Scuola di Dottorato di Ricerca in Medicina Molecolare
Università di Siena, Dipartimento di Medicina Molecolare e dello Sviluppo

RELAZIONE DI FINE ANNO DI DOTTORATO
IN MEDICINA MOLECOLARE
CICLO XXXII, aa 2016/2017

Borsa di Studio Pegaso con finanziamenti POR F.S.E. 2014/2020, messi a disposizione della Regione Toscana

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Anno: **I**

The initial proposal for my project is to investigate genes involved in the differentiation of human induced pluripotent stem cells (hiPSCs) to the endoderm lineage, the embryonic germ layer that contributes to a number of critical organs including the thymus, lungs, liver, pancreas, and intestine. The goal is to investigate if these genes are required for a) endodermal differentiation and b) for the formation of pancreatic ductal epithelium. The latter is a cell type of interest because it is where pancreatic ductal adenocarcinoma (PDAC) originates, the most common type of pancreatic cancer and one of the most lethal human cancers. Taking advantage of iPSCs to develop cancer models, I hope to unravel the molecular mechanism responsible for cancer initiation and progression. Besides modeling

cancer, further applications of the iPSC system include high-throughput drug screening, epigenetic reprogramming of cancer cell state, immunotherapy and regenerative cell therapies.

The choice of the genes to study is an important strategic decision. I started by selecting candidate genes which are known in the literature to be essential for endodermal cell differentiation. In the near future, I also intend to mine a list of candidate genes generated from *single cell transcriptomics analysis* of hiPSC differentiation to endodermal progenitors *in vitro* (W. Skarnes, unpublished).

At first, I focused on the transcription factor Small Mother Against Decapentaplegic 4 (SMAD4) gene. SMAD4 has a key role in the transforming growth factor (TGF)-β signaling pathway by regulating gene expression following activation of TGF-β receptors. Different scientific papers showed alterations in the TGF-β pathway genes, particularly SMAD4, revealed that these genes play crucial functions in maintaining tissue homeostasis and suppressing tumorigenesis. In fact, SMAD4 is known to be an important tumor suppressor gene in many human cancers, in particular in PDAC where loss of SMAD4 expression has been associated with poorer prognosis. Furthermore, signaling by TGF-β family ligands plays key roles in cell differentiation and proliferation, and is important in the regulation of human embryonic stem cell self-renewal and pluripotency.

To investigate the role of SMAD4 protein in endodermal differentiation, I will make use of a newly described method by Amanda Andersson-Rolf and her colleagues[1] to generate a conditional gene knockout (cKO). This CRISPR/Cas9-based method allows us to generate bi-allelic and reversible gene knockouts in one step in diploid cells. The strategy uses an invertible intronic cassette (FLIP-FlpE) plus two flanking flippase recognition target (FRT) sites to excise the cassette and revert the mutation. Once CRISPR/Cas9 has cleaved double-stranded DNA, the cells try to repair the double-strand break by the Non-Homologous End Joining (NHEJ) pathway or by homology-directed repair (HDR) pathway: in the first case resulting in insertions and/or deletions (InDels) which disrupt the targeted locus, and in the second allowing for precise replacement. In order for the strategy to operate as intended, clones are screened for the presence of frameshift InDels in one allele (by nuclease-mediated NHEJ) and the integration of the FLIP-FlpE cassette in the other allele by homology-directed repair.

Under the supervision of Prof. William Skarnes, at The Jackson Laboratories, I began by designing a conditional targeting vector for the SMAD4 gene, taking into account several criteria to identify an appropriate FLIP-FlpE cassette insertion site. To design the guide RNA for Cas9 (crRNA), I used the WTSI Genome Editing (WGE) tool, a CRISPR database for genome engineering[2], considering only guide sequences with a low off-target score. It is important that the CRIPSR/Cas9 target sequence is near or overlapping the insertion site of the FLIP-FlpE cassette in order to destroy the CRISPR site once the cassette is integrated. For SMAD4, I identified two crRNA in exon 2 and selected the one with the highest activity based on the T7EN assay. To generate the targeting vectors, I designed the primers to create the gene-specific homology arms for the FLIP-FlpE cassette: the donor vector contains the *puro*^{R} FLIP-FlpE cassette flanked by ~1-kb homology arms up- and downstream of the insertion site identified. The cloning strategy is based on GoldenGate assembly using the SapI restriction enzyme; for this reason, I chose genomic sequences without SapI restriction enzyme sites and I added the appropriate SapI sites to the PCR primers for the cloning. I set up the PCR using a high-fidelity polymerase and amplified the homology arms from genomic DNA extracted from KOLF2-C1 cells (the iPSCs used in this experiment). After the PCR, I purified the homology arms and I inserted them into the plasmid

backbone containing the FLIP-FlpE cassette to generate the final targeting vector. To verify our vector was correct, I used restriction digest analysis and, subsequently, Sanger sequencing.

Nucleofection (Amaxa 4D) was used to introduce CRISPR-Cas9 and the donor plasmid into KOLF2-C1 cells. Briefly, a single cell suspension of 2 x 10^6 iPSCs were nucleofected with uncut, supercoiled plasmid DNA and pre-assembled Cas9 ribonucleoprotein complex (Cas9 RNP). Cas9 RNPs consist of purified recombinant Cas9 protein (purchased from IDT) in a complex with synthetic crRNA/tracrRNA (purchased from IDT), which are assembled in vitro and delivered directly to the cells. After two days, the cells are selected with the puromycin: critically, the non-mutagenic orientation of the FLIP/flpE cassette expresses a puromycin resistance gene (puroR), allowing selection of correct nuclease-assisted targeting into the exon of one allele and simultaneous enrichment of cells that inactivate the second allele by nuclease-mediated NHEJ. Following puromycin selection, all cells on the control dish (non-transfected) died and iPSCs colonies formed on the transfected dish. At that point, I picked 48 colonies under a dissecting microscope and divided the cells into two wells of a 96-well plate. One set of 48 clones were archived and the duplicate set of clones were lysed for genotyping.

In the coming months, I will genotype clones and select correctly targeted conditional clones for my experiments. To permit loss-of-function studies during cell differentiation, the next step it is to introduce CreERT2 into the adeno-associated virus (AAVS1) locus. This locus allows stable, longterm transgene expression in many cell types, including embryonic stem cells[3]. The Cre-ERT2 fusion protein consists of Cre recombinase fused to a triple mutant form of the human estrogen receptor, which does not bind its natural ligand (17β-estradiol) at physiological concentrations but will bind the synthetic estrogen receptor ligands 4-hydroxytamoxifen (OHT). Only in presence of OHT Cre recombinase is active causing the inversion of the FLIP-FlpE cassette and loss of wild-type gene function.

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EDUCATIONAL ACTIVITIES:

1) I "Zebraday". Stella Maris, 30 Novembre 2016;
2) Cellule staminali e medicina rigenerativa, Relatore Michele De Luca; Sant'Anna, Scuola Universitaria Superiore di Pisa, 22 Marzo 2017;
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PUBBLICATION:

Pizzino G, Irrera N, Galfo F, Oteri G, Atteritano M, Pallio G, Mannino F, D'Amore A, **Pellegrino E.** Aliquò F, Anastasi GP, Cutroneo G, Squadrito F, Altavilla D, Bitto A. Adenosine Receptor Stimulation Improves Glucocorticoid-Induced Osteoporosis in a Rat Model. *Front Pharmacol.* 2017 Sep 5;8:558

SATISFACTION LEVEL OF PhD PROGRAM:

I am fully pleased with how this PhD program is enriching my scientific background and provides the required tools for the advancement of my research project, also thanks for the opportunity of the experience abroad.

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