1994	Ph.D. - University of Göttingen, Germany
1994 - 1997	PostDoc - Institute for Biochemistry I, Heidelberg University, Germany (Prof. Felix Wieland)
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Walter Nickel

Unconventional Protein Secretion

Goal

To reveal the molecular components and mechanisms involved in unconventional secretion of Fibroblast Growth Factor 2 (FGF2) from tumour cells, to elucidate the relevance of the FGF2 secretion system for other unconventionally secreted proteins such as HIV-Tat and Interleukin 1β and to identify small molecule inhibitors of these processes to develop novel classes of anti-cancer drugs targeting tumour cell survival.

Background

The vast majority of extracellular proteins is secreted through the classical ER/Golgi-dependent secretory pathway. However, numerous extracellular proteins have been identified that do not carry signal peptides and whose export from cells is not blocked by Brefeldin A, an inhibitor of ER-to-Golgi trafficking. Among this group of extracellular proteins are mediators of physiological processes with biomedical relevance such as angiogenesis and inflammation, among others. For example, FGF2 is of critical importance for processes such as embryonic development, tissue regeneration, wound repair and hematopoiesis. Beyond its function in normal cell growth and differentiation, FGF2 plays critical roles under pathophysiological conditions. This is particularly evident in the context of cancer with FGF2 being a major mediator of tumour-induced angiogenesis. In addition, FGF2 acts as a survival factor that inhibits tumour cell apoptosis by an autocrine secretion-signalling loop. This process is believed to represent a frequent cause of tumour cell resistance against anti-cancer therapies.

The molecular machinery mediating FGF2 transport into the extracellular space has been elucidated through a combination of genome-wide RNAi screening approaches and biochemical reconstitution experiments. It consists of four *trans*-acting factors, ATP1A1, $PI(4,5)P_2$, Tec kinase and heparan sulfate proteoglycans, all of which are physically associated with the plasma membrane (Fig.1).

Key steps in unconventional secretion of FGF2 are sequential interactions with ATP1A1, Tec kinase and the phosphoinositide $PI(4,5)P_2$ at the inner leaflet, $PI(4,5)P_2$ triggered oligomerization and membrane insertion as well as extracellular trapping of FGF2 mediated by cell surface heparan sulfate proteoglycans. Key intermediates in this process are membrane-inserted FGF2 oligomers that form membrane pores, a process that is regulated by Tec kinase dependent tyrosine phosphorylation of FGF2. FGF2 remains folded

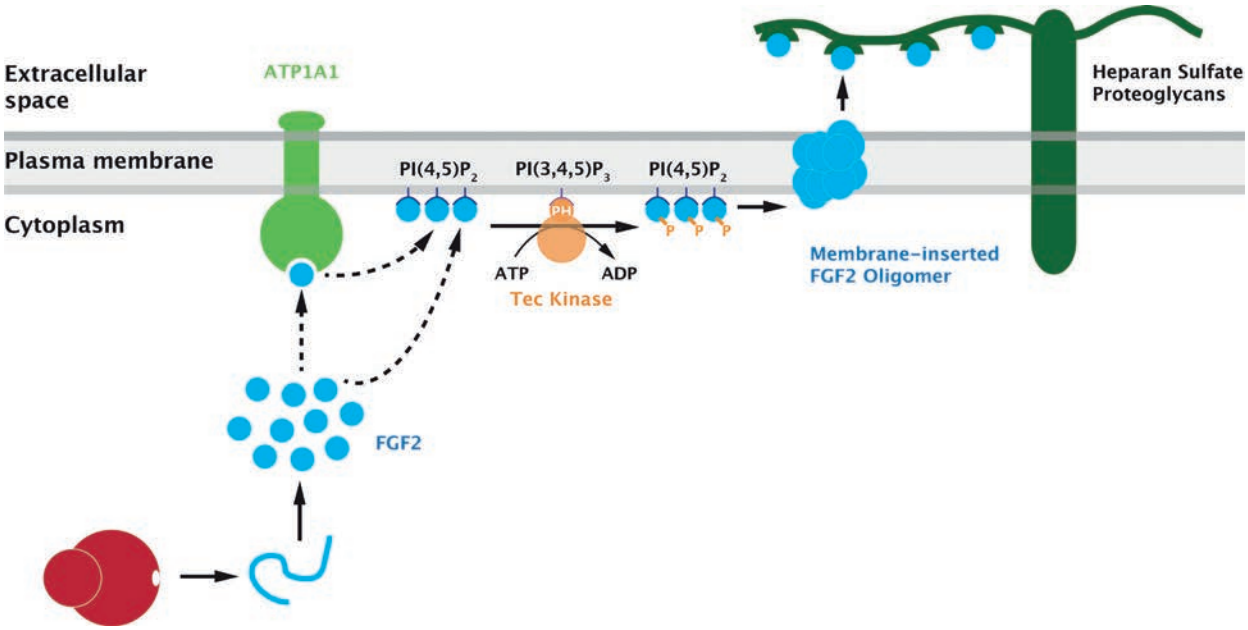

Fig. 1: Molecular components and mechanisms involved in unconventional secretion of FGF2 from tumour cells (La Venuta et al, 2015)

through all steps of this pathway, a property that is linked to specific requirements such as binding to $PI(4,5)P_2$, the formation of an oligomeric structure as part of a toroidal membrane pore and binding to heparan sulfates on cell surfaces. These steps are reflected by requirements for *cis*-elements in FGF2 that mediated binding to $PI(4,5)P_2$ (K127/R128/K133), binding to heparan sulfates (K133) and tyrosine phosphorylation (Y81). In conclusion, FGF2 is secreted through direct translocation across the plasma membrane, a novel type of protein translocation across membrane where the cargo molecule (FGF2) forms its own translocation intermediate through oligomerization and membrane insertion.

Research Highlights

A recent discovery has been the identification of a new *cis*-element required for FGF2 secretion from cells. It is composed of two cysteine residues (C77/C95) on the molecular surface of FGF2 that are absent from all FGF family members carrying signal peptides for ER/Golgi-dependent secretion, suggesting a specific role in unconventional secretion of FGF2. These cysteines form intermolecular disulfide bridges and are critical for FGF2 oligomerization and membrane insertion. Intriguingly, the four *cis*-elements in FGF2 required for unconventional secretion (K127/R128/K133 for binding to $PI(4,5)P_2$, K133 for binding to heparan sulfates, Y81 as the target of Tec kinase and C77/C95 required for oligomerization and membrane insertion) are transplantable. Following removal of the signal peptide of FGF4, a structural relative of FGF2, the hybrid protein was redirected into the unconventional secretory pathway of FGF2 resulting in its exposure on cell surfaces. These findings establish the core machinery mediating FGF2 secretion with four *trans*-acting factors and four *cis*-elements.

The relevance of the core features of FGF2 secretion for other unconventionally secreted proteins was elucidated through studies on HIV-Tat and Interleukin 1β secretion. On the one hand, we found that HIV-Tat is capable of forming membrane pores in a $PI(4,5)P_2$ dependent manner. Along with studies from other laboratories that demonstrated $PI(4,5)P_2$ dependent secretion of HIV-Tat from T cells, these findings strongly suggest that FGF2 and HIV-Tat share a common

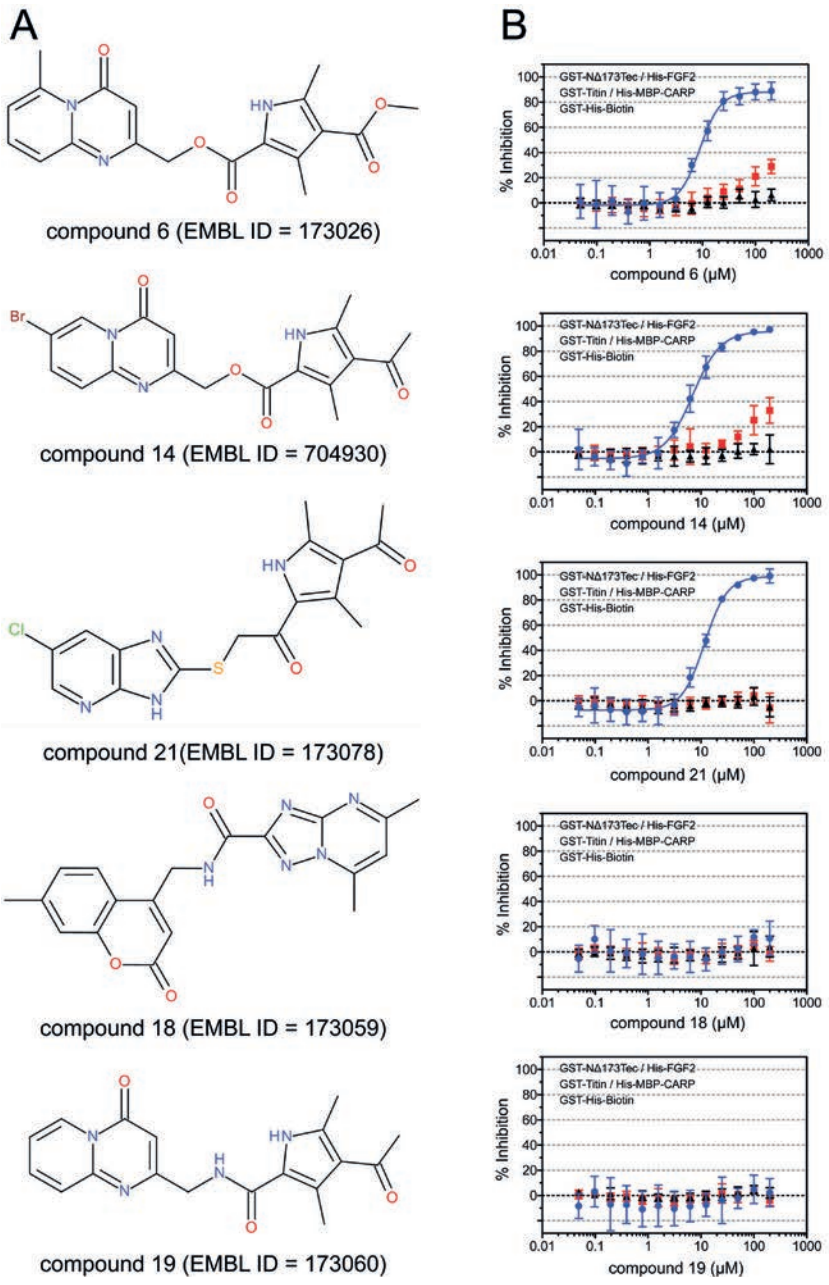

Figure 2: Identification of small molecule protein-protein interaction inhibitors of the Tec/FGF2 complex (La Venuta et al, 2016).

(A) *Chemical strcutures of active compounds (cpds 6, 14 and 21) and inactive derivatives (cpds 18 and 19).*

(B) *IC50 profiles of the compounds shown in panel A as determined with the AlphaScreen protein-protein interaction assay.*

mechanism of unconventional secretion. Furthermore, in collaboration with Pablo Pellegrin (Murcia, ES) and David Brough (Manchester, UK), we could demonstrate that inflammasome-dependent secretion of Interleukin 1β depends on $PI(4,5)P_2$ dependent membrane pore formation at the level of the plasma membrane. These findings imply that at least three major examples of unconventionally secreted proteins rely on a similar mechanism of transport into the extracellular space with FGF2 being the prototype cargo molecule.

Finally, another recent breakthrough was the development of a first generation of small molecule inhibitors targeting unconventional secretion of FGF2. While we address this aim in several ways, a first success has been the identification of compounds that prevent the interaction between FGF2 and Tec kinase (Fig. 2 and Fig.3). These compounds inhibit Tec kinase dependent tyrosine phosphorylation of FGF2 both in vitro and in cells. Intriguingly, the first generation of these inhibitors block FGF2 secretion from cells with an IC50 in the low micromolar range making them excellent lead compounds for drug development targeting tumour cell survival.

In conclusion, in recent years, the molecular mechanism and the molecular components required for unconventional secretion of FGF2 have been elucidated in great detail. Future challenges will be to fully reconstitute this process with purified components, to obtain structural insight into membrane-inserted FGF2 oligomers as the key intermediates of this process, to analyse the spatio-temporal coordination of this process in living cells and to develop drug-like inhibitors that prevent autocrine FGF2 signalling in the context of tumour cell survival strategies.

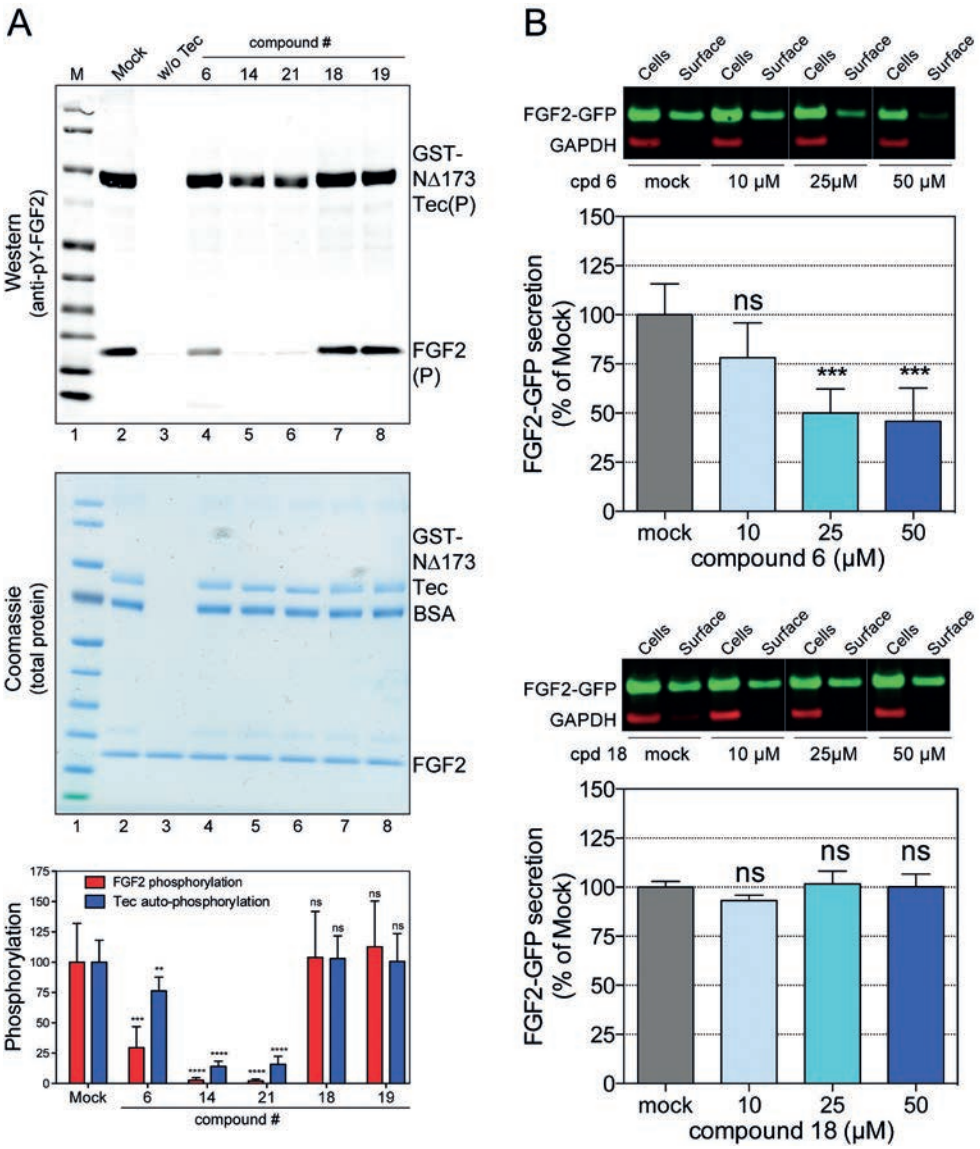

Figure 3: Small molecule protein-protein interaction inhibitors of the Tec/FGF2 complex block unconventional secretion of FGF2 (La Venuta et al, 2016).

(A) *Inhibition of Tec kinase mediated tyrosine phosphorylation of FGF2 in the presence and absence of active (cpds 6, 14 and 21) as well as inactive compounds (cpds 18 and 19).*

(B) *Inhibition of FGF2 secretion from cells in the absence and presence of compound 6 (active) and 18 (inactive).*

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Steringer JP, Müller HM, Nickel W (2014) Unconventional Secretion of Fibroblast Growth Factor 2-A Novel Type of Protein Translocation across Membranes? J. Mol. Biol. 427:1202-1210

Patent applications

Patent Application PCT/EP2015/073591: Nickel W (2015) "Assay for FGF2 Secretion and Signalling"; WO2016/055664

Patent Application Number GB1605173.2: Nickel W and Lewis JD (2016) "Inhibitors of the Unconventional Secretion of Fibroblast Growth Factor 2 (FGF2) by Tumour Cells and Uses Thereof"; 29 March 2016

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