Gene silencing approaches for functional angio-genomics

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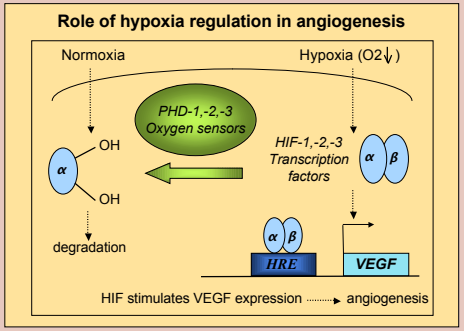
INTRODUCTION

This project focuses on applying RNA interference approaches to suppress gene expression *in vitro* and *in vivo* to study functional angiogenomics and regulation by hypoxia. Angiogenesis is the physiological process involving the growth of new blood vessels from pre-existing vessels. New vessels in the adult arise mainly through angiogenesis, although vasculogenesis also may occur. The Vascular Endothelial Growth Factor (VEGF) is known to play a major role in physiologic as well as pathologic angiogenesis, it is the major contributor to increase the number of capillaries in a given network. When challenged by hypoxia, cells increase the expression of proteins involved in the physiological adaptation to low oxygen environments. This compensatory response depends on the activation of heterodimeric transcription factors, designated hypoxia inducible factors (HIF). Decreased oxygen levels result in the stabilization of HIF-1α and the activation of a transcriptional complex leading to the expression of target genes such as VEGF. Prolyl hydroxylation is critical in the regulation of HIF-1α steady-state levels. In mammalian cells, three HIF Prolyl-Hyodroxylases (PHD1, 2 and 3) were identified and shown to hydroxylate HIF-1α subunits.



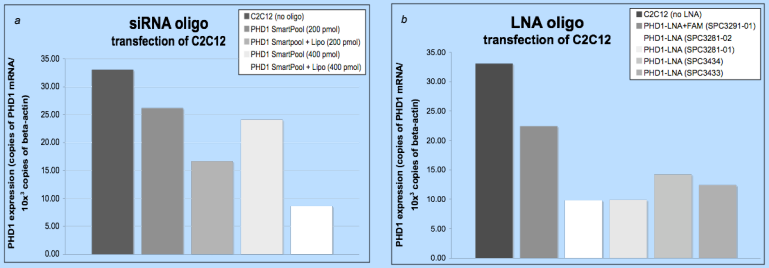
Our laboratory recently found that loss of PHD1 (by gene targeting), but not of its family members PHD2 or PHD3, provides specific protection against ischemic skeletal muscle necrosis in a model of hind limb ischemia in adult mice. This myo-protective effect was not due to enhanced angiogenesis, erythropoiesis or glycolysis, but attributable to a reduced production of reactive oxygen species via upregulation of protective genes (Aragones et al, unpublished results). To date, we have developed constructs for expression of shRNAs against Prolyl Hydroxylase-1 (PHD1). The purpose is to silence PHD1 to further study the protective effect of PHD1-deficiency in skeletal muscle ischemia.

AIMS

To examine whether RNAi-based time-restricted silencing of PHD1 in adult mice would provide a similar muscle protection phenotype. Therefore, antisense RNA oligos to block PHDs were designed (conventional siRNA oligos and Locked Nucleic Acid (LNA*) oligos) and tested *in vitro*. Then shRNA expression cassettes were cloned in plasmid or viral vectors and further tested in *in vitro* as well as *in vivo* settings.

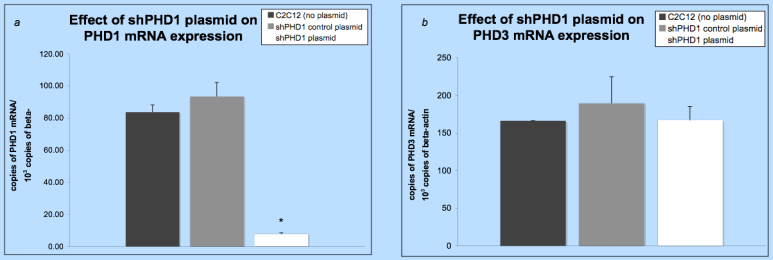
IN VITRO VALIDATION OF PHD1 ANTISENSE APPROACHES

1 - Silencing efficacy of PHD1-specific antisense oligos in vitro



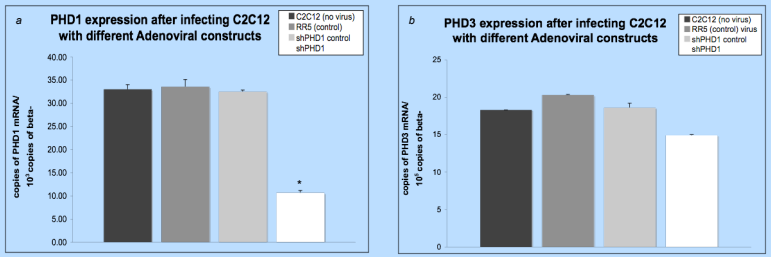
Validation of specific downregulation of PHD1 mRNA by transfecting two different types of synthetic antisense RNA oligonucleotides in C2C12 cells. PHD1 mRNA levels were quantified by real time quantitative RT-PCR. (*a*) Different amounts of siRNA oligo (SmartPool, Dharmacon) were used to transfect C2C12 cells, the efficiency of the targeted gene silencing was determined 48h post-transfection. 400 pmol of PHD1 SmartPool oligo reduced the PHD1 mRNA level by 74%. (*b*) Different Locked Nucleic Acid (LNA, Santaris*) antisense oligonucleotides targeting different sites in the PHD1 sequence were transfected into C2C12 cells to determine the sequence causing maximal PHD1 mRNA knockdown with minimal undesired effects (toxicity, aspecific effects). Two PHD1 LNA oligos (SPC3281-02 and SPC3281-01) inhibited the PHD1 level by 70%.

2 - Silencing of PHD1 in C2C12 cells transfected with shPHD1 expressing plasmids



Effects of shPHD1-expressing plasmid on gene expression in C2C12 cells. To enable long-term silencing in future *in vivo* experiments, an shPHD1-expressing plasmid was constructed and was transfected in C2C12 cells to evaluate its silencing efficacy. (*a*) A plasmid expressing a 21 base pair hairpin (sequence as in 1a) was constructed and tested in C2C12 cells, yielding specific silencing of 91% (N=5, *P*<0.001) 48h post-transfection. An irrelevant control shRNA-plasmid, used in parallel, gave no expression reduction. (*b*) Specificity towards PHD1 silencing was confirmed by the lack of reduction of PHD3 expression by the shPHD1-plasmid. Mean values ± SD are shown (N=5).

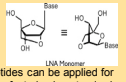
3 - Silencing of PHD1 in C2C12 cells transduced with shPHD1 expressing adenoviral vectors



PHD1 shRNA expressed from a recombinant adenoviral vector specifically reduced the endogenous PHD1 gene expression. Adenoviral vectors were applied to C2C12 cells at a multiplicity of infection (MOI) of 200. Three days post-infection, expression of the target (PHD1) gene and a control (PHD3) gene was analyzed by real time quantitative RT-PCR. (*a*) The PHD1 gene expression level after infection with shPHD1 adenovirus was reduced by 70% (N=3, *P*<0.01) relative to cells that were not infected with adenovirus. No reduction was observed after using control adenoviruses. (*b*) Specificity towards PHD1 silencing was confirmed by the lack of reduction of PHD3 expression by the shPHD1-adenovirus. Mean values ± SD are shown (N=3).

IN VIVO APPLICATION OF PHD1 ANTISENSE APPROACHES

1 - Locked Nucleic Acid (LNA) antisense oligonucleotides



LNA is a novel type of nucleic acid analog that contains a 2'-O, 4'-C methylene bridge. This bridge locks the moiety in the 3'-endo conformation restricting the flexibility of the ribofuranose ring and also locking the structure into a rigid bicyclic formation, conferring enhanced hybridization performance and exceptional biological stability.

LNA oligos possess unprecedented binding affinity toward complementary DNA and RNA. LNA oligonucleotides can be applied for efficient gene silencing *in vitro* and *in vivo*. Custom LNA oligos can be commercially purchased, can be transfected using standard techniques, are non-toxic and lead to increased target accessibility.

Preliminary results of the LNA approach in skeletal muscle *in vivo* showed us that the LNA oligos were successfully taken up by cells. Based on the proprietary rules from Santaris*/Proligo we had designed LNA oligos to knockdown the PHD1 expression in skeletal muscle (see in vitro results - 1a), among which the most efficacious sequence was identified for large scale synthesis. This PHD1 LNA oligo will then be applied to mimic the PHD1-KO model in order to further study the protective effect of PHD1-deficiency in skeletal muscle ischemia.

In a first experiment, the transfection efficiency of LNA oligos in tissue *in vivo* was tested. Therefore, a FAM-labeled LNA oligo was administered by direct multiple injections in the hindlimb skeletal muscle (50 µg injected in 3x 20 µl aliquots into the gastrocnemius) of WT mice. The results showed high uptake and nearly uniform distribution of the LNA oligo (see figure). The *in vivo* experiments to test the efficacy of PHD1 expression reduction by LNA oligos in WT mice is ongoing (no data shown).

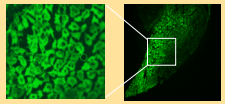
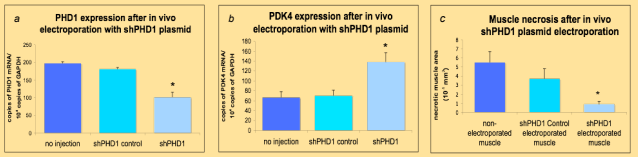


Figure legend : Fluorescence microscopy of cryosections of skeletal muscle, dissected 48 hours after FAM-LNA injection.

2 - Silencing PHD1 expression in skeletal muscle in vivo using electroporation of shPHD1 expressing plasmids

One of the alternative approaches to deliver plasmids more efficiently and long-term, *in vivo* for both gain- and loss-of-function studies, is by *in vivo* electroporation. Whereas the nonviral techniques for gene transfer *in vivo* are limited by the relatively low expression levels of the transferred gene, *in vivo* electroporation yields enhanced levels of plasmid expression. We have used this method to mimic the PHD1-knockout model. Therefore we directly injected shPHD1 specific plasmid in the hindlimb skeletal muscle of WT mice (70 µg plasmid, injected in 3 x 20 µl aliquots). Immediately afterwards, a pair of electrodes was placed against the sides of the gastrocnemius (GCN) to directly deliver electric pulses (8 alternating pulses of 120 V, duration 20ms at 1/sec intervals) (see right panel; Illustration shows results with a GFP plasmid). Skeletal muscle-targeting by *in vivo* electroporation is an efficient way to get transgenes or oligomers delivered for prolonged effects, it induced minimal damage of the skeletal muscle fiber and a mild degree of influx of inflammatory cells. However, no dysfunction of the lower limb was observed in preliminary experiments. The results indicated that *in vivo* electroporation increased the number of muscle fibers taking up plasmid DNA and/or the copy number of plasmids introduced into the cells as compared to injection without electroporation (data not shown). Delivery of PHD1-specific shRNA constructs was performed five days prior to induction of limb ischemia.

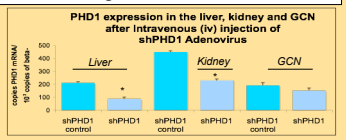
Electrodes
(Tweezertrodes)



(*a*) RT-qPCR expression analysis 5 days after electroporation showed a significant reduction by 50% of the PHD1 expression (N=4, *P*<0.005) after using the shPHD1 plasmid but almost no reduction after use of the control plasmid. (*b*) To verify if the knockdown of PHD1 was functional, we determined the expression of the Pyruvate Dehydrogenase Kinase-4 (PDK4) gene, which is upregulated after loss of PHD1 in baseline conditions. RT-qPCR data showed a 2-fold upregulation of PDK4 expression. *(c)* Histological analysis at two days after femoral artery ligation revealed that short term PHD1 silencing indeed significantly protected the myofibers against ischemic necrosis and oxidative damage, suggesting that specific PHD1 inhibition might offer novel treatment perspectives for muscular disorders characterized by oxidative stress.

3 – Silencing PHD1 in skeletal muscle in vivo using shPHD1 adenoviral vectors

An alternative method to inhibit specifically the expression of a gene in a variety of organs is the use of shRNA adenovirus, injected intravenously (into the tail vein) in a small volume and with a normal pressure. Preliminary experiments showed significant PHD1 gene suppression in the liver and kidney (*P*<0.05), and also - but only moderately - in the gastrocnemius (GCN). Further optimization of dosage and timing is ongoing.



CONCLUSIONS & PERSPECTIVES

- Our target gene expression data after administration of siRNA, LNA, shRNA plasmids and viral shRNAs, show that RNAi is an effective method to suppress gene expression *in vitro* and *in vivo*.
- Application of the approaches has already revealed that transient, local gene silencing of PHD1, initiated at adult stages in skeletal muscle of mice, suffices to provide protection against ischemic muscle damage, similar to PHD1 deficiency caused by classical gene inactivation, i.e. continuous deficiency throughout embryonic and adult life. This opens up new opportunities for the development of therapies for muscle ischemia.
- The gene silencing approaches will now also be further developed for

  i) gene knockdown in ES cells to generate animal-models with custom genetic alterations

  ii) further development of the approaches with shRNA expression plasmids in combination with *in vivo* electroporation as an alternative for gene silencing by viral vectors (local use).

  Optimized strategies will be validated and applied to study genes involved in angiogenesis.

(*) LNA oligos were kindly provided by Santaris Pharma A/S, Denmark.