. *Ugdh* (UDP-6-glucose-dehydrogenase) is required for the synthesis of GlcA and GlcNAc residues. *Extl3* (exostosin-like glycosyltransferase) attaches the first GlcNAc molecule to a linkage tetrasaccharide composed of glucuronic acid-galactose-galactosexylose. GlcA and GlcNAc residues are then alternately added to the HS side chain by exostosin glycosyltransferase *Ext1* and *Ext2* [47]. The extending side chain undergoes further processing such as removal of acetyl groups followed by addition of

sulfate groups by N-deacetylase-N-sulfotransferases (*Ndst1-4*) [48] and addition of sulfate groups by uronyl-sulfotransferases (*Hs2st*) and glucosaminyl-sulfotransferases (*Hs3st1-6*, *Hs6st1-3*) [49]. Structural and hence functional heterogeneity exhibited by HSPGs are in part attributable to these enzymes since modifications only occur in some of the GlcA and GlcNAc units, which varies considerably depending on the type of HSPGs and developmental processes. This HS side chain precursor is then transported to the cell surface or ECM where it undergoes further processing by 6-O-Endosulfatases (*Sulf1* and *Sulf2*) which remove specific 6-O sulfate groups [50]. HSPGs and HS chains can also be cleaved extracellularly by proteases and heparanase, causing their release into the extracellular matrix [51]. The specificity of each of these enzymatic modifications translates to a differential regulation of FGF morphogen gradient and signaling.

3.1. HSPGs control FGF morphogen gradient formation by regulating their diffusion

One of the mechanisms by which a morphogen gradient can be established in the developing embryo is simple diffusion. Several mathematical models and biological theories have supported the existence of simple diffusion to explain gradient formation of various morphogens [52,53]. However, the speed and extent of such diffusion must be controlled carefully to ensure proper morphogenesis. HSPGs are abundantly expressed in the extracellular matrix, which creates ample opportunities for them to interact with signaling molecules. The strength of such interaction, however, varies greatly due to the enormous structural heterogeneity of HSPGs, making HSPGs ideally suited to shape the morphogen gradient.

Experimental evidence first suggested that HSPGs function as ‘reservoirs’ for FGF ligands. Germinal *in vitro* studies established that HSPGs bind FGF ligands to prevent their degradation and proteolysis, thereby increasing their radius of diffusion [54–57]. More recently, analysis of FGF gradient spanning the embryonic mouse midbrain, however, suggests that FGF protein is immobilized by its interaction with the cell surface HSPGs [58]. Live imaging of single-molecule dynamics in gastrulating Zebrafish embryos show that the majority of FGF8 exists as freely diffusible single molecules, but a small fraction of FGF8 are HSPG-bound that move significantly slower than predicted for Brownian motion [59]. Enzymatic degradation of HSPGs extended the range of FGF signaling domain, mimicking the gain-of-function FGF phenotype. Further single cell imaging shows that the movement of FGF2 in the extracellular matrix can be slow or fast, short or long range, depending on the translocation of FGF2 molecules from one HSPG to another [60]. The importance of FGF–HSPG interaction is also revealed by the dynamics of FGF dimerization. Only homodimers of FGF9 and FGF20 are effective in binding to HSPGs, which restrict their diffusion [61]. A natural occurring mouse mutation disrupting FGF9 dimerization allows FGF9 to diffuse long distances, leading to ectopic FGF9 signaling [62]. Finally, explant studies on lacrimal gland and submandibular gland branching morphogenesis showed that FGF10 and FGF7 promotes bud elongation or branching respectively, which correlates with their ranges of diffusion in the presence of HSPGs [63]. A single amino acid change in FGF10 that reduces its affinity to HSPGs converts FGF10 into a FGF7-like ligand, resulting in extensive branching of the glands. But what happens when all HSPGs are removed to allow unfettered diffusion of FGF? This question is addressed in a study of lacrimal gland development where the gene

encoding a key biosynthetic enzyme *Ugdh* is knocked out to ablate the synthesis of all glycosaminoglycans including HSPGs [64]. *Ugdh* null embryos display excessive dispersion of FGF10 in mesenchyme and failure of lacrimal gland budding, resembling FGF10 loss-of-function phenotype. Therefore, whereas modest reduction in FGF–HSPG interaction extends the signaling range of FGF, a complete loss of HSPGs abrogates the morphogen gradient of FGF.

More recently, details of the specific core protein and HS side chain of HSPGs that regulates the binding and subsequent release of FGF ligand are being elucidated. A recent study on the collective cell migration in Zebrafish lateral line noted that the loss of HS side chains of Syndecans and Glypicans arising from mutations in *Extl3* and *Ext2* resulted in increased diffusion of FGF ligands into the surrounding tissue [65]. Similarly, local retention of FGF4 and FGF8 ligands by HS side chains was shown to be important for the activation of FGF signaling in mouse extraembryonic ectoderm [66]. Moreover, this study showed that FGF ligands are more likely to bind and interact with trans-membrane type of cell-surface tethered HSPGs such as Syndecans than with secreted type HSPGs such as Perlecans. It was previously noted in lung development that HS low in sulfation are expressed in the mesenchyme surrounding the prospective bud while the highly sulfated HS are present in basement membranes of the epithelial tubules, suggesting that the dynamic pattern of HS modification may be important for branching morphogenesis [67]. Interestingly, lacrimal gland development is normal when HS 2-O and 6-O sulfation are genetically ablated in the mesenchyme, but it is abolished by deleting *Ndst* genes, which not only affects HS N-sulfation but also the overall level of HS sulfation [64,68]. Hence at least for FGF10, the density instead of the position of sulfation groups in HS is likely to play a more dominant role in restraining FGF10 diffusion.

3.2. Shedding of HSPGs and HS side chains regulate distribution of FGF ligands

Proteases and endoglycosidases cleave HSPGs and/or HS side chains respectively from the cell membrane or the extracellular matrix to facilitate the distribution of FGF ligands. HtrA1 is a serine protease that cleaves membrane bound HSPGs such as syndecans and glypicans, which facilitate the long-range dispersal of FGF ligands to affect mesoderm formation and neuronal differentiation in developing *Xenopus* embryos [69]. Similarly in mouse, proteolytic cleavage of FGF-bound HS side chains has been shown to facilitate and extend the non-cell autonomous FGF signaling range in the extra-embryonic ectoderm [66]. Heparanase cleaves ECM HSPG perlecan to release FGF10, which is required for submandibular gland branching morphogenesis [70]. Heparanase is also required for the ectodomain shedding of syndecan-1 to release HS fragments that can bind and activate FGF2 signaling during tissue repair [71]. While HSPGs maintain local FGF gradient by binding FGF ligands, these results show that the cleavage of HS side chains or core proteins by endoglycosidases and proteases can also aid in the release of FGF ligands.

3.3. HSPGs act as co-receptors to modulate FGF signaling gradient

Pioneering *in vitro* studies first suggested the role of HSPGs as co-receptors in FGF signaling [33–36,72,73]. In this role, HSPGs function by physically approximating FGF ligands to FGF receptors and by simultaneously binding FGF ligands and receptors to

induce a conformational change required for FGF signal activation. Genetic manipulation of HS biosynthesis enzymes provides evidence that there exists heterogeneity in the manner by which HSPGs influence FGF downstream signaling [74–79]. In addition, the action of 6-O-Endosulfatases *Sulf1* and *Sulf2* on HSPGs lead to negative regulation of FGF signaling [80,81]. The differential distribution of HSPGs in tissues could plausibly establish a gradient of FGF co-receptors and in turn elicit a graded FGF signaling. This has been observed in mammalian lens development, where HSPGs are expressed in an anterior^{high}–poster^{low} pattern in the lens [82]. Ligand and carbohydrate engagement (LACE) assay reveals that the *in situ* assembly of FGF/FGFR complexes in the lens becomes increasingly stronger from the anterior to the posterior side. This corresponds to the spatial gradient of FGF signaling necessary to promote proliferation of the anterior epithelial cells and differentiation of the posterior fiber cells [83]. Indeed, genetic ablation of HSPGs by deleting *Ndst1* and *Ndst2* disrupted FGF signaling in the lens, leading to the cell proliferation and differentiation defects [82]. Thus HSPGs can utilize their co-receptor function to actively shape the FGF gradient in the signal receiving cells.

4. FGF gradient is controlled by the secretion and internalization of FGF ligands and receptors

The secretion of FGF ligand from the signaling cells is also a crucial step in establishing FGF morphogen gradient. Studies have described the intracellular trafficking of FGF ligands and function of HSPG to act as a ‘molecular trap’ for the translocation of secreted FGF2 across the cell membrane [84,85]. It has been recently discovered that subcellular trafficking of Drosophila FGF branchless in flight muscle can be redirected from surface to T-tubules, which is necessary to promote tracheal invasion [86]. In Zebrafish embryos, the migrating lateral line primordium forms a microluminal structure, which locally traps and concentrate freely diffusible FGF ligands [87]. These results demonstrate the importance of tissue cavities in regulating FGF diffusion.

According to a source-sink model, FGF gradient also relies on the process of endocytosis for the removal of FGF ligands and receptors [59]. Evidence for the role of HSPGs in internalizing FGF initially came from *in vitro* studies [88–90]. However, following internalization, presumably by endocytosis, the fate of FGF ligands in the cell is widely debated [91]. While some studies indicate that FGF follows a lysosomal degradative pathway after HSPG-mediated endocytosis[92,93], others argue that the internalization of HSPG-bound FGF is likely to be protected from lysosomal proteases [88]. While there is some evidence that internalization of FGF2 by HSPGs act as a shuttle for nuclear transport [94], a more recent study found that syndecan-4 internalized FGF2 and FGFR1 by the process of macropinocytosis to form functional cytoplasmic endosomes [95].

The availability of FGF receptor on the cell surface is important for sensing FGF gradient. Studies show that *Src* and *Esp8* mediate FGF receptor endocytosis by recruiting activated FGFRs into clathrin-coated pits in a dynamin-dependent pathway [96,97]. Ubiquitination of FGFRs is required for lysosomal sorting of FGFRs, causing them to be degraded or recycled to affect FGF signaling [98–102]. Extended-synaptotagmin related membrane protein ESyt2

has been shown to act as an FGFR adaptor for clathrin-mediated endocytosis [103–105]. Alternatively, FGFRs can also be endocytosed in a clathrin- and dynamin-independent pathway depending on the type of FGFR [106]. While endocytosis of FGFR mostly follows a degradative pathway, sometimes FGFRs are subjected to intracellular trafficking. Signaling pathways and ligands that promote the recycling of endosomal FGFRs to the cell surface during crucial developmental processes have been described[107,108]. Taken together, the internalization and trafficking of FGFs and FGFRs modulate morphogen gradient formation during development.

5. Other mechanisms that influence FGF morphogen gradient formation

A gradual decay of FGF mRNA is yet another mechanism of gradient formation [109]. In the developing mouse embryo, *Fgf8* mRNA is produced in the posterior tip of the developing embryo. As the axial elongation of differentiated tissue proceeds toward the anterior portion of the embryo, *Fgf8* mRNA decays progressively in a similar fashion. This leads to the formation of an *Fgf8* mRNA gradient along the anterior-posterior axis of the developing embryo, which translates into FGF8 protein gradient that alters downstream FGF signaling in a dose-dependent manner.

FGF signal interpretation also depends on several modulators of the FGF signaling cascade. *Sprouty* was identified in Drosophila as an inhibitor of FGF pathway *via* its suppression of Ras-MAPK pathway [110,111]. *Spred* is a *Sprouty* related tyrosine kinase substrate that also acts as an inhibitor of FGF signaling [112]. Actions of both *Sprouty* and *Spred* are essential for FGF signal interpretation during the development of *Xenopus* mesoderm [113]. Another modulator of FGF signaling – *Sef* is positively regulated by FGF in *Xenopus* and Zebrafish models. It regulates FGF signaling *via* a feedback mechanism by binding FGFR and affects various aspects of vertebrate development [114–118]. Recently, serpin protease nexin-1 (PN1) has been identified in developing *Xenopus* embryos as another feedback regulator of FGF signaling. Activated by FGF signaling, PN1 binds to and inhibits serine protease Htra1, thereby preventing the degradation of Syndecan 4 leading to an altered FGF signaling [119].

6. Perspectives

In summary, the role of FGF signaling molecules as morphogen has been established in various systems. Largely regulated extracellularly by HSPGs, FGF morphogen concentration gradient across the developing embryo induces sub-cellular signaling in a dose-dependent manner. FGF gradient is also controlled by other mechanisms such as mRNA decay, ligand trafficking and secretion, endocytosis of ligand and receptor, tissue cavity and feedback mechanisms by inhibitors of FGF pathway (summarized in Fig. 2).

While various mechanisms influencing FGF morphogen gradient have been described, the recent discovery of microluminal structures to confine FGF ligand movement [87] suggests new paradigms of gradient formation are still being revealed. Further studies are needed to determine if microluminal structures are formed during mammalian embryogenesis as well. The availability and ease of genetic manipulations in various model systems have enabled us to delve further into the specifics of HSPGs in creating a FGF concentration gradient. The

molecular details of FGF movement within the extracellular matrix itself still remains to be elucidated, which will likely be addressed by technical advances in super resolution microscopy and synthesis of heparan sulfates with defined structure. Resolving how FGF morphogen gradient is controlled may provide important insights for future development of pharmacological interventions.

Acknowledgements

The authors thank members of the Zhang lab for discussions. The work was supported by grants from NIH (EY017061 and EY018868 to XZ). XZ is supported by Jules and Doris Stein Research to Prevent Blindness Professorship.

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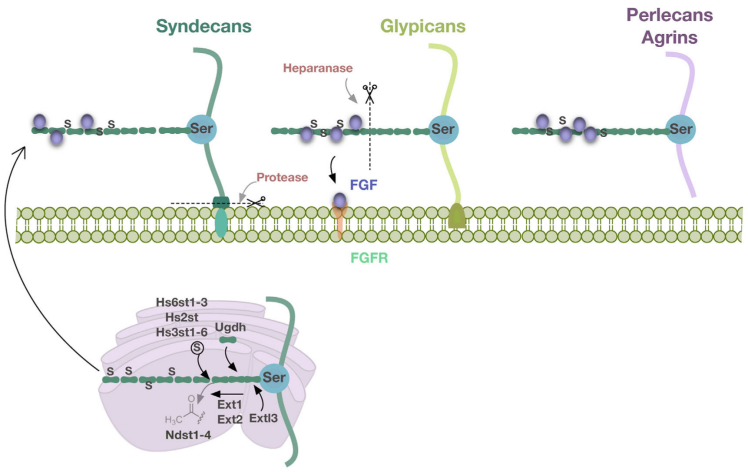

Fig. 1.

Biosynthesis and structural diversity of HSPGs: biosynthesis of HSPGs begins in the Golgi apparatus within the cytoplasm. Specific serine residues on the core protein undergo xylosylation that allows for the incorporation of repeating units of GlcA and GlcNAc residues. *Ugdh* is an enzyme early in the biosynthesis cascade that is required for the synthesis of GlcA and GlcNAc residues. *Extl3* is an exostosin-like enzyme that attaches the first GlcNAc to the linkage tetrasaccharide on the core protein. GlcA and GlcNAc residues are added to the side chain by exostoses *Ext1* and *Ext2*. Enzymes *Ndst1-4* add N-sulfation groups following the removal of acetyl groups. Additional O-sulfation groups at various positions are added to the chain by enzymes *Hs2st*, *Hs3st1-6*, *Hs6st1-3*. The precursor molecule is then transported to the cell surface or ECM where it undergoes further processing. Heparanase cleaves HS side chains while specific serine proteases cause shedding of HSPGs. Shown here for three kinds of HSPGs: transmembrane type (Syndecans), GPI anchored type (Glypicans) and secreted type (Perlecans, Agrins).

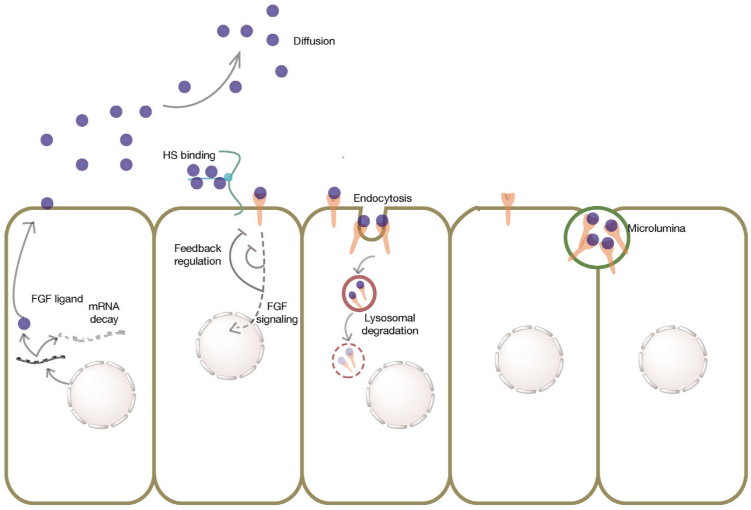

Fig. 2.

Summary of mechanisms regulating FGF gradient formation: FGF ligands from the source cell are bound by HSPG side chains to prevent their diffusion farther away from the source tissue. FGF ligands bound to the receptors initiate FGF signaling cascade that is subject to feedback regulation at various levels, mainly by FGF inhibitors. Alternatively, FGF mRNA can undergo decay as cells move away from the local expression domain. FGF ligands and receptors can also be endocytosed to follow a mostly degradative pathway. FGF ligands can also be locally sequestered by the formation of microluminal structures.