UNIVERSITY OF LIVERPOOL

Institute of Integrative Biology

Interactions of fibroblast growth factors with glycosaminoglycan brushes and the pericellular matrix.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of doctor in Philosophy

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September 2017

Author's declaration

I declare that the work in this dissertation was carried out in accordance with the regulations of the University of Liverpool and the CIC BiomaGUNE. The study described is original and have not been submitted for any other degree. All aspects of the experimental design and planning for the study were conducted by me under the guidance and agreement of my supervisors, Professor David G. Fernig, Dr. Ralf P. Richter and Dr. Edwin Yates. The experimental work in this study was undertaken by me, with specific contributions that are indicated in the core of the thesis when appropriate.

Any views and hypothesis presented in this thesis are from the author and to not represent those of the University of Liverpool. This thesis has not been presented to any other university for examination in the United Kingdom or overseas.

Acknowledgments

I would like to express my gratitude to the people who contributed by their support, guidance and advice to this study and my training.

This work would not have been possible without the input of many people but first and foremost, I would like to express my thankfulness to my supervisors, Professor **David G. Fernig**, Dr. **Ralf P. Richter** and Dr. **Edwin Yates** for their guidance and support throughout this four years of training in conducting research and personal development.

I am grateful to my assessors who guided me throughout this journey: Pr. **Olga Mayans**, Dr. **David Adams** and Dr. **Igor Baruskov**. Special thanks to Pr. **Jeremy Turnbull** and Pr. **Catherine Picart**, my internal and external examiners respectively for giving their final review to this work.

I would like to thank the people who I have worked along and who advised me in multiple occasions in the biosurfaces laboratory at the CIC BiomaGUNE and the biochemistry laboratory at the Institute of Integrative Biology: **Yong Li**, **Elisa Migliorini**, the group members and supporting staff of both laboratories. Thanks to Thao Bui, Pawin Ngamlert, Dunhao Sun and Zaid Alghrair for their daily support and friendships.

Thanks to my friends and family for their continuous support on this journey. I could not possibly be thankful enough to my mother, **Dianessy Hawa**, for always being by my side and supporting my will of studying science.

"Success is not an end in itself. It is a journey that is best judged by the number of times that the traveller has been able to stand up after he/she has fallen down."

H.E Ameenah Gurib-Fakim

Abstract

The components of the extracellular matrix (ECM) are produced *in situ* by cells and are either completely secreted from the cell into the ECM or remain associated with the cell membrane. Amongst them are polysaccharides of the glycosaminoglycans (GAG) family, which are either free or covalently bound to proteins to make proteoglycans (PGs). These form a highly-hydrated compartment in which the proteins are embedded. At the molecular level, all ECM components are structured to execute their function and have been implicated in regulating intercellular communication. The sulfated GAGs interact with a wide range of proteins and their structure and tissue localisation is related to their function. Thus, certain GAGs may be particularly enriched in specific tissues, *e.g.*, dermatan sulfate in skin, but they are found in all tissues; and heparan sulfate (HS) has the widest range of interacting protein partners. These partners include both the permanent ECM residents and the transients, such as the fibroblast growth factors (FGFs), which transmit signals from one cell to another in paracrine signalling involved in tissue development, differentiation and homeostasis. The aims of this thesis are (1) to use a simple biomimetic model of ECM in the form of a GAG brush to determine if FGF binding leads to different supramolecular structures. (2) To determine if these supramolecular arrangements allow FGF mobility as observed *in vivo*. The model GAG brush was assembled layer by layer by one-end grafting of biotinylated GAGs on a streptavidin monolayer, itself attached to a supported lipid bilayer. The structure of these brushes was probed using different recombinant human FGFs (FGF1, HaloFGF1, FGF2, HaloFGF2, FGF4, HaloFGF6, FGF9, FGF10, HaloFGF10, FGF17, FGF18 and HaloFGF20) with well characterised HS binding sites (HBSs) and where “Halo” refers to an N-terminal Halotag fused to the FGF for fluorescence labelling. Rigidification of soft and highly hydrated films was assessed by quartz crystal microbalance with dissipation monitoring (QCM-D), spectroscopic ellipsometry (SE) was used to quantify the biomolecules at the surface, and fluorescence recovery after photobleaching (FRAP) was employed to assess the lateral diffusion of the GAGs and the (Halo)FGFs. FGFs showed a preference in binding stoichiometry for specific disaccharide structures, and the ensuing interactions led to different supramolecular

organisations of the brush/FGF films. Upon binding to the brushes, FGFs possessing multiple HBSs ('multivalent' FGFs) with acidic borders delimiting their HBSs were able to immobilise the GAG chains; some of these FGFs, *e.g.* HaloFGF1, remained mobile, whereas others were trapped in the film, *e.g.* HaloFGF2. Monovalent FGFs, and multivalent FGFs with no acidic borders around their HBSs, were found to not cross-link the brushes and remained mobile.

To test the idea that acidic borders on the protein surface play an important role in determining the ability of an FGF to cross-link HS chains and thus regulate mobility of the FGF in the matrix, the behaviour of fluorescently labelled Halo-FGFs were measured in the native pericellular matrix of fixed human keratinocytes. HaloFGF2 was immobile in HS brushes and in the pericellular matrix of HaCaT cells. This indicates that although the other components of the pericellular matrix may also play roles in determining the diffusion dynamics of FGF2, HS would be the main director of it.

Interactions between growth factors such as FGFs with components of the ECM are specific to their molecular features and can be precisely monitored in biomimetic models. These interactions trigger supramolecular structures that can be characterised by their stiffness. It is also possible to assess the mobility of these growth factors using a fluorescent label. Interestingly, the mobility of at least HaloFGF1, HaloFGF2 and HaloFGF10 in HS brushes was reproduced in pericellular matrix of HaCaT cells. A key difference is that the local supramolecular arrangement of the pericellular matrix components will be heavily influenced by the interactions of the HS chains with endogenous HS binding proteins. This will form a network of binding sites for FGFs, which at least in the case of HaloFGF2, did not prevent the immobilisation of the growth factor. However, in the case of other FGFs, e.g. HaloFGF6 and HaloFGF20 we detected reduced mobility. Thus, bridging the gap between the analyses on the HS brush model and on pericellular matrix may require the elaboration of a more complex *in vitro* model, incorporating other molecules into the HS brush, such as collagens and fibronectin, which have multiple HBSs and would be expected to present to the FGF already cross-linked HS chains and a reduced number of available binding sites.

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List of abbreviations

BSA: Bovine serum albumin

CS: Chondroitin sulfate

CS-A : Chondroitin sulfate A

CS-C : Chondroitin sulfate C

CS-D : Chondroitin sulfate D

CS-E: Chondroitin sulfate E

DMEM: Dulbecco's modified Eagle medium

DMSO: Dimethylsulfoxide

DOPC: 1, 2-Dioleoyl-sn-Glycero-3-phosphocholine

DOPE-CAP-B: 1, 2-Dioleoyl-*sn*-Glycero-3-phosphoethanolamine-cap-biotin

DTT: Dithiothreitol

dp: degree of polymerization

DS : Dermatan sulfate

ECM : Extracellular matrix

EXT: exotosin

EXTL: exotosin-like

FCS: foetal calf serum

FGF: Fibroblast growth factor

FGFR: fibroblast growth factor receptor

FRAP: Fluorescence recovery after photobleaching

GAGs: glycosaminoglycans

Gal: Galactose

GalNAc: N-acetylgalactosamine

GlcA: Glucuronic acid

GlcNAc: N-acetyl glucosamine

GlcNS: N-sulfated glucosamine

HA: Hyaluronic acid

HaloTag: haloalkane dehalogenase tag

Halo-FGF: FGF fused to an N-terminal HaloTag

HB: HEPES buffer: 150 mM NaCl, 10 mM HEPES, pH 7.4

HBS: Heparin binding site

Hep: Heparin

HS: Heparan sulfate

HSPG: Heparan sulfate proteoglycans

IdoA: Iduronic acid

IPTG: isopropyl β-D-1-thiogalactopyranoside

LB: Lysogeny broth

MMPs: matrix metalloproteases

NDST: N-deacetylase/N-sulfotransferase

OD600: optical density at 600 nm

ON: overnight

OST: O-sulfotransferase

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate-buffered saline

PDB: Protein data bank

PFA: paraformaldehyde

PGs: Proteoglycans

QCM-D: Quartz crystal microbalance with dissipation

ROI: region of interest

RT: room temperature

SAv: Streptavidin

SDS: Sodium dodecylsulfate

SE: Spectroscopic ellipsometry

SLB: Supported lipid bilayer

SUV: Small unilamellar vesicles

SPDBV: Swiss PDB viewer

TEMED: N, N, N', N'-Tetramethylethylenediamine

Tris: Tris (hydroxymethyl) methylamine

TPA: Time-resolved profile analysis

UDP: Uracil diphosphate

Xyl: Xylose

1 Introduction

1.1 Cell communication in multicellular organisms.

In multicellular organisms, intercellular communication processes are the foundation of their growth and expansion, development, function, integrity and regeneration. Intercellular communication happens via various modes, the cell can target itself, a process that is call “autocrine” signalling or a neighbouring cell by direct molecular contacts (juxtacrine signalling) or cytoplasm contacts through gap junctions (1). Paracrine signalling is the local communication between cells without a direct molecular physical contact. Chemokines, cytokines, growth factors and many morphogens are paracrine signalling agents. They are secreted into the pericellular matrix of the source cell, and travel throughout the interstitial matrix towards the target cells. On the target cell, the paracrine effector binds to a receptor that triggers downstream signalling within the cell (2). This is the case of fibroblast growth factors (FGFs) signalling via tyrosine kinase receptors (3) and transforming growth factors family members such as the bone morphogenetic proteins that bind to the bone morphogenetic protein receptor type II in development processes (4). Intercellular communication can happen over longer distances, in which case the secreted effector travels through the endothelial barrier and is transported in the vascular blood flow throughout the body, but only acts on cells expressing the cognate receptor. This is the endocrine system and concerns hormones (Figure 1.1). The extracellular matrix (ECM) is the extracellular environment in contact with the cell; it is implicated in controlling cell fate decisions. During development, morphogen gradients are shaped by ECM components, whereas in homeostasis the ECM takes part in tissue regeneration and immunity by controlling the activity and transport of proteins regulating cell growth, migration and differentiation. Besides the components, the physical characteristics of the ECM are also relevant. It was shown that the elasticity of the ECM controls the differentiation of stem cells (5) and the polarisation of fibroblasts has been related to the stiffness of ECM (6). Stiffness and elasticity are both determined by the supramolecular structure of ECM, which in turn

depends on its molecular composition and the interactions of the component molecules.

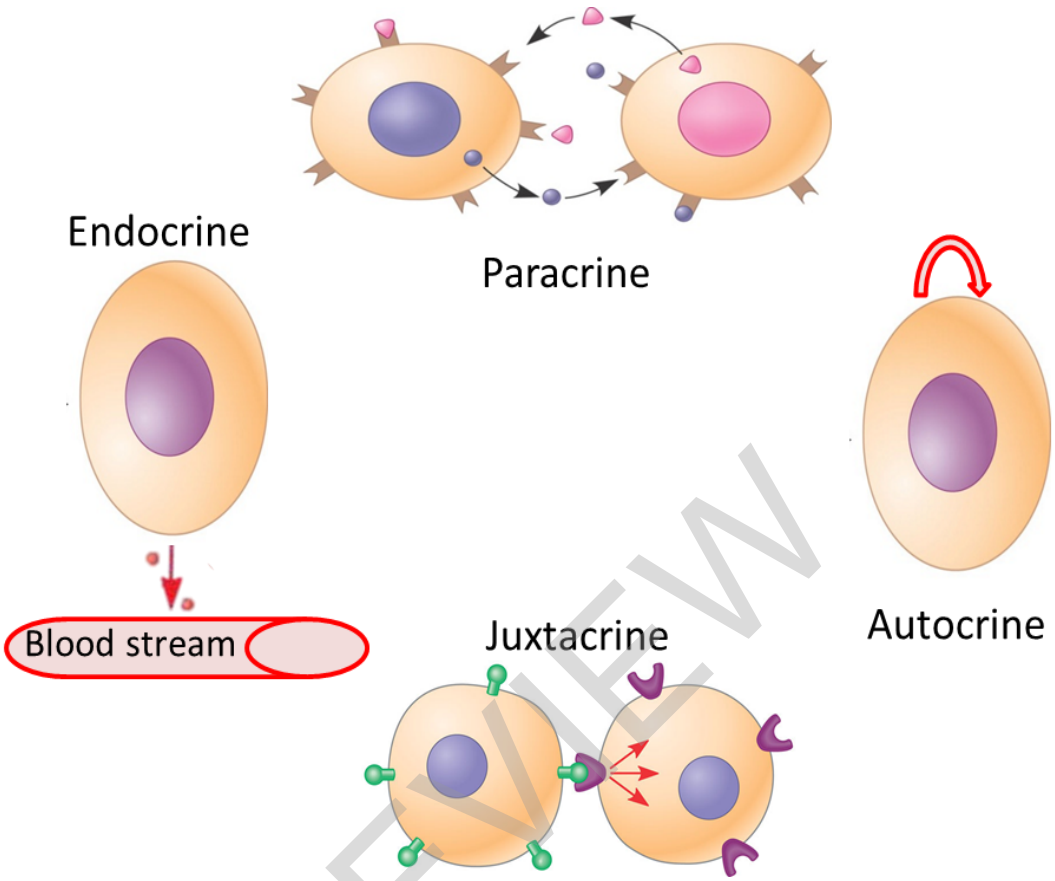

Figure 1.1: Forms of signalling in cellular communication.
Endocrine signalling corresponds to the intercellular communication that occurs via transport of the effector by the blood stream. Juxtacrine communication involves contact between the signalling and the targeted cell as described in the figure. Autocrine signalling depicts the signalling of a cell on itself and the paracrine signalling the one to a neighbouring cell. In paracrine and autocrine signalling, the effector molecule is transported in the immediate microenvironment of the cells that is the extracellular matrix.

1.2 Extracellular matrix

The ECM has distinct domains, the pericellular matrix is immediately adjacent to the cell surface, extending 1-5 µm in some tissues (7). Further away in mesenchymal tissues is the interstitial matrix. In epithelial tissues and the vasculature, a specialised ECM is found beyond the pericellular matrix, the basement membrane, so called due to its molecular density causing it to be heavily stained in a number of classic histological preparations. Basement membrane separates these compartments from

the underlying mesenchyme (8) (Figure 1.2). At the molecular level, all ECM have reasonably well-studied components and these are mainly fibrous proteins such as collagens, and the polysaccharides of the glycosaminoglycan (GAGs) family.

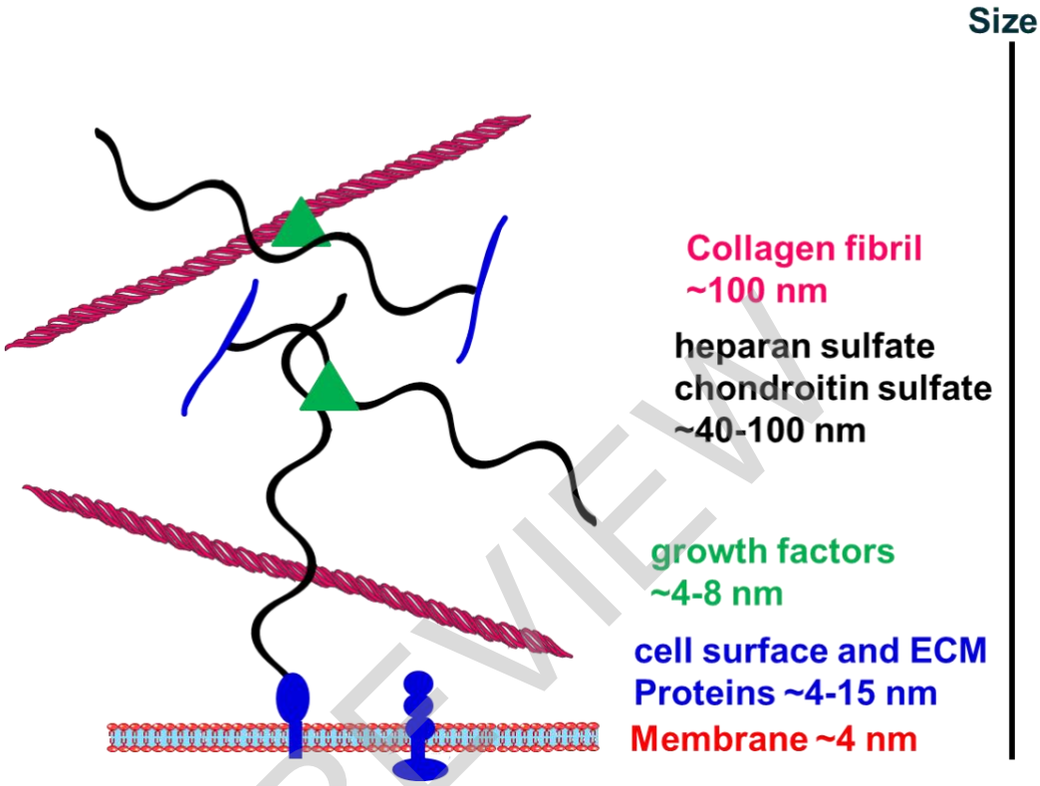

Figure 1.2: Schematic representation of the extracellular matrix proteoglycan organisation. Here schematized are the ECM with collagen fibrils and GAGs. HSPGs core proteins (blue) carrying HS chains (black) are sitting in the ECM or anchored in the cell membrane, with bound growth factor (green).

Collagens are the scaffold of extracellular matrices. They are tissue specific, organised in fibrils and provide resistance to shear and pressure (9). Type I and type III collagens sit in the interstitial matrices of soft tissues, *e.g.,* the dermis (10). In bones, collagen I fibrils can represent up to 90% of matrix protein components (11). Collagen type IV is the fibrous components of the basement membranes and is connected to the interstitial matrix via collagen VI, thus the fibrous scaffolds of basement membrane and the interstitial matrix form interconnected networks (12). Along with collagen type IV, fibronectins, laminins, nidogen and perlecan are the main constituents of the basement membrane. Laminins are connected to collagen IV and perlecans by nidogens (13). The ECM is a complex network where the main