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Hyper-functional factor IX improves liver-targeted AAV gene Therapy for hemophilia B

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Abstract

The development of the next-generation gene therapy vectors for hemophilia requires the use of lower and thus potentially safer vector doses, while augmenting their therapeutic efficacy. We have validated a computational strategy that led to the identification of hepatocyte-specific transcriptional cis-regulatory modules (CRMs) that were employed to obtain high levels of factor IX (FIX) in hemophilia B mice at low vector doses. Vector efficacy could be enhanced further in a dose-dependent fashion, by combining these hepatocyte-specific CRMs with a synthetic codon-optimized hyper-functional FIX (i.e. Padua R338L) transgene. This hyper-functional mutation further boosted FIX activity up to 7-fold, with no apparent increase in thrombotic risk. This combination approach resulted in sustained supra-physiologic FIX levels (400%) and correction of the bleeding diathesis at clinically relevant, low vector doses ($5x10^{10}$ vg/kg) that are considered safe in subjects undergoing gene therapy. Moreover, liver-restricted expression of the hyperactive FIX Padua R338L resulted in the induction of immune tolerance that precluded induction of inhibitory antibodies to FIX upon immunization with recombinant FIX protein.

Nanobody Stabilization of Active-state Beta2 Adrenergic Receptor

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Abstract

The past decade has witnessed a gradual increase on the number of structures of G-protein coupled receptors (GPCRs) being resolved but most of these structures only represent those of inactive state or antagonist bound state or structures in which stabilizing mutations have been introduced to compensate for the inherent instability of this receptor family when isolated from their natural environment. The discovery of a particular camelid single chain antibody fragment known as Nanobody80, a G-protein mimetic which increases agonist binding affinity to beta2-adrenergic receptor (β_2AR) was successful in the structural elucidation of an active state β_2AR. In this thesis, we investigated the effect of Nb80 on ligand binding properties in both membrane preparations and in detergent solubilized β_2AR expressed in sf9 cells using the Baculovirus Expression System. Our results show that under the control condition, agonist e.g. epinephrine exerts only 'low' affinity binding ($K_{i=}$ 3.5E-06M) to β_2AR. However when Nb80 was added, about 87% of these low affinity sites were converted to higher affinity state ($K_{i\,=}$ 2.2E-08). A

similar pattern was observed in 1% DDM solubilized β_2AR. On the other hand Nb80 was found to have apparently no effect on neither the silent antagonist (alprenolol) nor on the inverse agonist ICI118.551. This results show that, like the Gs-protein, Nb80 selectively binds and stabilize a high agonist affinity 'active-state' conformation of β_2AR.

We were also interested to know if Nb80 could thermo-stabilize detergent solubilized β_2AR by measuring antagonist binding as well as agonist binding with Nb80. For the antagonist (^{3}H-DHA) binding, a rapid receptor inactivation occur above 30 ˚C and no difference was observed between the control and Nb80 sample. Interestingly binding of ^{3}H-epinephrine to solubilized β_2AR was only measurable at 4 °C and in the presence of Nb80. Incubation of the 3H-epinephrine bound receptors at 37 °C resulted in a time-wise decline of the binding indicating only a relative thermostabilisation by Nb80.

PRESENCE, REGULATION AND FUNCTION OF GLP-1 RECEPTORS IN MACROPHAGES

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Abstract

Glucagon-like peptide (GLP-1), a gut hormone with insulinotropic effects, was recently shown to have immunomodulatory actions. The presence of GLP-1 receptor (GLP-1R) has been reported in diverse types of cells/tissues, most importantly, in immune cells such as macrophages. Therefore, the aim of this thesis is to determine the presence, regulation and function of GLP-1R in mouse macrophages since these cells play an important role in immunity.

First, the presence of GLP-1R was investigated in mouse macrophages using RT-PCR. The study showed that GLP-1R mRNA is also expressed in the macrophage cell lines J774 and RAW 264.7 and ex vivo macrophage PECs. MIN6 has the highest expression of GLP-1R mRNA, J774 and RAW 264.7 were comparable, while PECs has the lowest. The results of radioligand binding with 125I-GLP-1 were in parallel with the gene expression. cAMP measurement was done to determine the functional presence of the receptors; however, the currently available method using a bovine adrenal cortical cAMP binding protein was not sufficiently sensitive to detect GLP-1R agonist-mediated cAMP accumulation in MIN6 cells. The modulation of the GLP-1R expression by the proinflamma cAM tory compounds LPS and IFNg, and the GLP-1R ligands GLP-1 and exendin-4 (EX-4) was also determined. RT-PCR results showed that IFNg decreased the expression of GLP-1R in all the cells. However, this downregulation could not be confirmed by radioligand binding, likely because of relatively low cpm values. Furthermore, the ligands reduced the receptor mRNA expression in macrophage cells but not in MIN6.

The expression of M1 markers (NOS2, TNFa, IL-12 and IL-1B) and M2 markers (Arg1 and IL-10) was investigated. NOS2 and TNFa mRNA expression as well as the NO and TNFa production were in general increased after LPS and/or IFNg treatment in the two macrophage cell lines as well as ex vivo cells. Also, in all cell types, IL-12 and IL-1B were generally upregulated by LPS and/or IFNg. Regarding the M2 markers, Arg1 expression was increased in RAW 264.7 cells and PECs but not in J774 cells, after

stimulation with LPS and/or IFNg. Also, LPS treatment in RAW 264.7 and PECs increased the expression of IL-10.
Lastly, the influence of GLP-1 and EX-4 on cytokine expression (M1 and M2 markers) was explored in macrophages activated with LPS/IFNg. For the M1 markers, both GLP-1 and EX-4 reduced NOS2 mRNA expression in RAW 264.7, J774 and PECs under basal conditions. However, a different pattern emerges after macrophage activation with LPS and/or IFNg. All these modulations were not accompanied by changes in NO production. TNFa expression was reduced by GLP-1R ligands in RAW 264.7 and J774 but not in PECs under basal conditions. The modulation of LPS/IFNg-induced TNFa mRNA and protein expression by the GLP-1R ligands was not evident. With IL-12 and IL-1B, expression was marginally or not affected by the GLP-1R ligands under basal conditions and after activation with LPS and/or IFNg. The expression of M2-related genes, e.g., Arg1 and IL-10 was found to be unaffected or very modestly regulated by incubation of the cells with the GLP-1R ligands.

ALLOSTERIC PROPERTIES OF AN ENGNEERED KANAMYCIN BINDING BETA-LACTAMASE

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Abstract

BlaKr is an engineered kanamycin-binding TEM-1 β-lactamase, which is regulated by binding of kanamycin or other aminoglycoside antibiotic to its allosteric aminoglycoside binding site (Van de Water et al., 2011; Volkov et al., 2011). The structure of BlaKr is well known (Volkov et al., 2011), unlike the structure of the BlaKr/kanamycin complex. Moreover, the yield of BlaKr isolated from Escherichia coli (E. coli) expression hosts is relatively low.

The first aim of this study was to maximize BlaKr expression, and increase the yield of purified protein. For this purpose, we selected two E. coli strains, phage-resistant BL 21 and C 43, to carry out BlaKr expression studies. Initially we constructed a master plate containing cultures of E. coli BL 21 and C 43 cells transformed with pET24(ompA)BlaKr plasmid. E. coli BL 21 cells were selected to carry out further expression studies after showing better BlaKr expression then the C 43 cells. However, the master plate approach was abandoned due to the lack of sustainable BlaKr expression level. Following that, we tried expressing BlaKr using fresh transformation with the BlaKr-containing plasmid under different culturing and incubation conditions (incubation temperature, time, and IPTG concentration). In addition, we acquired the active BlaKr directly from the periplasmic space through osmotic shock technique. This allowed us to identify the optimal BlaKr expression conditions (incubation at 20 °C for 24 hours, followed by application of osmotic shock). We managed to purify the obtained BlaKr expressed in LB medium.

The second aim of this study was the structural characterization of residues involved in the BlaKr/kanamycin complex formation. To achieve that, we carried out the expression of isotopic labelled BlaKr in M9 minimal medium under the same optimized incubation conditions. This resulted in a BlaKr yield which is 10 fold lower than that obtained in LB medium. Nevertheless, the amount of the purified protein was sufficient enough for two dimensional (2D) NMR experiments. The acquired spectra of the free BlaKr and BlaKr/kanamycin complex are being analyzed to solve the structure of the BlaKr-kanamycin complex.

Finally, we constructed BlaKr mutants with cysteine residues on their surface near the enzyme active site. The clones were obtained by site-directed, PCR-based mutagenesis. The presence of the inserted cysteine residues was verified by the DNA sequencing. Paramagnetic probes can be attached at the site of the inserted cysteine residues. Based on NMR spectroscopy, these paramagnetic probed BlaKr mutants will be provide an enhanced precision in detecting the structure of the BlaKr-kanamycin complex.

EXPRESSION, PURIFICATION AND CRYSTALLIZATION OF TWO PROTEINS INVOLVED IN BACTERIAL PERSISTENCE

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Abstract

Persister cells constitute an antibiotic-tolerant subpopulation of planktonic cultures and biofilm communities of several pathogenic bacteria, such as *Pseudomonas aeruginosa* and *Escherichia coli.* These cells survive prolonged exposure to high doses of antibiotics and are believed to seriously hamper effective treatment of infections. Mechanisms of persistence are currently poorly understood, and only few genetic determinants have been shown to be involved in persistence[1].

Based on a previously published high-throughput screening[2], our collaborators in Leuven identified new genes involved in persistence, and two of these gene products will be studied in current thesis:

EdpA is the first gene and is involved in the synthesis of an extracellular determinant of persistence. As its function is unknown, four constructs were received and tested for expression and protein production (pET24-EdpA-C-His tag, pET24-EdpA-N-His tag, pET24-EdpA-42-end-C-His tag and pET24-EdpA-42-end N-His tag). One of these is pET24-EdpA-C-His tag and was partially soluble but it formed large aggregates. The other two (pET24-EdpA-N-His tag and pET24-EdpA-42-end N-His tag) constructs were not expressed as soluble and the last (pET24-EdpA-42-end-C-His) was not expressed at all. Therefore, new constructs were generated starting from amino acid 60 (just after the hydrophobic helix) and a second one only containing the LmbE-like domain (starting amino acid 178). The constructs were tested for expression, the LmbE-like domain construct showed no sign for expression. The construct which started from a.a.60 showed low expression and probably not binding on a column.

The second gene is obgE. Previously four constructs were generated for obgE; full length-C-terminal His tag, full length-N-terminal His tag, 1-341a.a C-terminal his tag and 1-341a.a.N-terminal His tag,

The C-terminal domain which corresponds obgE-342-end is predicted to be intrinsically disordered. Therefore this construct was used for X-Ray crystallography analysis. Previously structure of 1-341N-terminal his tag solved with GDP and our goal to solve structure of 1-341-C terminal with GTP analogues.

ObgE341stop C- terminal His tag construct was purified and crystallized in the presence of GDP.AlF_x and GTPγS. These crystals diffracted to 3.4Å and 2.8Å respectively. However is not clear if any of the ligands is bound or not.

POST-TRANSLATIONAL MODIFICATION AND PROTECTING ACTIVITY OF ARABIDOPSIS DEHYDRIN ERD14 IN RESPONSE TO ABIOTIC STRESS

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Abstract

ERD14 is an Arabidopsis dehydrin which belongs to group 2 of LEA proteins. The increase in level of ERD14 expression in cells during abiotic stresses such as water deficit, cold, and high salinity has been indicated (Kiyosue et al. 1994, Nylander et al. 2001). In vitro experiment showed that ERD14 behaved like a chaperone protecting some model substrates including lysozyme, alcohol dehydrogenase, firefly luciferase, and citrate synthase against enzymatic activity loss or protein aggregation due to high temperature (Kovács et al. 2008). However, the precise function of ERD14 inside plant cells as well as its mechanism has not yet been elucidated, which intrigued us to conduct this study.

Wild type, ERD14 overexpression (C line) and ERD14 knock-out (KO5.14 line) Arabidopsis plants have been used as our materials. From our results, we confirmed that the C line were strongly overproduced ERD14 and that the KO5.14 line was a complete knock-out of ERD14. In wild type Arabidopsis, ERD14 was expressed under untreated as well as stress-treated conditions. ERD14 expression level depended on plant developmental stages. The accumulation of ERD14 was high in young seedlings and lower at the flowering stage. The treatment of plants with 180 mM Mannitol for 3 days was able to provoke enhanced expression of ERD14 in these plants. Meanwhile, 4oC and 100 mM NaCl treatments did not alter the degree of ERD14 abundance.

In both wild type and ERD14 overexpression plants, during normal as well as mannitol-constraint conditions, we found that ERD14 has been phosphorylated but not N-glycosylated nor sumoylated with Arabidopsis SUMO1. Still, there is a possibility of ERD14 O-glycosylation and sumoylation with other SUMO isoforms, which has not been examined. We suggest that phosphorylation plays a key role in the function of ERD14 and stress conditions might be involved in the modulation of this modification through which the activity of ERD14 will be adjusted.

After 1 hour of cold treatment, ERD14 overexpression plants have higher content of alcohol dehydrogenase with unknown reasons. Enhanced content of ERD14 was also directly or indirectly able to rescue alcohol dehydrogenase from denaturation due to high temperature in planta while ERD14 knock-out mutants and wild type plants could not. This could suggest that chaperone activity of ERD14 in vitro holds true under the physiological condition inside plant cells.

The protecting effect of ERD14 against dehydration condition was revealed in our drought-tolerance tests. The results support that overexpressing ERD14 confers plants enhanced water deficit tolerance while knock-out of ERD14 leads to severe drought-sensitivity. However, the exact role of ERD14 during dehydration in planta still remains unclear. The mechanism could be via chaperone activity of ERD14 which protects other macro molecules from the dehydration-mediated denaturation. Finally, loss of ERD14 triggered the delay in germination of seeds, suggesting an effect of ERD14 towards seed germination, possibly through its role during dehydration period of seed development.

METHIONINE SULFOXIDE REDUCTASE A (MsrA) IN THE REPAIR OF OXIDIZED PROTEINS IN THE PATHOGENIC ACTINOMYCETE CORYEBACTERIUM DIPHTHERIAE

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Abstract

Methionine sulfoxide reductase A (MsrA) is an oxidoreductase, which specifically reduces the S-epimeric form of methionine sulfoxide (Met-SO). The catalytic mechanism of the Met-S-SO reduction has been shown for both E. coli and bovine MsrA. So far, not much is known about MsrAs of Actinomycetes, as only a few experiments have been described. This study focuses on the characterization of MsrA of the pathogenic Actinomycete Corynebacterium diphtheriae. We cloned and purified Cd-MsrA and showed that for the reduction of L-Met-SO recombinant Cd-MsrA is coupled to the thioredoxin/thioredoxin reductase (Trx/TrxR) pathway, which suggests that Cd-MsrA uses the Trx/TrxR pathway in vivo. With reversed phase chromatography and fluorometry, we showed that the reaction is associated with structural changes in Cd-MsrA. Cd-MsrA has four cysteines and we constructed four single Cys mutants (C52S, C87S, C206S and C215S). In kinetic assays, we showed that three cysteines Cys52, Cys206, and Cys215 are essential for the reduction of L-Met-SO, while the absence of Cys87 has no effect on the catalytic reduction of L-Met-SO. Apart from that, we crystallized Cd-MsrA and solved its structure to a resolution of 1.9 Å. In the active site a disulfide bond is formed between the nucleophilic Cys (Cys52) and the first resolving Cys (Cys206). Further, in the active site a cacodylate molecule was observed. This molecule mimics the L-Met-SO substrate by forming the same stabilization interactions with the active site residues as described for the crystal structure of Neisseria

meningitidis MsrA with AcMetSONHMe bound. Furthermore, in vitro experiments showed that after reducing L-Met-SO, Cd-MsrA reacts with mycothiol, the low molecular weight thiol of Actinomycetes, which suggests that the mycothiol-mycoredoxin1-mycothione reductase pathway might be used as an alternative reducing system next to the Trx/TrxR pathway. All in all, our results suggest that Cd-MsrA is using a disulfide relay mechanism (Cys52-Cys206, Cys206-Cys215) to expose the oxidative equivalents, ready for Trx to be reduced, and we trapped Cd-MsrA during the first step (Cys52-Cys206) of its resolving mechanism.

GENETIC AND FUNCTIONAL STUDY OF RSPO1, 2 AND 3 IN BONE FORMATION

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Abstract

Osteoporosis is a well-known medical and socio-economic burden characterized by low bone mineral density, reduced strength and increased susceptibility to fragility fractures. Despite the availability of effective medications that manage to slow down the process of bone loss, mainly anti-resorptive drugs, the research focuses towards other therapeutic approaches. These approaches are mainly targeting components of the canonical WNT signaling pathway involved in bone formation. In this respect, studies performed in the field of sclerosing bone dysplasias, characterized by abnormal bone formation, allowed the identification of potential targets in the treatment of osteoporosis. For instance, mutations in the low density lipoprotein receptor-related proteins (LRP5 and LRP4) and sclerostin (SOST) genes have been identified by studies of these monogenic bone disorders. Anti-sclerostin monoclonal antibodies are already in clinical trials phase III with promising results as a future osteoporosis treatment strategy. Recently, the R-spondins (RSPOs), a novel protein family was associated with activation and regulation of the WNT pathway. Several studies attest their role as extracellular agonists of this pathway, but their involvement in bone has not yet been studied in detail. Therefore, within the current research, we focused on the RSPO gene family and we aimed to elucidate the effect of RSPOs on the canonical WNT signaling pathway, a major pathway in bone formation.

Mutation analysis on high bone mineral density (BMD) affected patients resulted in the identification of several variations in intronic regions that are not likely to be disease causing. However, we cannot exclude that large deletions or mutations in non-coding regulatory regions which result in a decreased expression of either RSPO1, 2 or 3 are disease causing. Association studies of the SNPs between two Odensee Androgen Study Young (OASY) cohorts with most extreme BMD values did not reveal any statistically significant difference ($p < 0.05$) in their genotype frequencies. However, there was one SNP in RSPO3 exon 1, rs140821794, p.M16V (MAF = 0.0233) that occurred only in one member of the low BMD cohort. Functional study of the RSPOs in bone formation revealed that RSPOs seem to partially rescue the signaling pathway from the effect of sclerostin in both HEK293T and Saos-2 cells. Furthermore, our results prove RSPO3 to be the most powerful enhancer among the RSPOs leading to the

activation of the canonical WNT pathway and subsequently to bone formation in both investigated models.
The present study contributes to a better understanding of the pathway and the role of RSPOs in bone formation. However, further research is needed to fully understand this subject and the exact mechanism through which the RSPOs act.

CHARACTERISATION OF AVIAN PATHOGENIC Escherichia Coli (APEC) ISOLATES INVOLVED IN POULTRY COLIBACILLOSIS

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Abstract
Colibacillosis in poultry is an infection causes by Avian Pathogenic Escherichia coli (APEC). Its prevalence among poultry is increasing. The aim of this study was to have a better insight in the population of APEC infecting Belgian poultry. O-antigen serotyping was done using 31 different antisera. Positive strains were grouped among the APEC group (O1, O2 and O78) and the "Other" APEC group. Strains negative for all these serotypes were not typeable (NT). The isolates were grouped into A, B1, B2 and D phylogroups. PCR were performed to detect virulence genes. Antimicrobial susceptibility was tested by micro-broth dilution (TREK diagnostics sensititre plates) and using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints. Multi drug resistance (MDR) was described as resistance to more than three classes of antimicrobials. Isolates, 76, could be classified to a single serogroup, of which 39 belonged to the APEC group (O1, O2 and O78), 37 to "Other" APEC and 33 to NT APEC. APEC strains mostly belonged to phylogroups D (38%) and A (36%); 20% belonged to B1 and 6% to B2. Phylogroups were all distributed across NT APEC and "Other" APEC whereas the APEC group was composed of only phylogroups A and D. Phylogroups A and D were equally distributed among poultry (37%). Genes coding for adhesions factors (fimA, fimH, fimAvMT78), iron acquisition systems (iutA, iucA, iroC, fyuA and irp2), papC and felA genes were present and highly distributed among serogroups and phylogroups. They were mostly detected among the APEC group and phylogroup D (respectively 10 and 11 genes showed prevalence higher than 30%). PapGI and prsGIII genes were not detected at all. Most of the isolates were resistant to ampicillin (63%), ciprofloxacin (56%) and nalidixic acid (52%); they all remain susceptible to florfenicol. MDR represented 51% of isolates (n=56), of which 24 belonged to phylogroup A, 22 to D, 7 to B1 and 3 to B2; 22 belonged to NT APEC, 21 to APEC group and 13 to "Other" APEC; 28 were observed among broilers, 16 among breeders and 7 among layers. In conclusion, APEC strains showed high prevalence of virulence factors that might be associated with greater pathogenic potential. APEC strains belonging to group B2 and D phylogroups were identified; these phylogroups are associated with urinary tract infections in humans. These bacteria are potentially zoonotic and may act as reservoirs of resistance and virulence genes. With the high antimicrobial resistance, it is important to not only preserve our current reservoir of antibiotics but also to research into new potential alternatives.
Keywords: APEC, serotype, phylogroup, virulence gene, antimicrobial susceptibility.

Characterization of activity-modulating nanobodies against the translation-inhibiting proteins Doc and MazF

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Abstract

Toxin-antitoxin (TA) modules are small operons that are ubiquitous in bacterial genomes and are involved in stress response and persister cell formation. In this thesis, the effects of nanobodies raised against two TA toxins are studied: MazF from Staphylococcus. aureus (MazFsa) and Doc from the E. coli bacteriophage P1. The MazFsa toxin of the S. aureus MazEF TA module is a specific endoribonuclease that cleaves mRNA and modifies ribosomes to translate preferentially leaderless mRNA. It is neutralized by its corresponding antitoxin MazEsa antitoxin. The Doc toxin of the phd/doc addiction module of bacteriophage P1 on the other hand inhibits translation through phosphorylation of the elongation factor Tu. Nanobodies against Doc and MazFsa were produced and characterized. ITC results show a high binding affinity between these toxins and the respective nanobodies.

Three nanobodies against MazFsa were characterized, all of which at least partially inhibit the ribonuclease activity of MazFsa and none are able to activate this activity in presence of MazEsa. A single anti-MazFsa nanobody binds per monomer of the MazFsa homodimer. The crystal structure of one such co-crystallized complex shows that anti-MazFsa nanobody 9(Nb9MazF) occupies part of the region of overlap of the MazEsa and the RNA binding sites on MazFsa, partially inhibiting MazFsa endoribonuclease activity as observed in the MazFsa RNase assay. Several complexes were crystallized and the corresponding crystal structures will provide more understanding of the nanobody molecular epitopes on the MazFsa proteins in rationalizing the inhibitory properties of these antibodies.

Similarly, of the seven anti-Doc nanobodies tested, two are capable of complete inhibition of Doc phosphorylation activity, but again none are activating. ITC results show a stoichiometry (n) of 1 depicting a single anti-Doc nanobody binding of the Doc. The observed Doc-anti-Doc binding affinity ranges between KD = 2.5 - 377 nM. This however is much lower than the Doc-Phd52-73 binding affinity (KD = 0.0017 nM) obtained in the same study. Again, Doc could be crystallized in presence of several of the nanobodies. As there is currently no crystal structure available of Doc in a state where it is not inhibited by Phd, the non-inhibiting nanobodies may for the first time allow us to observe the active site of Doc in its free state.

DNA DAMAGE IN HESC CULTURED ON FEEDER-FREE CONDITIONS: THE IMPACT OF CELL DENSITY

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Abstract

The capability of human embryonic stem cells (hESC) to differentiate to all kind of cell types holds a very promising future for clinical applications. However, hESC's potential is hampered by the genetic aberrations which repeatedly reported in hESC. We investigated the correlation between cell density and DNA breaks in hESC kept in a feeder-free culturing system. The experiments were conducted in two feeder-free systems, Laminin-521/NutriStem® and MatrigelTM/NutriStem®. We found that the feeder-free culturing systems induce fewer DNA damage in the cells as compared to the feeder-based system. Cell density also had no effects to DNA damage in feeder-free system while it did in feeder-based system. Furthermore, accumulation of lactic acid and decreasing pH did not correlate with DNA damage in the feeder-free system as it did in the feeder-based system.

OPTIMIZATION OF THE EXPERIMENTAL SET-UP FOR THE STUDY OF THE SELECTIVE ADVANTAGE AND DIFFERENTIATION CAPACITY OF CHROMOSOMALLY ABNORMAL HUMAN EMBRYONIC STEM CELLS

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Abstract

To carry out the study of the selective advantage and differentiation capacity of chromosomally abnormal hESCs, preparatory experiments and optimizations of the techniques for analyses need to be conducted. In this thesis, we carried out several of these preparatory work. HESC sublines carrying 18q deletion and 20q11.21 duplication were inserted with GFP gene through lentiviral transduction to be used to study the dynamics of culture take-over and spontaneous differentiation capacity of these chromosomally abnormal hESCs. A set-up for cell competition assay was designed and tested, and differentiation of hESCs both spontaneously (mixed embryoid body formation) and lineage directed [towards osteoprogenitor like (OPL) cells] were tried. Also, techniques such as immunofluorescent stainings for GFP (to enhance detection), and for the detection of protein expression pluripotency markers NANOG and POU5F1 in

fresh hESCs and OPLs, and combination of GFP with POU5F1 and NANOG with POU5F1 in paraffin embedded hESCs and tissue were optimized.

Chromosomally abnormal hESC lines expressed GFP few days after lentiviral transduction but its expression was not always detectable under the microscope. Immunofluorescent staining enhanced GFP detection but did not fully discriminate GFP positive cells from the negative cells. An alternative way for analysis was used to analyze samples from cell competition assay. Percentage of hESCs carrying 18q deletion showed increasing trend in every passage indicating its ability to take-over the culture because of its acquired selective advantage. Spontaneous differentiation of mixed hESCs to EBs and optimization of the immunofluorescent staining for analysis were not successful. While the immunofluorescent staining protocols for analyses of the protein expression of the pluripotency markers NANOG and POU5F1 were optimized for fresh hESCs and OPLs and for paraffin embedded hESCs and tissue.

FUNCTIONAL ANALYSIS OF ARABIDOPSIS THALIANA TETRASPANIN GENES IN PLANT DEVELOPMENT

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Abstract

Approximately 35,000 genes have been discovered by genome sequencing in Arabidopsis thaliana completed in 2000, yet a number of genes have still uncharacterized functions. Our project was about the investigation of TETRASPANIN genes function in plant development using Arabidopsis. TETRASPANINS contain four transmembrane (TM) domains, which delimit cytoplasmic amino and carboxy terminal domains, an intracellular loop, small extracellular loop (SEL) of 13-30 amino acids, and a large extracellular loop (LEL).These domains are crucial sites of interactions during biosynthesis and assembly of the network known as the "TETRASPANIN Web". In animal, partnerships between proteins can be formed with the extracellular or the intracellular domains of the interacting molecules. During Immune signalling formation the CD81 is known to be necessary for the reorganization induced in response to B-cell receptor (BCR) and interact with CD19/CD21 complex, both are member of immunoglobulin superfamily. The tetraspanin protein family contains 33 members in humans, 37 in Drosophila and 17 in Arabidopsis. The phylogenetic tree of Arabidopsis TETRASPANINS revealed that most genes are duplicated. The aim of this project was to study gene TET function in plant development using insertion mutants requested from NASC grown in normal conditions. Homozygous lines were identified by segregation analysis and genotyping and studied with a focus on root, leaf and flower development. Interestingly, our analysis of TET2 showed a pronounced leaf phenotype implying a function in leaf development, while TET10 and TET13 control primary and lateral root development. For TET3, TET4 and TET5 genes, no phenotypes in single mutants were observed. Double mutants were used

to study gene redundancy of TET5 and TET6 and showed significant effects on primary and lateral root development. In conclusion, TET gene function needs to be studied not only by single mutants but also by double and/or triple mutants, and a wider range of phenotypic assays has to be used in the future.

MAIZE GENOTYPES, B104, F047, F052, F7, MO17, CML91, H99, W153R, MINIMAIZE: COMPETENCE FOR AGROBACTERIUM TUMEFACIENS INFECTION, HYGROMYCIN AND PHOSPHINOTHRICIN SELECTION, IN VITRO REGENERATION AND DEVELOPMENTAL ANALYSIS

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Abstract

The maize transformation platform at the department of plant systems biology generates transgenic maize lines for basic, translational and biotechnological research. Agrobacterium tumefaciens mediated transformation is carried out on the maize genotype B104 and the bar selectable marker gene is used for subsequent selection of transformed embryogenic calli on phosphinothricin. Currently the platform uses only one genotype and selectable marker, thus there is need to enlarge the toolbox for maize transformation by screening for additional genotypes and selectable markers. The hpt selectable marker gene was analyzed in this study as well as several genotypes.

Analyzing the hpt selectable marker gene entailed first selecting non-transformed immature embryos derived from B104 on media containing different concentrations of hygromycin to see which concentration efficiently inhibited their growth. It was established that an ideal selection scheme was one where embryos were selected on media that had a gradual increase in the concentration of hygromycin because more calli survived compared to when selected on media where the hygromycin concentration was drastically increased. The next step was to subject immature embryos transformed with the hpt gene to the selection schemes determined in the first step and compare the frequency of transformation which was calculated as the total number of hygromycin resistant T0 shoots per total number of co-cultivated immature embryos. This varied between 4 and 8% depending on the selection scheme. Strikingly, the frequency of transformation for the hpt gene was higher than that of the bar gene and thus the hpt gene is a suitable selectable marker for use at the maize transformation platform.

Seven genotypes were tested alongside B104 to determine which of these could replace it in field trials. First, their competence to Agrobacterium infection was determined and those that were found to have very low competence were eliminated from further analysis. The remaining genotypes were tested for their regeneration capacity and again those that had a good frequency of regeneration were used for the final part of the experiment which involved carrying out stable transformation with the bar selectable marker gene. None of the genotypes selected for stable transformation produced transgenic T0 shoots.

Thus a better alternative for field trials would be to use hybrids derived from these genotypes and transgenic B104 because on-going research using such hybrids shows that they perform much better in temperate climate.
B104, which is used for phenotyping at the phenomics platform at PSB, grows to a height that is too tall to fit in the imaging cabins thus analysis at advanced stages is challenging. Conversely, the genotype mini-maize is fast flowering and grows to a maximum height of approximately 30 cm. Therefore, in this study, B104 was crossed with mini-maize and developmental analysis of B104, mini-maize and the F1 hybrids derived from crossing these two genotypes was carried out to determine whether the hybrids could replace B104 for phenotyping. However, the F1 hybrids were found to grow to a height that was almost the same as that of B104 and thus they did not provide a solution to the phenomics platform.

CHARACTERIZATION OF GRRP1 A NOVEL GENE INVOLVED IN EMBRYONIC STEM CELL DIFFERENTIATION

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Abstract

Dramatic transformations take place after the implantation of the mouse embryo into the uterine wall. The primitive streak (PS) formation is a fundamental step in the initiation of gastrulation. In gastrulation the three primary germ layers are clearly differentiated in ectoderm, endoderm and mesoderm. Gene expression changes are involved in every step of embryo development, and continuous developmental research points at functional characterization of new genes discovered. Embryonic Stem cells (ES cells) are used as a tool in several developmental biology studies. Pluripotency and self-renewal capacity of ES cells provide a source for specific mutations and functional approach studies.
In order to get a better understanding of the changes in gene expression that drive to the induction, formation and patterning of mesoderm, numerous studies have been performed by the Cell Genetics Laboratory of the Vrije Universiteit Brussels. Genes involved in the induction of mesoderm were identified by microarray analysis [Dakou, 2009]; and eight genes selected from this study were localized in the mouse embryo through whole-mount in situ hybridization (WISH) [Amys, 2010]. Among these genes, Glycine/Arginine Rich Protein 1 (Grrp1) was observed at 6.5 days post coitum (d.p.c.) in the region of PS formation, but there is no molecular function, biological process or cellular component localization described yet.
In the present study ES cell cultures were performed in order to characterize Grrp1 expression. The formations of Embryoid bodies (EBs) allowed determining the expression of Grrp1 by means of qRT-PCR. Results on time course experiments showed that Grrp1 started at day 3 and reached a peak at day 4 of EB differentiation, and when EBs size was manipulated Grrp1 is expressed differentially according ES cell fate triggered by different EB sizes.
Finally, signalling pathways play an important role in ES cell differentiation, previously in Willems (2007) studies, he probed that Wnt/β catenin, Nodal/Activin and BMP pathways were involved in

mesoderm induction and patterning. In this study, ES cell differentiation in monolayer cell culture with chemical activators resulted in the activation of Wnt/β catenin, Nodal/Activin and BMP pathways. Data demonstrated that Grrp1 expression is only upregulated in the induction of mesoderm triggered by Wnt/β catenin pathway activation.

MOLECULAR EPIDEMIOLOGY AND EVOLUTION OF HUMAN CYTOMEGALOVIRUS

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Abstract

Human cytomegalovirus is known to establish persistent but asymptomatic infections in the infected host. As a prerequisite, such a lifestyle can be established following an exquisite adaptation of the virus to the host. In order to evaluate the molecular mechanisms that have shaped the evolution of HCMV genomes, we have implemented an approach that makes use of full genome sequences obtained from high-throughput sequencing of 107 clinical samples (urine and nasopharyngeal swabs). Through-put sequencing using NGS platforms generates ambiguities in the length of homopolymeric tracts especially around heterogeneous regions of the HCMV genome. Using Sanger sequencing, we were able to determine the most abundant length of each homopolymer tract as well as the continuity of the sequence over the homopolymer.

With growing evidence suggesting that extensive cell culture passaging results in ORF disrupting mutations in genes that are inhibitory or non-essential for growth in that cell type, we examined ORFs revealed to contain ORF disrupting mutations from our dataset of complete HCMV genomes by sequencing directly from clinical isolates. All analysed mutants were confirmed as being already present in the clinical isolate. Our data therefore did not identify any ORF disrupting mutation that occured during limited cell culture passaging

Of the 170 HCMV genes examined, more than 95 % had a dN/dS ratio of amino acid substitutions less than 0.6, which indicates the level of purifying selection on the HCMV genome. Overall, HCMV genomes are under negative selection with some level of positive selection acting on individual sites within 14 genes (8.2 %), most of which have host immune modulatory functions. Similarly, genome wide phylogenetic analysis suggests that recombination has contributed to the divergence of the HCMV species and most of the circulating HCMV strains already exist as recombinants ($\Phi = 0.00$).

These data thus provide evidence that the molecular evolution of HCMV, a ubiquitous virus with a large dsDNA genome is kept stable under purifying selection within the host. Our results further offer insights into the virus evolution, which is shaped by recombination. In the future, linking the described viral diversity and the variation in coding capacity to clinical data from the infected host could lead to novel

insights in determinants of viral pathogenicity. These results could be valuable for clinical diagnostics and the development of antivirals and vaccines.

IN VITRO AND IN VIVO CHARACTERIZATION OF HUMAN IgG1 Fc TARGETING NANOBODIES

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Abstract

Advances in antibody engineering have made it possible to produce chimeric, murine-human or fully human monoclonal antibodies of different isotypes. A number of these chimeric antibodies have been approved by food and drug authority (FDA) in treatment and prophylaxis of numerous clinical disorders including tumor. Therefore it would be of great importance to develop a tool for monitoring the preclinical pharmacokinetics and in vivo targeting potential of these humanized antibodies towards their targets. This could be approached by use of nanobodies, a single domain fragment derived from camelid heavy chain antibody. As the nanobodies can be selected to specifically targeting Fc fragment of these antibody isotypes. On the other hand nanobodies can easily be radio-labeled with a short half life radionuclide contrary to direct antibody labeling which would require use of long half life radionuclide due to their slower clearance rate.

This study focuses on generation and characterization of nanobodies that can specifically bind to humanized antibodies of IgG1 subtype, applied in clinical cancer therapies. Trastuzumab, which targets the breast cancer marker HER2 was used as model. In a first phase of the study the VHH gene segments of the 5 presumable human IgG1-specific nanobodies were recloned from the pHEN4 phagemid vector to the pHEN6 plasmid vector. In a next phase, the 5 presumable human IgG1 binders and the control nanobody BcII10 were produced in bacteria, extracted from the periplasm and further purified via IMAC and gel filtration in PBS and most of produced nanobodies had a good yield.

In vitro studies such as ELISA, surface plasmon resonance analyses and quickpick IMAC capturing experiments were performed to investigate the ability of produced nanobodies to specifically target Trastuzumab. The result indicated that most of the nanobodies could bind to Trastuzumab very well. SPR epitope mapping experiments showed that all the nanobodies competed with each other, as they all targeted a nearby (overlapping) epitopes. In vivo studies were also performed to test 99m-Tc labeled nanobodies ability to track Trastuzumab, intravenously injected into C57BL6 mice. This study showed a good nanobody clearance in naive mice (without Trastuzumab pre-injection) and a reduced clearance rate in mice that received 20 µg of Trastuzumab pre-injection before labeled nanobody administration. Finally measurement of the ability of Trastuzumab to target HER2-expressing tumors was also evaluated, however the results were not very conclusive.

SITE-SPECIFIC LABELING OF NANOBODY® BASED IMAGING PROBES VIA SORTASE TECHNOLOGY

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Abstract

In this master thesis the sortag LPETGG was introduced at the C-terminal end of Nbs, upstream of the His6-tag. This allowed the further use of the His6-tag for convenient purification of the Nbs, while the His6-tag was lost upon conjugation. Site-specific sortase-mediated conjugation of Nbs was optimised with the bifunctional chelators GGGYK-CHX-A''-DTPA and GGGYC-Maleimide-DTPA (functionalized with tri-glycine). These chelators are suitable for radiolabeling with the radionuclide 111In, which is used for SPECT. A proof-of-concept was generated in the human epidermal growth factor receptor type 2 (HER2) cancer model.

After optimization of the reaction conditions, the HER2-targeting Nb 2Rs15d was successfully conjugated to GGGYK-CHX-A''-DTPA and GGGYC-Maleimide-DTPA. The new constructs were first biochemically characterized in vitro. Recombinant HER2 antigen was bound with equal nanomolar affinity as the unconjugated 2Rs15d. The conjugated Nb labeling with 111In was efficient and obtained high radiochemical purity. HER2-positive BT474M1 cells could be specifically bound by both 2Rs15d-CHX-A''-DTPA-111In and 2Rs15d-Maleimide-DTPA-111In. As CHX-A''-DTPA is described to form more stable complexes with 111In with higher kinetic inertness compared to normal DTPA, the functionality of Nb 2Rs15d-CHX-A''-DTPA-111In was finally evaluated in vivo in a biodistribution study in BT474M1-xenografted mice. The HER2-targeting Nb showed a significantly higher tumor uptake with 2Rs15d-CHX-A''-DTPA-111In (5.03 ± 3.36 %IA/g) compared to the control Nb cAbBcII10-CHX-A''-DTPA-111In (0.35 ± 0.25 %IA/g) at 5h post injection. Moreover high T/B ratio and T/M ratios were obtained. The Nb retained thus its functionality as an optimal imaging tracer after site-specific labeling via sortase.

These results provide a start for the further development of a general applicable strategy for the site-specific labelling of Nbs for use in molecular imaging.

TRANSCRIPTIONAL REGULATION OF ARGININE TRANSPORT GENES IN *Escherichia coli*

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Abstract

wo of the three L-arginine specific uptake systems in E. coli (arginine specific transport, and the Arginine-Ornithine system) are encoded by the artJ-artPIQM gene cluster which expression is negatively regulated by arginine, through ArgR mediated transcription repression. Genome-wide studies indicate that Lrp plays a role in transcription regulation of artJ and artP and that ArgR is involved in control of lrp expression. Our in vitro binding experiments indicate that binding of Lrp to the artJ and artP control region is sequence-specific and that exogenous L-leucine reduces the affinity of Lrp for both control regions. Identification of protected zones in the artJ and artP control regions by binding of Lrp and binding interference assays between ArgR and Lrp indicate that in the case of artJ, ArgR prevents Lrp from binding to the control region, while in the artP control region both proteins were able to bind the same DNA molecule. A high resolution map of the ArgR binding site in the lrp control region was established by enzymatic and chemical footprinting experiments and missing contact probing. Mapping lead to the identification of two 18 bp ARG boxes separated by 3 bp located from +27 to +66 downstream of the transcription start. This is an unusual location for the ArgR binding site. All previously identified ArgR binding sites are located upstream of the transcription start, mostly overlapping the -10 and/or -35 promoter elements. In vivo single-copy reporter gene analysis indicates that Lrp is negatively autoregulated. L-leucine had an opposite effect on Lrp autoregulation, reducing the negative effect exerted by lrp expression. Negative autoregulation thus appears to be of the reciprocal type. ArgR effects on lrp expression were not observed.

POSSIBLE ROLE OF THE RETROGRADE (RTG) REGULATORY PATHWAY IN THE TOLERANCE OF Candida albicans TO FLUCONAZOLE

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Abstract

Candida albicans is the cause for the majority of candidal infections in humans causing mucosal (superficial) infections such as oral or vaginal candidiasis and invasive (or life threatening systemic) infections. C. albicans infections are commonly treated with Fluconazole, a triazole fungistatic agent which blocks the biosynthesis of ergosterol by a high selective inhibition of the enzyme Lanosterol 14-α-demethylase (Erg11p). Resistant strains have also emerged and one of the mechanisms that lead to this resistance is via activation of stress responsive pathways. Some of these pathways are closely related to the retrograde pathway which is hypothesized to be involved in C.albicanstolerance to Fluconazole, This hypothesis was put forth by Fiori in 2012 who observed that over expressing the retrograde pathway genes (RTG1 and RTG3 genes) enhance fluconazole suppression.

We therefore in this study investigated the effect of Fluconazole on C. albicans strains in which the RTG1or RTG3 gene has been knocked down or knocked out.

We started with knock down experiments and we used the MET3 promoter to knock down single alleles of these genes. Results showed no phenotypic difference between knock down strains and wild type.

Next, we proceeded to knock out experiments. We attempted to delete the RTG3 gene via Li Acetate transformation but we didn't succeed to obtain a clone in which the gene was deleted despite several attempts and screening of 99 colonies. Never the less, using knockout strains obtained from a different laboratory, we observed no significant difference in susceptibility between the knockout mutants and the wild type in spot test experiments we performed. These results were strongly coherent with MICs tests that we also carried out.

In as much these observations were similar to those of another research team who had conducted a similar study on these genes, the results were contrary to our expectation.

We suggest that more careful and intensive studies be carried out on these particular transcription factors in order to ascertain the conclusions on their characteristics especially taking into account the fact that another research team though having contrary observations to our observation, did not carry out enough studies (based on their publication) to supplement their observation.

SELECTIVE GLUCOCORTICOID RECEPTOR MODULATOR EFFECTS ON HSP70 IN MULTIPLE MYELOMA CELLS

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Abstract

Multiple myeloma (MM) cells are malignant plasma cells that produce abundant monoclonal antibodies. Multiple myeloma is incurable disease and current treatments offer only an average survival of 3 years. Current treatments involves glucocorticoids (GCs) (dexamethasone) which is used as a chemotherapy alone or in combination with other drug such as bortezomib, lenalidomide and thalidomide. Classical GCs acts in both transactivation and trans-repression but has many side effects. Transactivation results into most undesirable effects while trans-repression produce desirable effects.

Researchers are continuously searching for better treatment to improve and increase survival of MM patients. To avoid or reduce side effects of GCs researchers are also looking for selective glucocorticoids, which will act only on trans-repression but not on transactivation. The discovery of CpdA which is a selective glucocorticoid receptor (GR) modulator that acts only on trans-repression caught the researchers' attention. CpdA was thought to regulate Hsp70. Hsp70 is a chaperone and also participate in a pathway that leads apoptosis. Because MM cells grow, divide and expand enormously, targeting the Hsp70 which may lead to apoptosis of MM cells will be an ideal strategy to reduce MM cells.

In this thesis we performed several experiments to determine the effects on CpdA on MM cells. MTT assay was performed to check for cell viability. Knowing that, CpdA was able to reduce cell viability with concentration of above 10-5 µM CpdA, we then performed western blot to analyze the effects of CpdA on protein expression level of glucocorticoid receptor (GR), because when CpdA diffuse into the cell, it binds to GR and mediate is effects. We also performed western blot on Hsp90, because Hsp90 supports tumor cell growth, and western blot on Hsp70 in OPM-2(MM cells) and leukemia's sister cells CEM C7-14 and CEM C15-15. ELISA was performed on Hsp70 in OPM-2 cells was appeared to be upregulated. To analyze the effects of CpdA on genes coding for Hsp70, qPCR was performed on several gene that codes for Hsp70 on OPM-2, CEM C7-14 and CEM C1-15.

To learn more about Hsp70 regulation, we use Eg5 inhibitor (Dimethylenastron) which is kinetin mitotic arrest. OPM-2 and MM1.S (both are MM cells) were treated with 20mM Eg5 inhibitor and cell viability was determined by MTT assay. We then analyzed the effects of Eg5 inhibitor and CpdA plus Eg5 inhibitor on the protein expression levels of GR, Hsp90 and Hsp70 in OPM-2 and MM1.S cells by western blot.

In conclusion CpdA was able to reduce cell viability and upregulates Hsp70 on OPM-2 and MM1.S cells. Although CpdA showed the expected results it still not convincing to be used as a therapeutic drug because of its instability and high concentration dose. However, CpdA can be used as an experimental tool for research purposes. As for MM, there are couples of drugs which are in clinical trial as potential

MM treatment with promising results such as elotuzumab and Iomab-B which are monoclonal antibodies and they target a cell surface antigen.

"CONVERSION OF NANOBODIES TO FLUORESCENT PROBES WITH CONSERVED SIZE AND FUNTIONALITY"

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Abstract

Thanks to their well-known properties, Nanobodies are excellent candidates as targeting molecules in many bio-analytical disciplines. To be used as an analytical tool, they must be rendered traceable. One possibility is to make them fluorescent. This should be achieved in a controlled way and without loss of the Nbs' particular biological and physical properties, of which their specific affinity for an antigen combined with a minimal size is the most important asset. The classical way to render proteins fluorescent is to chemically conjugate them with fluorescent dyes, but difficulties to control this reaction in a site-directed way (to maintain binding properties) made us consider another approach. Indeed, a supplemental advantage of Nanobodies is that they are easily manageable for genetic modification, and recent publications report that the introduction of particular amino acid sequences in proteins can make them fluorescent. More particularly, the DW(L)-tag, coordinated with Terbium3+, has time-resolved fluorescence properties. Time-resolved fluorescence has the advantage that background fluorescence can be largely eliminated, leading to excellent signal-to- noise ratios. Moreover, the DW(L)-tag is bifunctional because it can be used for affinity chromatography of the protein as well.

We genetically converted Nanobodies to fluorescent terbium-binding probes. To this end, a five stage-strategy was followed, including: 1° the construction of a novel pDPMECS vector coding for the DW(L) affinity tag instead of the classical HA-His tags on Nbs; 2° cloning the well-known Nb models BCII10 and anti-HER2 into this newly constructed plasmid; 3° production and purification of the newly generated DW(L)-tagged Nbs by Terbium immobilized lanthanide affinity chromatography; 4° determining the specific affinity of the newly designed Nbs for their antigen and 5° characterization of the fluorescence properties.

As a result, an "empty" pDPMECS plasmid coding for the DW(L)-tag was generated, and four DW(L)-tagged Nbs were generated: one Nb, the BCII10, against lactamase and three anti-HER2 Nbs. The production and purification of these Nanobodies was proven to be comparative with the common IMAC procedure for classical Nanobodies, in both the ease and yield. Preliminary fluorescence measurements on the Nb-DW(L) using a time-gated method resulted in an expected excitation and emission spectrum together with a detection limit at 30 nM. Although further optimization and extension in clinical

applications are required, we have proof of principle for a bifunctional tag on Nbs, which could become an essential analytical tool in the future.

NANOBODY BASED PROXIMITY LIGATION ASSAY FOR HUMAN TOXOCARIASIS

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Abstract

Human toxocariasis is the disease caused by the dog worm Toxocara canis. Both man and dog get infected accidently by ingestion of the embryonated egg containing L2 larva stage which hatches in the intestine to release the motile larva. Only in the dog the larva is able to molt and develop into adult worm which lays eggs and continues the life cycle, while in man, the larva doesn't undergo any development or multiplication it remains at L2 stage and continue migration through the whole body causing disruption. The current method of diagnosis is the enzyme linked immunosorbent assay (ELISA) which depends on detection of the antibody in the patient serum using standardized antigen (TES) but the limitation of this method is the low specificity and sensitivity.

By using nanobody instead of antibody, this will improve the specificity and affinity. Using higher sensitivity technique like proximity ligation assay (PLA), will amplify the signal of the reaction and improve the sensitivity.

In this context, we need to produce nanobody in the form of proximity probe. For this purpose it is essential to tether an oligo-nucleotide to the target-specific nanobody and this linkage we are going to evaluate via three methods;

1. Azide alkyne cycloaddition
2. Thiol-maleimide coupling
3. The SNAP-tag coupling

In the Azide alkyne cycloaddition method a modified amino acid with a terminal alkyne group is inserted in the nanobody sequence. This can be achieved by making use of a special archaeal plasmid (pEVOL) which encodes for a mutated tRNA that can bind the modified amino acid and insert it at the amber stop codon in the mRNA reading sequence. The oligonucleotides coupled to azide group reacts with the nanobody with the terminal alkyne in presence of Cu (I) as a catalyst and results in formation of 1, 2, 3-triazoles.

Concerning the thiol-maleimide coupling method, additional terminal Cys should be inserted in the sequence of our nanobody as a source of –SH group. This can be achieved by recloning of the nanobody in pHEN25 vector. Due to the high reactivity of –SH group, it tends to dimerize with each other. Both monomers and dimers should be treated with a reducing agent just before reacting with maleimide to free the –SH group for the coupling reaction.

The SNAP-tag coupling method depends on modification of a human DNA repair enzyme called O6-alkylguanine-DNA alkyltransferase (AGT) to form SNAP-tag protein. The principle of coupling reaction

depends on formation of SNAP-tag fusion with the protein of interest followed by reaction with the substrate O6-benzylguanine (BG). The reaction results in removal of guanine as leaving group and formation of stable thio-ether bond between benzyl group and SH- from Cys residue at the active site of SNAP protein. Benzyl group can be linked to whatever required label as biotin, fluorophore, nanoparticle or DNA strand. The reaction is independent on the fused protein and has the advantages of being specific, stable, quantitative and irreversible with high reaction rate.

THE STUDY OF SPECIFIC METABOLIC PROCESSES AS INNOVATIVE TARGETS FOR COMBATING BACTERIAL BIOFILMS

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Abstract

Although bacteria are single celled organisms, they spend a substantial amount of their lives as part of complex multicellular communities, called biofilms. Being part of a community has many advantages for a cell because it provides protection against environmental stresses such as heat, desiccation, UV radiation and host immune system. Biofilms are not only composed of multiple cells, but also of extracellular polymers which form a highly adhesive matrix, making them very difficult to eradicate. Biofilms also play a role in antibiotic resistance, because they prevent antimicrobials from killing the cells inside the matrix. Hence, biofilms are important in clinical settings. It is estimated that biofilms are involved in approximately 80% of all bacterial infections, particularly in cystic fibrosis and hospital acquired infections (Steenackers et al. 2012). In addition, recent increase in drug resistance in less developing countries (e.g. LDC) is a cause of concern (Ahmed et al. 2006). The ability of Salmonella as well as E. coli to form biofilms is fundamental for their survival and transmission.

Aside from the clinical and industrial relevance, biofilm formation is fascinating on a purely fundamental level. The remarkable ability to switch back and forth from existence as independent single cells to multicellular colonies, provides a new model for studying bacterial development. The switch from single-cell mode to biofilm is complex and tightly controlled by conditions both inside and outside the cell.

To find efficient ways to eradicate bacteria in biofilm, it is very important to study certain metabolic processes which play an important role in the formation of biofilms. This thesis hypothesized that changes in the nucleotide pools may lead to changes in the pools of nucleotide derived secondary signaling molecules (such as c-di-GMP) and thereby alter/inhibits the biofilm formation process. To better understand the importance of nucleotide biosynthesis in biofilm formation, we used two approaches –

On one hand a global approach through this looked into the changes in transcription patterns of several biofilm genes under nucleotide starvation and excess conditions. For this approach we used reporter genes

in various strains with exogenously added purines and pyrimidines. And on the other hand a hypothesis driven approach was used to investigate several possible ways in which nucleotide biosynthesis could be important for biofilm formation, for example by affecting Quorum sensing, cyclic-di-GMP levels, ATP production or eDNA release. The most interesting findings of this thesis are- a) Lack of ATP is not causing reduced biofilm, b) Quorum sensing influenced biofilm development and c) E. coli BW25113 ΔcarA strain has a surprisingly high level of c-di-GMP production which influenced biofilm formation.

EVALUATION OF THE 18SSU PCR-RFLP TECHNIQUE FOR DIAGNOSIS OF TRYPANOSOMIASIS ON SAMPLES COLLECTED FROM SICK AND APPARENTLY HEALTHY ANIMALS

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Abstract

East Africa is one of the regions in the sub-Saharan African facing the scourge of African Animal Trypanosomiasis. Diagnostic laboratories often rely on the rather insensitive buffy coat technique (BCT) for detection and characterization of the trypanosomes in field samples. To-date there are many PCR techniques developed for the same purpose but hardly any of them have been adopted. Besides cost implication, low adoption rate is attributed to inadequate information regarding the performance of these novel DNA based tests. In this study the 18Ssu PCR-RFLP was chosen and compared with BCT. The PCR is robust with ability to detect those important animal trypanosomes and it is easy to interpret making it a candidate test for complementing BCT in diagnostic laboratories. Whole blood collected from sick domestic animals (in Uganda) and apparently healthy cattle (in Kenya) without prior knowledge of their trypanosomiasis status were co-examined by 18Ssu PCR-RFLP and BCT. Uganda samples were collected from 13 districts in central region and two in western whereas Kenya samples were from Kwale district which is at the coast. Of the sick animals examined there were 287 cattle, 40 goats, 29 dogs, 3 cats, 3 camels and an horse; and apparently healthy animals was made of 364 cattle. In sick animals, 18Ssu PCR RFLP revealed single infection with T. theileri (3.41%), T. congolense and T. brucei (1.7%) each, Leishmania species (26.21%) and Herpetomonas species (1.99%); and mixed infection with Leishmania species and Herpetomonas species (0.85%), Leishmania species and T. brucei (0.28%) and Leishmania species and T. congolense savannah (0.56%). BCT detected 0.56% T. congolense and 0.28% T. brucei. Cattle were the most affected species of the sick animals examined and T. theileri was the most predominant trypanosome detected. In apparently healthy cattle, the 18Ssu PCR RFLP revealed 15.11% infection of which there were single infections with T. theileri (5.12%), T. vivax (3.98%), T. congolense kilifi (3.13%) and T. congolense savannah (2.84%), Leishmania species (19.94%), Herpetomonas species

(1.61%), Paratrypanosoma (0.28%); and mixed infection with T. congolense kilifi and T. theileri (0.28%), and Leishmania species and T. vivax (0.28%). BCT detected 3.84% T. vivax and 1.92% T. congolense. The detection power of 18ssu PCR RFLP was better than BCT. More over the technique is versatile and could reveal infection with other potential animal pathogens in the family trypanosomatidae that is often ignored in most diagnostic laboratories in the sub-Saharan Africa. Therefore, whenever thorough diagnosis and species characterization is required, BCT should be complemented with 18Ssu PCR-RFLP. Further, comparative studies between novel tests should be encouraged in all geographical locations where the target disease is endemic to aid technician operating diagnostic laboratories make appropriate choice when faced with diagnostic challenge.

Keywords: African Animal Trypanosomiasis, BCT, domestic animals, 18Ssu PCR-RFLP

REVISITING HER2 SOMATIC MUTATIONS IN NON SMALL CELL LUNG CANCER

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Abstract

Background: HER2 kinase mutations have been documented in approximately 2% of NSCLCs. The majority of these mutations comprise of in-frame insertions/duplications in exon 20, which leads to continuous and constitutive activation of the HER2 receptor. Although literature has demonstrated that the major NSCLC-derived HER2 mutations potently transphosphorylate EGFR, the impact of their interaction with their HER3 dimerization partner has not been described. Recently, a Phase II clinical study initiated at the UZ Brussel to validate inhibitors for HER mutant NSCLCs revealed the presence of HER2 mutations in 2% of tumor samples with HER2 YVMA dup and HER2 GSP dup accounting for 63% and 13% of respective cases. Since there is currently no standard treatment protocol available to treat mutant HER2 NSCLCs, we therefore sought to revisit these mutations in order to address the putative role of HER3 in their oncogenic potential and inhibition.
Methods: Plasmids encoding the mutated forms of the HER2 receptor were generated by site-directed mutagenesis and transiently transfected into H292 NSCLC cells using lipofectamineTM 2000 as either mono-transfectants or as co-transfectants with wild-type HER3. Following transfection, cell viability analysis and protein analysis of HER receptor activity were performed on the transfectants to assess their oncogenic potential in the presence of serum. Additional functional analyses were performed on the H292 transfectants to assess growth and induction of apoptosis in the absence of serum. H292 co-transfectants

were thereafter analyzed for their response to acute neuregulin stimulation and to HER2 inhibition via growth and protein analysis of HER receptor activity.
Results: Our results show that transiently transfected H292 NSCLC cells overexpressing mutant HER2, acquire a modest growth advantage both in the presence and absence of serum when coupled with HER3. Furthermore, H292 co-transfectants carrying both mutant HER2 and HER3 increase active HER3 levels in the presence of serum when compared to corresponding mono-transfectants. Interestingly, additional cellular-based studies show that mutant HER2 YVMA dup and not wild-type HER2, strongly transphosphorylates HER3 in a neuregulin- independent manner. Pertuzumab, a monoclonal antibody that prevents HER2 from dimerizing with its other HER partners potently inhibits the growth of H292 HER2 YVMA dup cells while afatinib, a pan-HER tyrosine kinase inhibitor, is highly effective in suppressing both cellular growth and HER2/HER3 receptor phosphorylation in all the tested H292 co-transfectants.
Conclusions: Taken together, these data provide a rationale for using inhibitors targeting both HER2 and HER3 in the treatment of mutant HER2 NSCLCs.

MOLECULAR ANALYSIS OF PESTICIDE DRIVEN ADAPTATIONS OF THE BACTERIAL COMMUNITY IN AN ON-FARM BIOPURIFICATION SYSTEM AND THE ROLE OF MOBILE GENETICS ELEMENTS

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Abstract
Mobile Genetic Elements (MGEs) are considered to play a major ecological role in the rapid bacterial adaptations to survive and proliferate under highly selective conditions such as pesticide polluted environments. Particularly, two MGE: IS1071 sequences and the IncP-1 plasmids have been found to promote the transfer of pesticide-catabolic genes within the bacterial community members living in these restrictive habitats. In the present study we tested communal-trait adaptations towards pesticide degradation and the abundances of IS1071 and IncP-1 plasmids in two ecosystems: a recently installed on-farm Biopurification System (BPS) used to control the farming environmental pollution risks derived from the disposal of pesticide contaminated wastewater, and a microcosm experiment. Both environments showed an increased prevalence of catabolic gene markers and MGE due to high and continuous pesticide loads. Moreover, high abundances of the quantified genes were concomitant with an enhanced pesticide biodegradation capacity. We were able to monitor the establishment of the functional adaptations towards pesticide degradation in the BPS bacterial community using a Long Range PCR approach targeting the accessory genes of IS1071. The amplified LR-product showed observable differences in the abundance of "suspected IS1071 cargo genes" throughout the time. As such, the bacterial community seems to "genetically shape" after the operational start of the on-farm BPS system as it was indicated by the increased detection of high molecular products after the first load of pesticide wastewater to the system.

Our data suggest a temporal increasing prevalence of MGE linked to a high abundance of pesticide-catabolic genes. These observations might suggest the important role of IS1071 and IncP-1 as communal mediators of functional adaptations towards pesticide degradation.

CHARACTERIZING THE STRUCTURE AND FUNCTION OF THE MINIMAL BINDING REGION OF THE PLASMODIUM FALCIPARUM VAR2CSA PROTEIN USING NANOBODIES®*

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Abstract

Malaria is a major human parasitic disease. In *P.falciparum*-endemic areas, infections during gestation give rise to a form of malaria referred to as pregnancy associated malaria (PAM). During PAM, the sequestration of parasitized erythrocytes within the placenta leads to an inflammatory process with poor outcomes for the neonate. Implicated in this sequestration is the VAR2CSA protein, from the PfEMP1 family, which interacts with placental CSA. Observations have shown that multigravidae are less susceptible to PAM over successive pregnancies. Experimentally linked to a VAR2CSA targeted humoral immune response, this observation indicates promise for PAM vaccine development based on VAR2CSA. However, the VAR2CSA protein is structurally complex, presenting difficulites with recombinant large scale production. A strategy to circumvent this constraint is the derivation of fragments of the VAR2CSA protein which retain its ligand binding capacity (minimal binding regions) and which induce a cross-reactive adhesion inhibiting immune response.

This study focused on the development of a platform to interrogate the structural and functional properties of a fragment (DBL1-ID2a-His) derived from the recombinant VAR2CSA protein. The structural aspect of the study utilized circular dichroism spectroscopy to investigate secondary structure as well as stability of the DBL1-ID2a-His under different conditions. Results revealed no significant differences in secondary structure between reduced and non-reduced DBL1-ID2a-His. However, a dose and exposure-time dependent downward shift of melting temperature was observed. Molecular tools (Nanobodies®) were raised against the DBL1-ID2a-His which served to assess its functional properties under different conditions. These indicated a loss of epitopes as function of time of exposure to reducing conditions. Nanobodies® were further used to as probes to test the ability of the recombinant DBL1-ID2a-His to induce adhesion blocking antibodies against erythrocytes parasitized with the *P. falciparum* isolate FCR3. Results indicated a percentage drop in adhesion of parasitized erythrocytes to a CSA analog, chondroitin surface proteoglycan (CPSG) with 4 Nanobodies® (Nb2, Nb 6, Nb 27 and Nb 100) exhibiting the greatest inhibition. The results of this study indicate the development of a platform for the characterization of the structural and functional properties of the minimal binding region of a VAR2CSA protein. It is envisaged that the tools obtained from this platform will be expanded in order to fully characterize the minimal binding region of the VAR2CSA.