

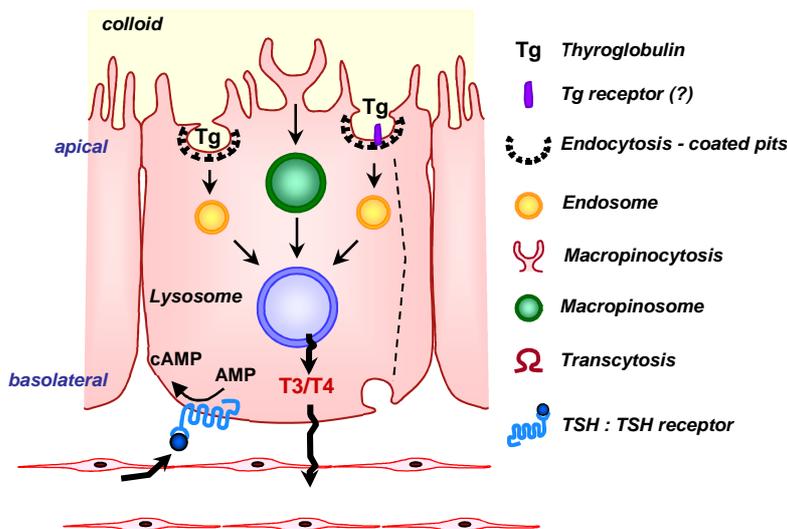
Cellular and tissular mechanisms for stem cell therapy of epithelial cells in a mouse model of cystinosis : coping with tissue heterogeneity by functional and targeted ultrastructural imaging combined with focal transcriptomic studies

First progress report (as of April, 2012)

A. Background and objectives

Cystinotic patients frequently suffer in the first decade of life from overt hypothyroidism with increased TSH, requiring hormonal replacement therapy, and eventually show complete gland atrophy (Chan et al. 1970). Thyroid hormone (T3/T4) production is a two-step process comprising: (i) apical secretion of disulfide rich (>100) thyroglobulin monomer (Tg) and iodination (iodinated-Tg) for storage in the colloid; then (ii) apical endocytosis of Tg followed by lysosomal degradation to release T3/T4 (1 Tg monomer comprises only 3 hormogenic sites, i.e. only 1% of its mass) and hormone secretion into blood. T3/T4 plasma level is tightly controlled by a TSH feed-back loop: TSH promotes Tg synthesis, its endocytic transport into lysosomes, and thyrocyte proliferation (Fig 1).

A. Thyroid hormone synthesis



B. Pituitary control

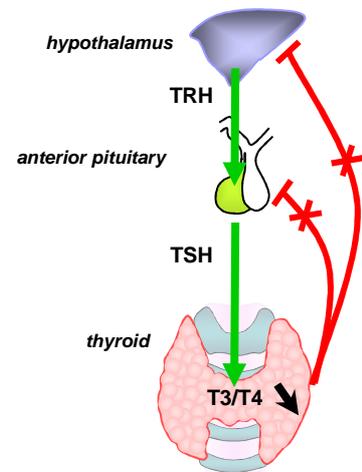


Figure 1. Thyroid hormone synthesis (A) and its hypothalamo-hypophyseal control (B). A. Hormonogenesis. Newly synthesized thyroglobulin is secreted at the apical membrane where it is iodinated, then iodo-thyroglobulin is stored in the luminal colloid as a reserve. Upon TSH stimulation, T3/T4 production is achieved by (i) apical endocytosis of iodo-thyroglobulin (either by macropinocytosis or by a still elusive receptor-mediated endocytosis mechanism : megalin ?); (ii) its subsequent lysosomal degradation to release T3/T4 hormones; which (iii) are specifically transferred across the basolateral membrane, then into blood. **B. Feedback control.** A low circulating plasma T3/T4 level is first sensed by the hypothalamus which responds by releasing the Thyroid Releasing Hormone (TRH), itself triggering Thyroid Stimulating Hormone (TSH) secretion by the anterior pituitary. T3/T4 plasma level is also sensed at this level. TSH promotes acute T3/T4 release and Tg synthesis (store replenishment) by existing thyrocytes, then proliferation of thyrocytes (new « workers ») and blood vessels (the thyroid gland is highly vascularized).

Both early alteration of lysosomal function in intact cells and late glandular atrophy could account for impaired thyroid hormone production in cystinosis. We previously reported to CRF that apical endocytosis is impaired in human and cystinotic kidneys prior to atrophy. In our second CRF grant, we proposed to address (i) the physiopathology of hypothyroidism in cystinosis using C57BL/6 *Ctns*^{-/-} mice and (ii) the impact of stem cell therapy on thyroid function using grafted *Ctns*^{-/-} mice (second grant spring 2011, for one year). For this first interim report of the second grant, two directions have been successfully followed. The **first objective** was to characterize the differentiation state of thyrocytes and associated microvasculature. The **second objective** was to investigate the thyroid hormonal function and pituitary adaptation. The **third objective**, aiming to evaluate whether grafting of hematopoietic stem cell (GFP⁺-HSC) could improve the thyroid function in dsRed *Ctns*^{-/-} mice model, is extremely encouraging, but is still being completed and will be more appropriately summarized in our next (already final) report.

B. Interim progress report

Objective 1. Characterization of the differentiation state of thyrocytes and associated microvasculature.

Thyrocytes are coupled to the microvasculature as a tight functional unit. Recent work from the team of Dr. Pierreux in our laboratory has revealed crucial paracrine communications between epithelial and vascular cells to control thyroid development (Hick et al, to be submitted to Development). Looking at both thyrocyte and blood capillaries is thus critical to characterize the overall differentiation state of thyroid.

Thyroids were first analysed by conventional histopathology at 6, 9 and 12 months. We found that *Ctns*^{-/-} mice systematically develop around 9 months multifocal thyrocyte hyperplasia (mimicking early papillary premalignant lesions upon activating mutations of the TSH-receptor), associated with complete focal colloid exhaustion and mesenchyme expansion (Figure 2).

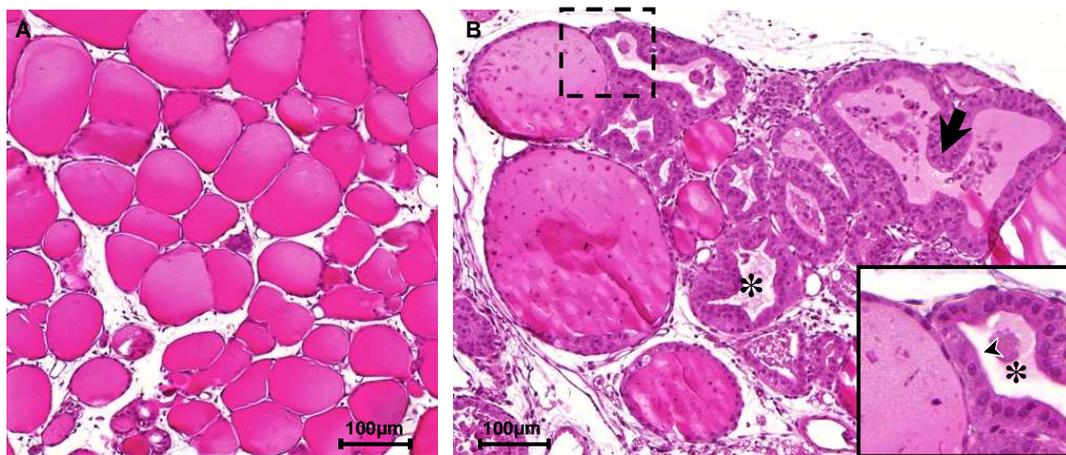


Figure 2. *Ctns*^{-/-} mice develop spectacular thyroid hyperplasia. Hematoxylin-eosin staining of 9 months-old WT(A) vs *Ctns*^{-/-} mice thyroids (B). In *Ctns*^{-/-} mice, thyrocytes are hypertrophic in most follicles (insert, arrowhead) and hyperplastic, so as to form papillae (thick arrow). Such lesions are systematically associated with colloid exhaustion (asterisks). Notice frequent shedding of cell remnants in the follicular lumen.

We next demonstrated by multiplex immunofluorescence that thyrocyte hyperplasia was associated with activation/proliferation of blood capillaries (Figure 3).

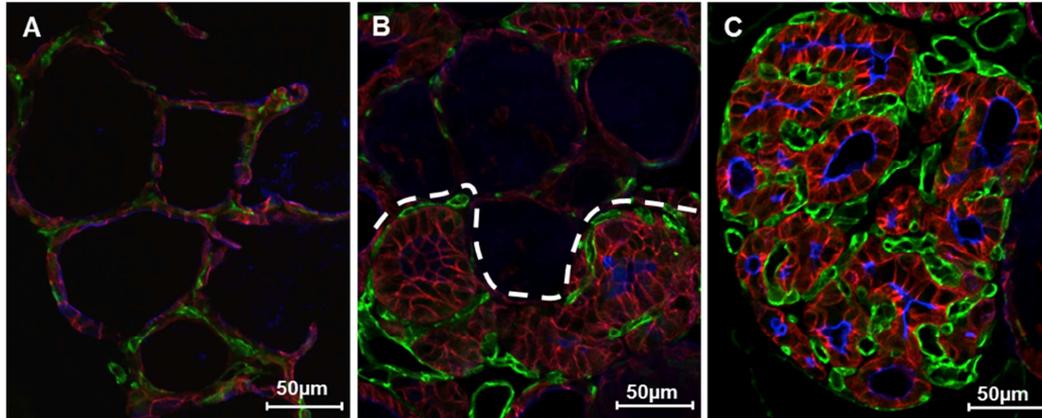


Fig 3. Activation/proliferation of thyrocytes and associated blood capillaries in *Ctns*^{-/-} mice thyroids : triple immunofluorescence for E-cadherin (red, thyrocyte baso-lateral membrane), ezrin (blue, thyrocyte apical membrane) and PECAM (green, blood capillaries) at 9 months. (A) WT mice. (B, C) *Ctns*^{-/-} mice. Increased TSH plasma concentration (not shown) causes follicular hyperplasia, colloid exhaustion and capillary proliferation. The unusual field at B shows an abrupt boundary (dotted line) between the few remaining intact follicles (top) vs hyperplastic follicles (bottom) with empty lumen and microvasculature proliferation (best seen at C).

Structural alterations of *Ctns*^{-/-} thyrocytes were finally characterized by electron microscopy (Figure 4). Remodelling of the apical surface in hypertrophic thyrocytes and increased number of inflated lysosomes, some of which contains cystine crystals, reflect accelerated endocytosis and strongly suggest altered thyroid hormone synthesis. Increased number of autophagic/residual bodies also supports altered metabolism in *Ctns*^{-/-} thyrocytes.

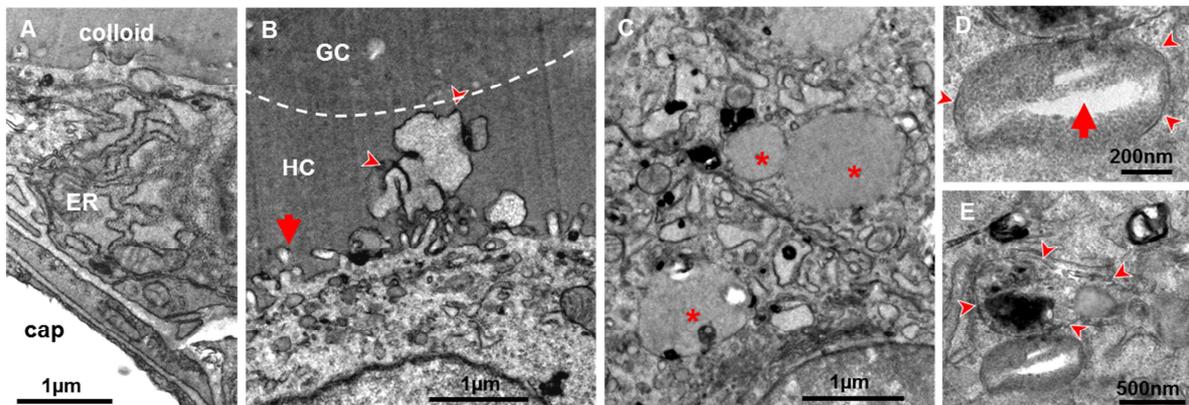


Figure 4. Ultrastructural alterations of *Ctns*^{-/-} mice thyroid. Representative electron microscopic views of 8 months-old WT (A) vs *Ctns*^{-/-} mice thyroid (B-E). (A, WT) Resting thyrocytes are mostly engaged in Tg synthesis (dilated endoplasmic reticulum, ER) while their apical surface facing a non-solicited colloid is essentially flat (top view). Cap, capillary lumen (empty since these samples were perfusion-fixed). (B-E; *Ctns*^{-/-}) (B) Remodeling of the apical surface reflecting accelerated endocytosis is evidenced by filipodia (thick arrow) and lamellipodia (arrowheads). The colloid itself is stratified : the dotted line indicates a boundary between the Homogenous peripheral Colloid ring (HC, containing newly synthesized Tg) and the central Granular Colloid (GC) including cell remnants (not visible in this view). (C-E) Alteration of the thyroid hormone production pathway is evidenced by the multiplicity of inflated lysosomes (asterisks at C), some of which contains cystine crystals (thick arrow at D where small arrowheads underline the lysosomal limiting membrane) and by the presence of numerous autophagic/residual bodies (one of the three visible is indicated by arrowheads at E).

Objective 2. Characterization of thyroid hormonal function and pituitary adaptation

Alteration of thyroglobulin processing in hypertrophic *Ctns*^{-/-} thyrocytes was further demonstrated by increased accumulation of thyroglobulin in the basal pole of thyrocytes as well as increased uptake/lysosomal accumulation of iodinated thyroglobulin (Figure 5). This could result in decreased circulating T3/T4 hormone and thus hypothyroidism.

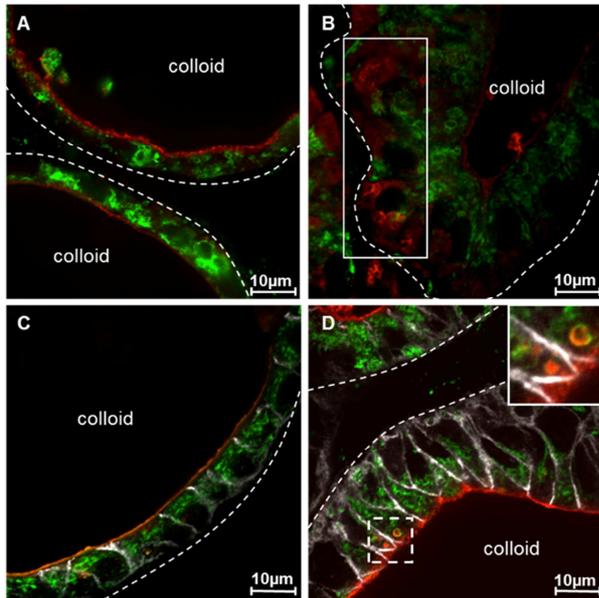


Figure 5. Altered thyroglobulin processing in *Ctns*^{-/-} thyrocytes. Triple immunofluorescence for LAMP-1 (green; lysosomes), E-cadherin (white; thyrocyte baso-lateral membrane) and either thyroglobulin (red; A-B) or iodinated-thyroglobulin (red; C-D) in 9 months-old mice thyroid. (A, C) Inactive thyrocytes in WT mice. (Iodo)-Tg is stored in the follicle colloid (lack of red signal in deep colloid is due to confocal Z-positioning artefact). (B, D) Alterations of *Ctns*^{-/-} thyrocytes. (B) Red signal accumulation at the basal pole (box) likely reflects enhanced Tg production (dotted line indicates position of the basement membrane). (D) Lysosomal accumulation of iodinated-thyroglobulin (red; insert) at the apical pole of *Ctns*^{-/-} thyrocyte.

Since increased TSH is a sensitive indicator of hypothyroidism, we measured its plasma concentration of *Ctns*^{-/-} mice. We observed that TSH plasma concentration was significantly increased at 9 months reflecting activation of compensatory hypothalamo-hypophyseal feedback (Fig 6A), associated with increased thyrocyte proliferation (Fig 6B), and likely explaining the resulting hyperplasia.

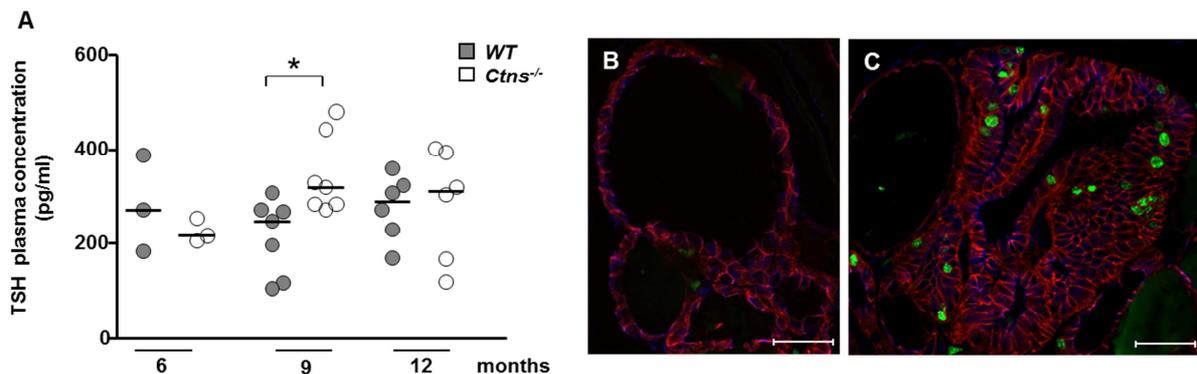


Figure 6. Increased TSH plasma concentration correlates with thyrocyte proliferation. (A ELISA). TSH plasma concentration is significantly increased in 9 month-olds *Ctns*^{-/-} mice. (B-C) Proliferation imaging using Ki67 immunofluorescence. Increased TSH induces thyrocyte proliferation. Double immunofluorescence for E-cadherin (red) and Ki-67 (green; proliferation, G1 to M phase of the cycle) in 9 month-olds *Ctns*^{-/-} thyroid.

In conclusion, altered thyroid hormone production in *Ctns*^{-/-} mice elicits a TSH response which causes a spectacular hyperplasia of thyrocytes and associated microvasculature.

C. Next plans within this grant and perspectives after August 2012.

Within few weeks, we will have collected sufficient data to complete the characterization of cystinosis physiopathology in the mice thyroid, as well as the benefit from GFP⁺-HSC grafting into DsRed *Ctns*^{-/-} mice. Preliminary data indicate that combination of paracrine interactions and probably occasional thyrocyte replacement results in frequent normalization of TSH plasma concentration.

Till the end of this grant, and hopefully after August 2012, we will further test the possibility (and examine the mechanism) of thyrocyte transdifferentiation from grafted HSCs. Four questions will be addressed by multiplex immunofluorescence, multiphoton and electron microscopy: (i) can we exclude the possibility that GFP⁺-cells inserted among thyrocytes are merely infiltrating interstitial (HSC) cells and instead conclude to truly transdifferentiated epithelial cells, (ii) are transdifferentiated GFP⁺-epithelial cells engaged in the thyrocyte differentiation program, (iii) have transdifferentiated GFP⁺-epithelial cells gained the ability to generate thyroid hormones and (iv) how do interstitial stem cells (grafted or endogenous) gain access into the thyrocyte monolayer across a continuous basement membrane.

A detailed manuscript on the physiopathology of cystinotic thyroid lesions and their correction by HSCs will then be assembled this year.