Zebrafish as a new model to study in vivo the functional consequences of human THRA variants

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1. INTRODUCTION

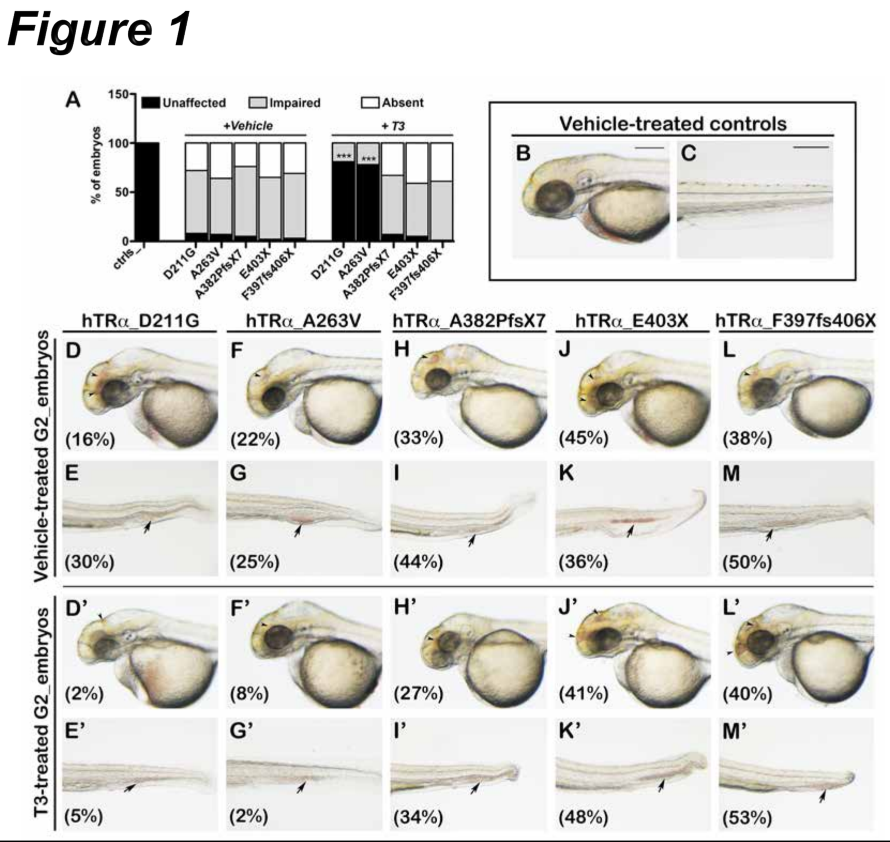
Since 2012, different heterozygous mutations in the *THRA* gene have been described in patients with Resistance to Thyroid Hormone alpha (RTHα). The associating symptoms are reminiscent of untreated congenital hypothyroidism (growth retardation, psycho-neuromotor disorders, delayed bone development and bradycardia) but with raised T3/T4 ratio and normal TSH levels. All genetic abnormalities act in a dominant negative (DN) manner against functional receptors due to reduced T3-binding or defective interaction with corepressors or coactivators of the ligand-binding domain (LBD). Therefore, RTHα patients present variable sensitivity to TH treatment. We previously described that zebrafish embryos expressing a DN form of *thraa* recapitulate the key features of RTHα, and that zebrafish and human receptors are functionally interchangeable (Marelli et al, 2016). In this work, we present a simplified model obtained by direct mRNA microinjection into zebrafish eggs of several human *THRA* variants (D211G, A263V, A382PfsX7, E403X and F397fs406X). Using a series of molecular and analytical approaches we studied the embryonic development of cardiovascular, skeletal and nervous systems, which are directly involved in the T3-dependent TRα action.

2. RESULTS

2.1 Blood circulation in hTRα-injected embryos

About the 80-90% of vehicle-treated TRα embryos showed a significant impairment or absence of blood circulation (Figure 1). Only the hTRα_D211G and hTRα_A263V injected embryos displayed a significant recovery of blood circulation (+87% and + 83% over the baseline, respectively) after T3 supplementation (A). Additionally, the vehicle-treated TRα embryos presented, in variable percentages, cerebral haemorrhages (D, F, H, J,) and blood stasis in the tail (E, G, I, K, M), which were dramatically reduced in T3-treated embryos injected with the missense variants (D'-E' and F'-G').

Figure 1



2.2 Heart morphology and function in hTRα-injected embryos

At 2dpf, controls and less than 50% of the vehicle-treated hTRα injected embryos presented a normal S-shaped heart with the ventricle positioned on the right of the atrium, indicating a correct D-looping process (Figure 2A and B). The remaining embryos displayed either a moderate phenotype with impaired looping (C), or absence of looping with a completely linear heart tube (D), or a reversed heart looping with the ventricle on the left of the atrium (E). Additionally, all vehicle-treated hTRα-injected embryos exhibited a 20-25% reduction of heart rate compared to controls (G). As reported in the histograms F and G, only the missense variants positively respond to T3 supplementation.

Figure 2

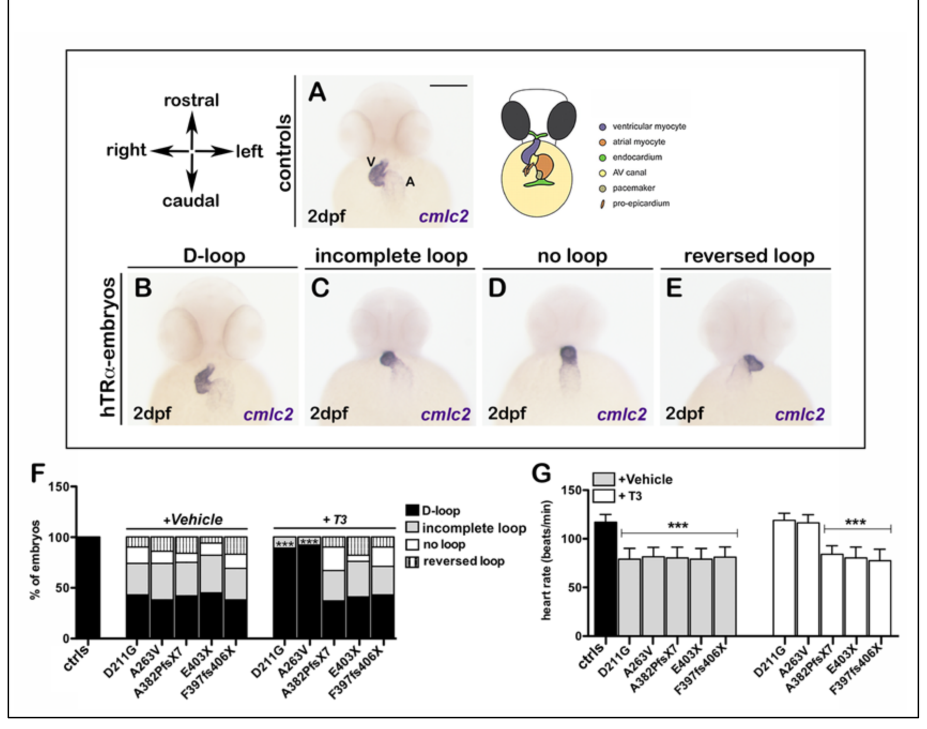
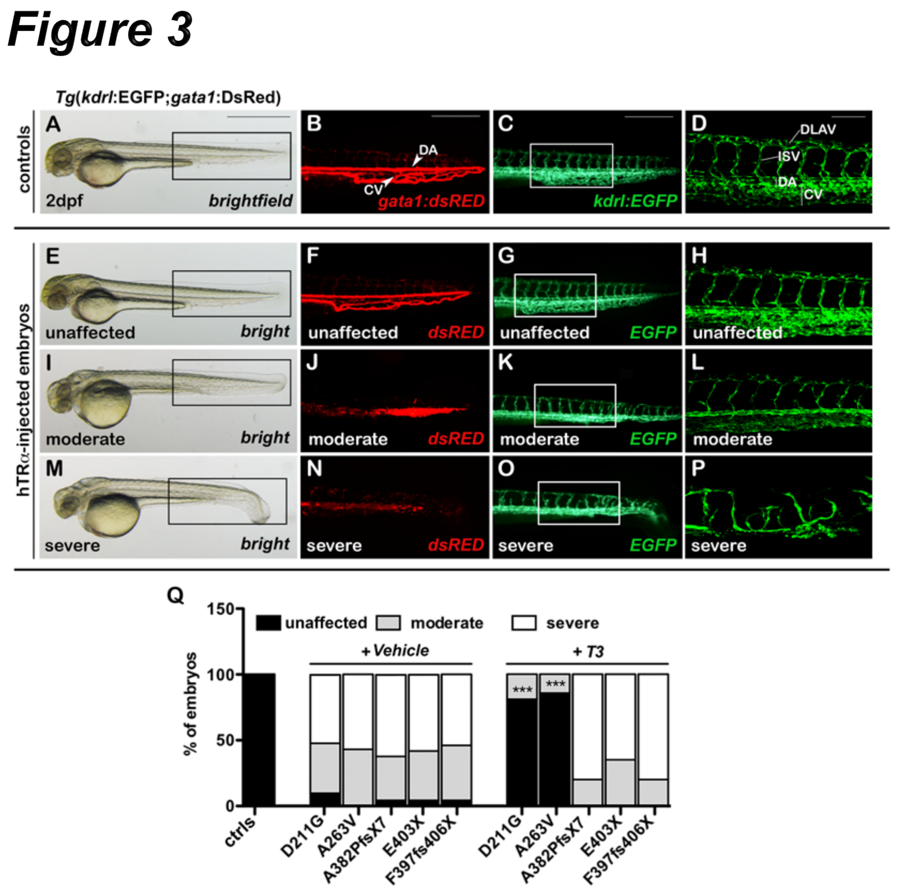


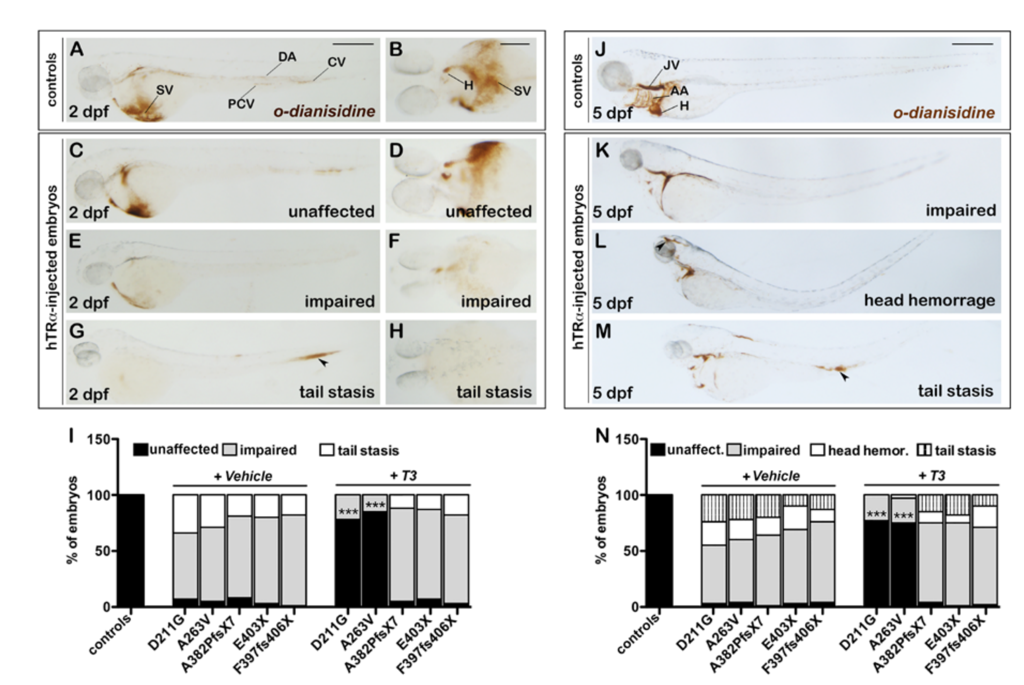
Figure 3



2.3 Vascular development in hTRα-injected embryos

To rule out that circulation defects could be caused by alteration of vascular development, we carried out hTRα injections in the tg(*gata1*:dsRed)sd2; tg(*kdrl*:EGFP)S843 double transgenic line (endothelial cells in green and erythrocytes in red) (Figure 3). The hTRα-injected embryos revealed variable defects in vascular development, comprehending embryos with moderate (K and L) or severe (O and P) abnormal development of the dorsal longitudinal anastomotic vessel (DLAV), of the intersomitic vessels (ISVs) along the trunk and the CV plexus, suggesting that these alterations were likely caused by angiogenesis abnormalities. The rate of embryos with these defects was reported in the histogram Q.

Figure 4



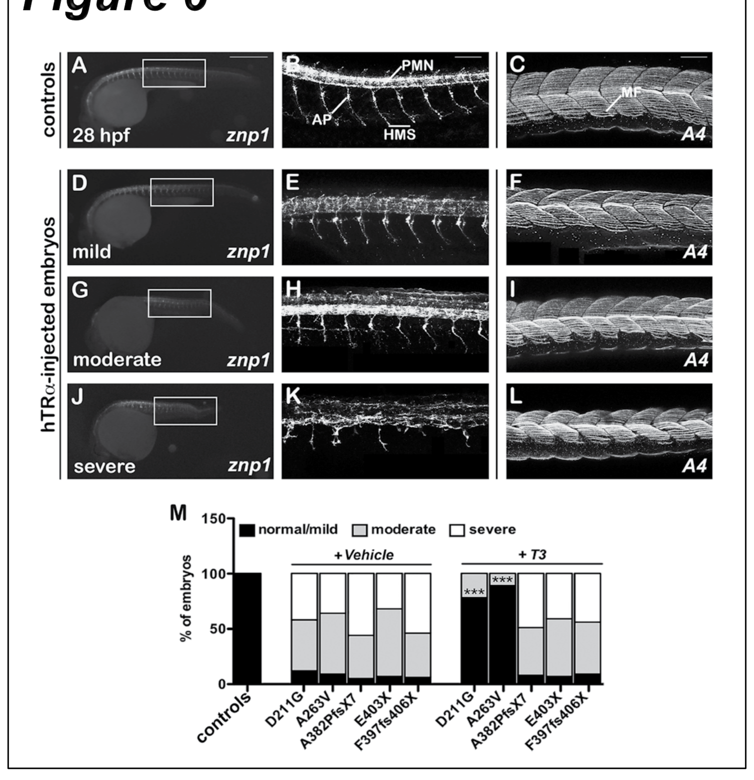
2.4 Haematopoietic process in hTRα-injected embryos

Haemoglobin hypochromia (*o*-dianisidine staining) was still evident at 2 and 5 dpf, when the >90% of the vehicle-treated hTRα-injected embryos exhibited an impairment of circulating erythrocytes (Figure 4 E-F, G-H and K-M), which are detectable in normal embryos at 2dpf in the heart (H), sinus venosus (SV), main axial vessels (DA and PCV) and in the CV plexus (A-B and C-D), and in the jugular veins (JV), in the aortic arches (AA) and in the heart at 5 dpf. As expected, only the T3-treated embryos injected with the missense variants showed a significant recovery of the defective phenotypes (I and N).

2.6 Motorneuron development in hTRα-injected embryos

All injected embryos showed early progressive motility defects leading to complete inability of autonomous swimming in later stages. At 28 hpf, *znp1* labelled the primary motorneurons (PMN), the axonal projections (AP) that extend ventrally from the spinal chord reaching the horizontal myoseptum (HMS) (Figure 6 A-B) into somatic muscle fibers (MF) in controls (C).
The axonal pathfinding resulted impaired in hTRα-injected embryos displayed a moderate to severe disorganization of both dorsal and ventral motorneurons, that appeared mis-orientated or truncated (D-F, G-H). The presence of normal development of the muscle fibers (Fig. C, F and I) indicated that motor axons do not innervate to the muscle fibers properly.

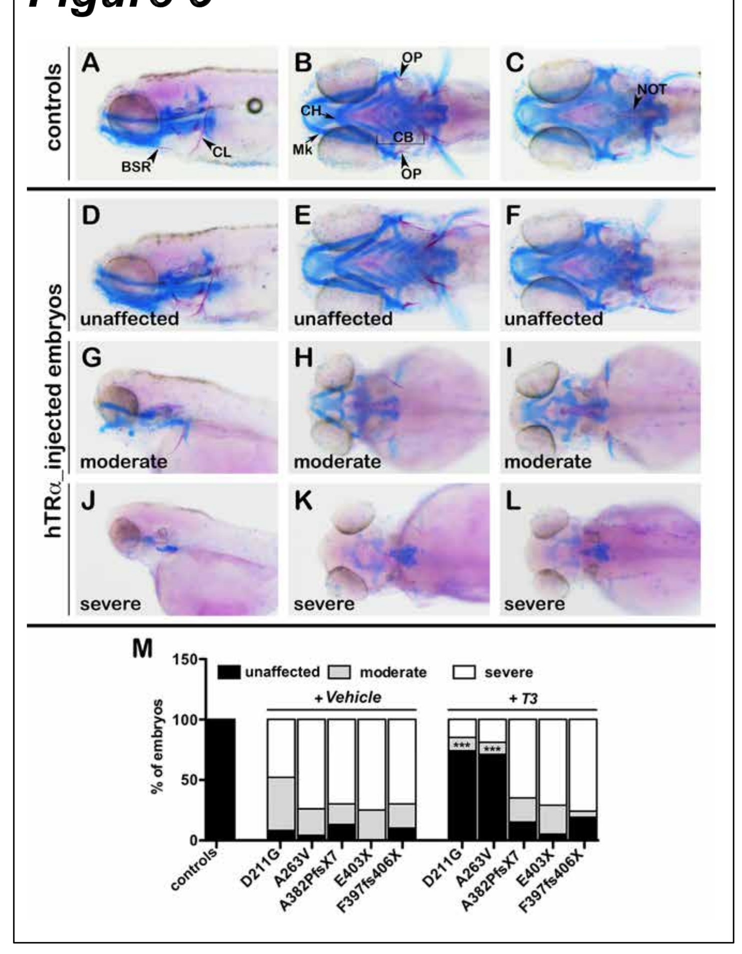
Figure 6



2.5 Skeletal maturation in hTRα-injected embryos

Alcian Blue and Alizarin Red staining was used to reveal cranio-facial cartilage and bone patterning of 5 dpf larvae (Figure 5).
In hTRα-injected embryos most of pharyngeal and neurocranium cartilage and bone elements were malformed or severely reduced (G-L) compared to controls (A-C). Those defects includes alterations in the first and second arches cartilages (Meckel's, Mk and ceratohyal, ch) and in the five ceratobranchial arches (cb) together with impaired mineralization of brachiostegal rays (brs), operculum (op) and notochord (not).
The rate of embryos with these defects was reported in the histogram M.

Figure 5



3. CONCLUSIONS

In conclusion, here we demonstrate that the injection of the human *THRA* mutated transcripts is able to counteract with the zebrafish TRs thus recapitulating the biochemical and clinical features of RTHα patients. Furthermore, we described for the first time the involvement of TRα in angiogenic processes. Indeed, zebrafish represents a powerful model to shed light on the molecular mechanisms and functional consequences of the newly discovered human *THRA* variants. Our "tailor made" models could be also useful to test new compounds that are able to overcome the TH resistance of each specific mutation avoiding thyrotoxic effects.