

## **ESPE Sabbatical Leave Programme-Final Report**

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### **ESPE Sabbatical Leave Programme Committee**

#### **Chair:**

Prof. Dr. Raimo Voutilainen (till 2010) and Prof.dr. Jan-Maarten Wit (from 2011)

European Society for Paediatric Endocrinology

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#### **Time period:**

1 year- August 2010-August 2011

#### **Host:**

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#### **Rationale:**

In this era of advances in molecular endocrinology, it is exiting to observe how molecular and genetic research offers explanations for physiologic and pathologic conditions. Therefore, I decided to combine my clinical experience with molecular studies and chose to focus my research on bone and mineral metabolism. I had performed molecular genetic studies in patients with hypophosphatemic rickets and pseudohypoparathyroidism type Ib. However, molecular biology and animal experiments are an important field for the further development. I had a great chance to increase my experience in different molecular techniques in transgenic animals and cell culture studies with support of ESPE Sabbatical Leave Programme. This

collaboration is very important for me because it showed me the yield of the combination of clinical knowledge with experimental animal models and molecular biology studies.

### **Objectives:**

Pseudohypoparathyroidism (PHP) is characterized by hypocalcemia, hyperphosphatemia due to proximal renal tubular resistance to Parathyroid hormone. PHP type Ia (PHP-Ia) and PHP-Ib are caused by maternally inherited heterozygous mutations that affect the  $Gs\alpha$ -coding exons of the *GNAS* gene or the imprinting of this locus, respectively (1,2). In addition to  $Gs\alpha$ , *GNAS* gives rise to several gene products that show imprinted expression profiles (3-5). Among those is the extralarge  $\alpha$ -subunit (XL $\alpha$ s), a paternally expressed protein that shares exons 2–13 with  $Gs\alpha$  but uses a distinct upstream promoter and an alternative first exon (3, 4, 6). XL $\alpha$ s can mimic  $Gs\alpha$  function in transfected cells, since it is identical to  $Gs\alpha$ , with the exception of its N terminal portion, having most of the important functional domains of  $Gs\alpha$  activity (7-9). With its  $Gs\alpha$ -like activity, XL $\alpha$ s can be involved in some of the unexplained features of PHP. For example, PTH resistance in PHP develops primarily because  $Gs\alpha$  is paternally silenced in the proximal renal tubule and the maternal allele is inactivated by the inherited mutation. However, patients with PHP develop PTH resistance not during infancy, but later in childhood, and the factors preventing the development of PTH resistance remain currently unknown. We hypothesize that a delay in the paternal silencing (imprinting) of  $Gs\alpha$  and/or a compensatory mechanism that involves XL $\alpha$ s, which is not affected by the *GNAS* mutations causing PHP due to its paternal exclusive expression, allows sufficient PTH actions, thereby explaining the delay in PTH resistance.

### **Methods:**

According to our study plan, we first started breeding to obtain maternal (mat) or paternal (pat)  $Gs\alpha$  exon 1 knock out (E1KO) animals. We especially use E1KO animal since exon 1 is not involved in XL $\alpha$ s and we could avoid the direct KO effect of XL $\alpha$ s. We studied the mice at postnatal days 0, 3, 5, 7, 14, 21 and adult mice (2 months old).

To determine the allelic expression of  $Gs\alpha$  in the renal proximal tubule, the renal proximal tubule was isolated from mat-E1KO and pat-E1KO mice and their wild-type (WT) littermates at different ages by laser capture microscopy. The proximal tubules were labeled after injection of intracardiac Alexa-labeled albumin (invitrogen Alexa Fluor® 555). First, I developed an intracardiac injection method. This injection was very successful for the mice at postnatal days 3, 21 and adults. However, the injection of day 0 mice and older mice were not successful, so we decided to use the day 3 mice as the youngest mice examined.

I learned to make frozen section from renal tissues and then performed laser capture microscopy. Laser capture microscopy (in MGH Core Facility) was performed to collect labeled renal proximal tubules. RNA was isolated from collected proximal renal tubules by using Stratagene Absolutely RNA Nanoprep Kit.

The *Gsα* and *XLas* gene expressions were measured with real-time RT-PCR by using one-step Taq-man probes specific for these genes (QIAGEN® OneStep RT-PCR Kit).

### **Results:**

#### *Characteristic of mice population:*

The *matE1KO* animals had edema at postnatal first 3 days of life as described previously.

The *matE1KO* mice weighed significantly more than their WT littermates during the first 3 days of life due to the early postnatal edema. By postnatal day 5, edema resolved and weight became significantly lower than WT littermates. However, *matE1KO* mice gained more weight and weight equalized with littermate at postnatal day 21 and became significantly more at month 2. In contrast, *patE1KO* mice had a lean phenotype and weighed less than littermates at all time points from postnatal day 0 through 2 months of age, which was significant at postnatal days 5 and 14.

#### *The allelic expression of *Gas* and *XLas**

Our results showed that at postnatal day 3 the level of *Gas* mRNA in both the *patE1KO* and the *matE1KO* mice was ~53% of wild-type littermates, indicating that *Gas* expression is biallelic at this time. In 3-week old animals, whereas the *patE1KO* mice continued to show about 61% expression of the wild-type, the levels of *Gas* mRNA in *matE1KO* mice was 42% of the wild-type. Adult mice with the *patE1KO* also had *Gas* mRNA levels that were 66% of the wild-type, while adult mice with the *matE1KO* had only 33.4% of *Gas* mRNA compared to wild-type. *Gas* expression was significantly different in *matE1KO* from *patE1KO* at the ages of 21 day and 2 months. We could not obtain any *XLas* expression at renal proximal tubule at first 3 days of life either with Taq-man probe with one step qRT PCR or SYBR Green with two steps from c-DNA.

### **Conclusions:**

Our findings are consistent with the hypothesis that the paternal silencing of *Gas* is not established in the proximal tubule at birth, but instead, it develops gradually, which can explain the delayed and progressive PTH resistance in PHP patients. Additionally, *XLas* expression is not present at renal proximal tubules at postnatal period and seems to have no role in development of PTH resistance at proximal renal tubule.

### **Additional Projects:**

During the time of breeding and growing-up of mice, I worked on a cell culture project, in which I examined constitutively active, GTPase deficient Gs $\alpha$  and XL $\alpha$ s which were carrying point mutations that are analogous to Gs $\alpha$ -R201H, on bone differentiation by transfecting preosteoblastic cell lines (MC3T3-E1 cells) with Gs $\alpha$  and XL $\alpha$ s. We found that when transfected MC3T3-E1 cells were grown under osteogenic conditions for 5 days, transient expression of Gs $\alpha$ -R201H and XL $\alpha$ s-R543H significantly impaired osteoblastic differentiation, as judged by significantly lower alkaline phosphatase mRNA levels in these cells compared to control cells transfected with empty vector. These findings will be presented as oral presentation at ESPE 2012 (Glasgow) and are given as a part of a different paper, entitled “*The extra-long Gas variant XL $\alpha$ s escapes activation-induced subcellular redistribution and is able to provide sustained signaling*” which is accepted for publication at Journal of Biological Chemistry .

Additionally, I attended Endocrine Society meeting-2011 which was held in Boston, where I gave one oral and one poster presentation. I presented my work in 2 group meetings and one main talk to the Endocrine Unit.

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**Publications:**

Zun Liu, Serap Turan, Vanessa L. Wehbi, Jean-Pierre Vilardaga, Murat Bastepe. The extra-long *Gαs* variant *XLαs* escapes activation-induced subcellular redistribution and is able to provide sustained signaling. *Journal of Biological Chemistry*

The temporal profile of *Gαs* imprinting in the renal proximal tubule during the early postnatal period in mice- in preparation.

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