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"ROLE OF TYPE 3 DEIODINASE IN NORMAL SKIN AND IN WOUND HEALING PROCESSES"

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"All science would be superfluous
if the outward appearance and the essence of things
directly coincided"

Karl Marx

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ABSTRACT

The skin is a well-known target of thyroid hormone (TH). TH action is finely controlled by the deiodinase family of enzymes, responsible of the tissue-specific activation and inactivation of the prohormone thyroxine (T4).

We report that the type 3 deiodinase (D3), the principal terminator of TH action, is required for the normal epidermal proliferation. D3-depleted keratinocytes show a proliferative defect, associated with a trend toward early differentiation. K14CRE-Dio3$^{Fl/Fl}$ mice, in the absence of D3 only in epidermis, present an alterated epidermal phenotype characterized by a thinner epidermis, reduced levels of K14 (specific marker for the basal proliferating cells) and slowed T4 clearance. These manifestations affect on epidermal regeneration processes in which a proper balance between cellular proliferation and differentiation is strictly required. We found a massive induction of D3 during early phases of wound healing, when the cellular environment requires an increased proliferation necessary to repair the damage. Moreover, K14CRE-Dio3$^{Fl/Fl}$ mice show a defective wound repair compared to WT mice, with a delayed wound closure, caused by a decrease of proliferation.

In conclusion, we show that the D3 enzyme is employed to attenuate TH-signaling in skin, providing a striking example of how a circulating hormone can be tissue-specifically attenuated to influence local requirement.

Key words: type 3 deiodinase, thyroid hormone, skin, wound healing

INTRODUCTION

Thyroid hormones (THs) influence several cellular activities including proliferation, differentiation and apoptosis in virtually all mammal cells. The canonical action of TH is its binding to ligand-dependent transcription factors, namely, TH receptors (TRs). The latter enhance or inhibit the expression of target genes by binding to specific DNA sequences, known as TH response elements (TREs). An initial step in the activation of TH is its transport across the cell membrane. Indeed, TH uptake is specifically regulated by different types of TH-transporting proteins [1]. Among them there are organic anion transporters 2 and 3 (Oatp2 and Oatp3), L-type amino acid transporters (Lat1 and Lat2), and monocarboxylate transporters 8 and 10 (MCT8 and MCT10) [2-3]. These transporters are differentially expressed in several tissues in a developmental and cell-type specific fashion [4]. Moreover, although some transporters are able to carry different solutes (e.g. thyroxine (T4), triiodothyronine (T3), 3,3',5'-Triiodo- L-thyronine (rT3), 3,3'-diiodo-L-thyronine (T2)) [5] , other transporters have a high substrate specificity [6]. At nuclear level, TH action is triggered by its interaction with specific nuclear receptors, TRa and TRb, which directly bind the TRE sites on DNA and activate or repress gene expression. TRα and TRβ are encoded by the THRA and THRB genes, respectively [7]. Several cells and tissues express various isoforms of each TH receptor protein, for example receptors TRα1, TRβ1 and TRβ2. Among these, TRα1 and TRβ1 mRNAs and proteins are widely expressed in several tissues, however, their expression is locally modulated. For example in rats, TRα1 mRNA is mainly expressed in skeletal muscle and brown fat, whereas TRβ1 mRNA is mainly expressed in brain, liver and kidney. Ligand availability and the concentration of the TR-TH complex in the nucleus ultimately define the level of TR transcription activity. Upon binding to T3, TR undergoes a conformational switch that results in the release of the co-repressor and recruitment of multiple co-activator proteins to the TR-TRE complex in a sequential manner. Those cofactor changes lead, in TH-positively regulated target genes, to local chromatin remodelling and increase transcription [7].

A crucial step in the control of TH intracellular availability is given by the action of three thioredoxin fold-containing selenoenzymes, called "deiodinases". These enzymes metabolize THs in a stage- and tissue-specific manner by a mono-deiodination reaction that involves two distinct pathways. The type I and II deiodinases (D1 and D2) catalyze the outer ring deiodination thus converting the inactive precursor T4 to the active form T3 — a process that increases circulating T3 levels and the availability of the active hormone for the nuclear receptors [8]. In contrast, type III deiodinase (D3) inactivates TH by converting T4 and T3 to the inactive metabolites reverse T3 (rT3) and T2, respectively. All three deiodinases are integral membrane proteins that share a ~15 amino acid long conserved region within the active center coding for a selenocysteine. The subcellular localization varies among the three enzymes, and this affects their systemic versus cellular contributions to TH homeostasis [9]. In particular, D1 is an integral plasma membrane protein and D2 is an endoplasmic reticulum resident protein. D3 is mainly localized within the plasma membrane, and is recycled through the endosomes and is often found in other subcellular localizations [10]. In terms of enzymatic efficiency, D2 is considered the principal activator of TH action at intracellular level, whereas D3 is the principal terminator of TH action. Notably, the combined actions of D2 and D3 are viewed as a cell autonomous pre-receptoral mechanism that controls TH signalling in a time- and tissue-specific manner although it does not affect serum hormone concentrations. In contrast, D1 is an enzyme with a low kinetic efficiency. It plays a significant role in circulating T3 levels. In fact, D1-generated T3 equilibrates with plasma more rapidly than D2-generated T3 which has a much longer intracellular resident time [11]. The ability of D1 to dehalogenate iodine metabolites suggests that it could also act as a scavenger enzyme that recycles iodine to replenish the thyroid's iodine reservoir (Fig.1).

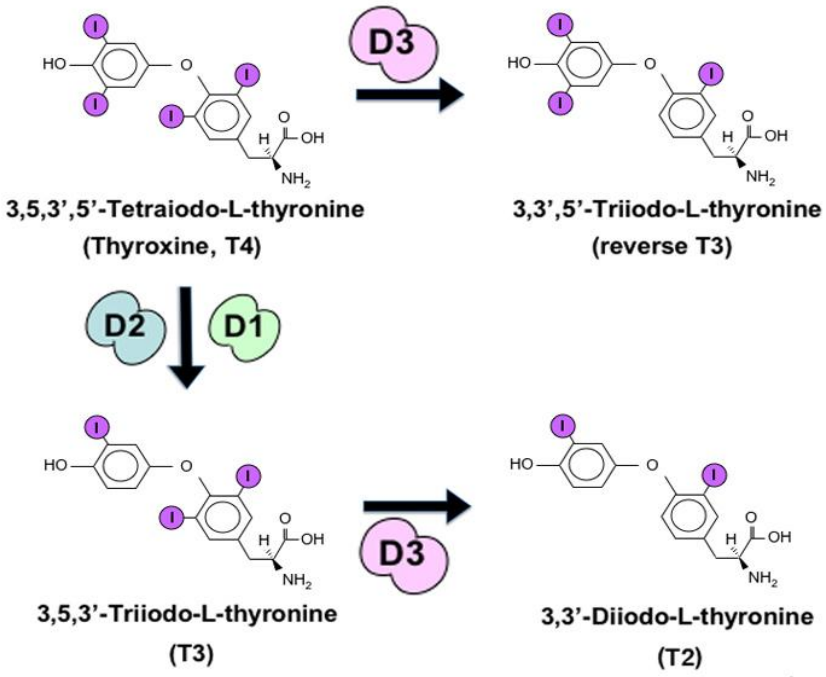

Fig.1: Structures of the iodothyronines differentially activated or inactivated by the deiodinases. 3,5,3',5'-tetraiodo-L-thyronine (thyroxine, T4) is converted into 3,5,3' triiodo-L-thyronine (T3) by two iodothyronine deiodinases, D1 and D2, by phenolic outer ring deiodination. T4 and T3 are respectively inactivated in 3,3',5'-triiodo-L-thyronine (reverse T3) and 3,3'-diiodo-L-thyronine (T2) by phenolic inner ring deiodination catalyzed by type 3 deiodinase, D3. (Sibilio A., Minerva Endocrinol 2012)

D3 - the main physiologic inactivator of TH - is thought to control TH homeostasis by protecting tissues from an excess of TH. It is abundantly present in fetal tissues and high D3 levels are expressed in the human placenta, where it blocks the excessive maternal-to-fetal transfer of T4. In contrast, during late neonatal and adult life, D3 expression is more limited, and under regulation by tissue specific agents. Thus far, D3 activity has been identified in only a limited number of postnatal tissues, i.e., skin, brain, and pregnant uterus [12-13]. Of note, several studies have revealed the re-expression of D3 in different pathophysiological conditions, among which cardiac hypertrophy, chronic inflammation, critical illness [14-15], hypoxia or ischemia [16] and cancer. We have recently demonstrated that D3 is up-regulated in basal cell carcinoma [17] and in colon carcinoma [18-19] as well as in hemangiomas [19-20], astrocytomas [21], pituitary gland adenoma [22] and glioblastoma [21]. Because of its

presence in fetal as well as in malignant tissues, D3 is referred to as an “oncofetal enzyme”.

The human type 3 iodothyronine deiodinase (DIO3) gene was cloned in 1995 [23] and subsequently localized to chromosome 14q32 [24]. In contrast to human DIO1 and DIO2 genes, which contain four and two exons, respectively, D3 genomic structure contains a single exon encompassing 1,853 nucleotides. Two other major features of the mouse Dio3 gene are that: i) its sequence in both the coding and 3′-UTR regions has been highly conserved among diverse species; and that ii) its promoter region, which is located within 78 bp of the translational start site, is highly GC enriched (80%), containing a TATA box that is critical for its function as well as CAAT and GC boxes. The human DIO3 gene is positioned 870 kb downstream of two imprinted genes, DLK1 and GLT2. In the mouse, D3 is the last imprinted gene at the telomeric end of the imprinted domain Dlk1/Gtl2, and is preferentially expressed from the paternal allele both in human and mouse [25-26].

The genetic mouse model of D3-deficiency has been instructive in understanding the role of D3 with regard to fertility and developmental processes. Both male and female D3KO mice showed impaired fertility, significant perinatal mortality and growth impairment [27]. In addition, the developmental programming of the thyroid axis is perturbed in the D3KO mouse, presumably due to the overexposure of the animal to excessive levels of TH in utero and during the first weeks of perinatal life. Early in life, D3KO mice have a delayed T3 clearance with markedly elevated serum T3 level. From postnatal day 15 to adulthood, D3KO mice demonstrate central hypothyroidism, with low serum levels T4 and T3, and modest or no increase in thyroid-stimulating hormone (TSH) concentration. The thyrotoxic status in the D3KO newborn evolves to hypothyroidism after 2 weeks of life, and this persists in adulthood. This is accompanied by tissue hypothyroidism, as demonstrated by a marked decrease in liver expression of T3-inducible genes. The nature of this hypothyroidism is clearly central, as serum TSH is suppressed, unchanged, or only slightly elevated in D3KO at different stages despite the low serum concentrations of both T4 and T3. In the adult, a 50% increase in TSH is observed in the presence of low T4 and T3 levels

[27]. Thus, the D3KO mouse manifests marked abnormalities in thyroid status and physiology, which underscores the critical role of the D3 enzyme in the development and in the function of the hypothalamic-pituitary-thyroid (HPT) axis (Fig.2). Interestingly, the abnormalities of the HPT resemble those observed in children born to mothers affected by hyperthyroidism during pregnancy [28-29]. However, refinement and optimization of the animal models such as the generation of a conditional Dio3- KO mouse will provide useful tools to advance our molecular understanding of D3's role in development and its specific role in local tissue physiology.

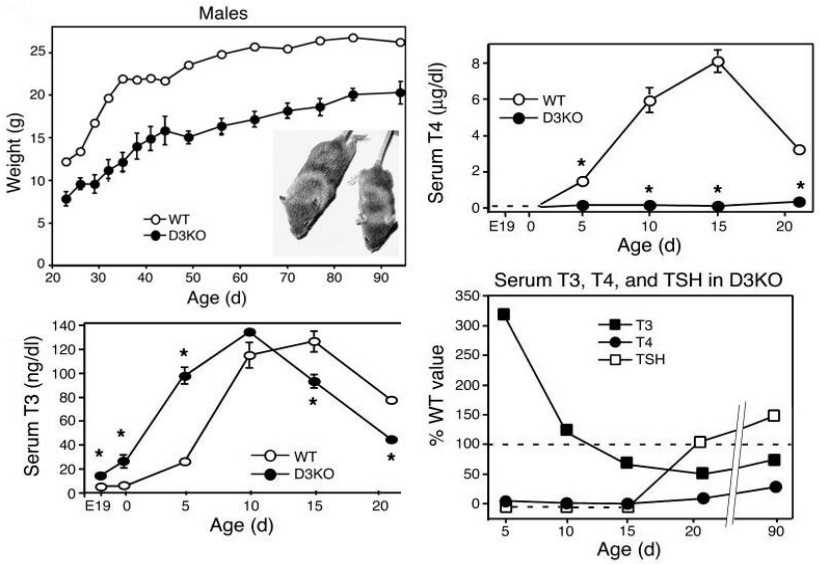

Fig. 2: Mouse D3 knockout model. D3KO mice exhibit growth impairment and alterated maturation and function of thyroid axis [27].

Thyroid hormone action and skin

The skin is a well-recognized target of thyroid hormone, which is an important regulator of epidermal homeostasis. The skin, which protects organisms from the external environment, is comprised of a stratified epithelium, the epidermis, separated by a basement membrane from the underlying connective tissue, the dermis. After birth, its role is to guard against infection, to prevent dehydration, and to undergo re-epithelialisation after wound injuries. To

accomplish these feats, the epidermis constantly replenishes itself by a process of homeostasis. During this process, dividing cells in the innermost (basal) layer continually execute a programme of terminal differentiation, move outwards and are sloughed from the skin surface. When expose to physical trauma and chemical assaults the epidermis must also protect itself, which it does by producing copious amounts of cytoplasmic heteropolymers known as intermediate filaments that are composed of keratin proteins. As cells exit from the basal layer and begin their journey towards the skin surface, they switch from the expression of keratins K14 and K5 to K1 and K10 [30-31]. This switch was discovered more than 30 years ago, and remains the most reliable indication that an epidermal cell has undergone a commitment to terminally differentiate. The first suprabasal cells are known as spinous cells, reflecting their cytoskeleton of K1/K10 filament bundles connected to robust cell–cell junctions known as desmosomes. These connections provide a cohesive, integrated mechanical framework across and within stacks of epithelial sheets. K6, K16 and K17 are also expressed suprabasally, but only in hyperproliferative situations such as wound healing [32-33].

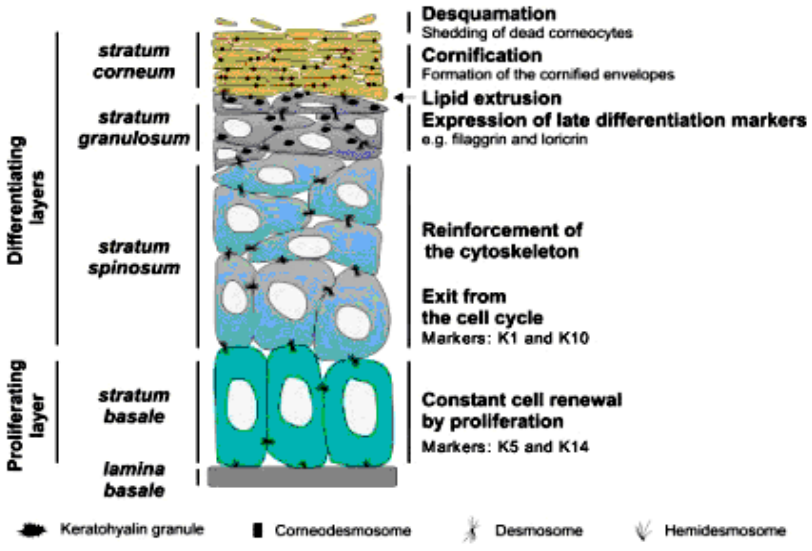

Fig. 3: Scheme of epidermal layers of the skin with the expression of the specific markers.

Normal TH levels are required for efficient epidermal homeostasis, function and regeneration. Moreover, TH action is crucial in the balance between proliferation and

differentiation in normal and pathological conditions, including epidermal regeneration and cancer [34]. In skin, TH exerts profound effects on fetal epidermal differentiation, barrier formation, hair growth, sebum production, wound healing, epidermal oxygen consumption, keratinocyte proliferation, and keratin gene expression [35-38]. Besides, several TH responsive genes have been identified in skin. For example, TH stimulates the K5, K14, and K17 gene promoters [36] and the hairless promoter is also differentially regulated by TH in keratinocytes [39]. Moreover, TH affects many cell cycle genes: cyclin D1, Mdm2, Myc, p21, p53 [40-41].

Direct thyroid hormone action on skin is mediated through TRs [42-44]. All three widely recognized thyroid hormone binding isoforms of TR have been identified in skin tissues. TRα have been detected in epidermal keratinocytes, skin fibroblasts, hair arrector pili muscle cells, other smooth muscle cells, sebaceous gland cells, vascular endothelial cells, Schwann cells, and a number of cell types that make up the hair follicle [42]. TRβ1 was the predominant form of TR expressed in the human hair follicle [43]. In addition, TRβ1 acts as a potent suppressor of tumor invasiveness and metastasis in chemical skin carcinogenesis. TR deficiency seems to inhibit benign tumor formation at early stages of skin carcinogenesis while increasing malignant progression at later stages [44]. Moreover, TRα and TRβ mediate effects of thyroid hormone on epidermal proliferation. The epidermis of knockout mice lacking the TH binding isoforms TRa1 and TRb shows reduced keratinocyte proliferation. Defective proliferation is associated with reduction of cyclin D1 expression and up-regulation of the cyclin-dependent kinase inhibitors p19 and p27 [45]. TRKO mice show decreased hyperplasia in response to topical application of 12-O-tetradecanolyphorbal-13-acetate (TPA), a well-known model for induction of skin hyper-proliferation and inflammation [45]. Anyway, available data in the literature show that the function of THs and of their receptors on cell proliferation is not homogenous. In fact, it strongly depends on the cell type, its developmental state (progenitor or differentiated), its patho-physiological state (normal or tumor cell), and the so-called ‘cellular context’. For example, T3 activates proliferation of hepatocytes after partial hepatectomy and is considered a primary liver

mitogen [46-47]. It induces proliferation of cultured bovine thyroid cells, of bone marrow pro-B cells [48], of pancreatic acinar cells [49-50], and of renal proximal tubular epithelial. Finally, it increases DNA synthesis in osteoblasts [51]. On the other hand, T3 treatment blocks proliferation and induces differentiation of oligodendrocyte progenitor cells of neuroblastoma N2a-β cells [52], and of erythroid progenitors [53]; it also prevents cycling of postnatal Sertoli cells [54] [55], as well as of mammary epithelial cells. Also in the skin the effects of TH on keratinocyte proliferation and epidermal differentiation are controversial, with divergent results [35]. Safer's group has found that systemic and topical TH administration cause diverging effects on keratinocyte proliferation. While topical T3 stimulates epidermal proliferation, hair growth, dermal thickening and accelerates wound healing in mice [56-58], systemic thyrotoxicosis leads to thinning of skin, hair and collagen loss [57]. The authors postulate that the different effects exerted by topical versus systemic TH treatment on epidermal cell can be explained by the T3-induced production of inhibitory factors from fibroblasts with systemic T3-treatment. In addition, Zhang B et al. have recently showed thyroid hormone analogue stimulates keratinocyte proliferation but inhibits cell differentiation in epidermis [59]. In contrast, Tiede et al. have demonstrated that THs reduce proliferation, Cyclin D1 expression and induce apoptosis of isolated K15-GFP+ cells (K15, marker of human epithelial hair follicle stem cells). THs reduce the viability of human hair follicle epithelial progenitor cells in vitro, caused by combination of cell death and growth arrest [60].

In keeping with the ability to modulate growth and differentiation, it is not surprising that TH might play a critical role in skin. Indeed thyroid dysfunction is correlated with alterations in skin architecture and homeostasis [61] and changes in circulating TH levels, particularly hypothyroidism, are often associated with an edema-like-associated skin condition caused by increased glycosaminoglycan deposition. The skin exhibits typical abnormalities in hyperthyroidism and hypothyroidism [37, 62]. In hyperthyroidism, skin changes include erythema, palmoplantar hyperhidrosis, acropathy, and infiltrative dermopathy; also, Graves' disease may be associated with generalized pruritus, chronic urticaria, alopecia areata,

vitiligo, and diffuse skin pigmentation. In hypothyroid humans the skin is cool and dry with a pasty appearance, the epidermis is thin and hyperkeratotic, alopecia may develop, and there is diffuse myxedema [61], showing that thyroidal status influence skin morphology and function.

Finally, conflicting evidence are present in the literature about TH action in epidermal regeneration processes, as wound healing. Healing of wounds is one of the most complex biological events afterbirth as a result of the interplay of different tissue structures and a large number of resident and infiltrating cell types. Indeed, wound healing is an interactive process that involves soluble mediators, extracellular matrix components, resident cells (keratinocytes, fibroblasts, endothelial cells, nerve cells), and infiltrating leukocyte subtypes, which participate differentially in the classically defined three phases of wound healing: inflammation, tissue formation, and tissue remodelling [63-64]. Safer's data affirm that the topical application of supraphysiologic doses of T3 accelerates wound healing in mice, but at the same time intraperitoneal injection of T3 do not have wound healing acceleration beyond that of control mice [58]. In addition, the L-thyroxine replacement therapy in hypothyroidism cases was beneficial with regard to wound healing [65]. In contrast, Pirk et al. noted no change in wound healing with euthyroid hamsters receiving ip T4 [66].

Type 3 deiodinase

As stated previously, TH signalling is also modulated locally by the deiodinases. The expression of D2 and D3 can be exquisitely cell-specific and change rapidly in response to a number of developmental, metabolic, and disease cues through different signaling pathways [14]. Because the expression of these enzymes can be turned on or off in discrete groups of cells, most of the time their actions do not affect circulating thyroid hormone levels, which are tightly controlled via the TRH/TSH axis. Thus, the actions of D2 and D3 are viewed as a cell-specific pre-receptor mechanism to control thyroid hormone signaling that cannot be predicted based on the levels of circulating TH [14].

The presence of D3 activity in skin is dating to 1985, when Huang TS et al. demonstrated that "skin is an active site for the inner ring monodeiodination of thyroxine to 3,3',5'-triiodothyronine" [67]. Subsequently, various studies showed that D3 protein is present in both mouse and human skin [17, 68-70]. D3 is first expressed in the mouse epidermis at E13.5; it is highly expressed in the epidermal layers and in hair follicle keratinocytes at E17.5. D3 expression is very high in anagen (growing) phase of the hair follicle cycle, as well as in the surrounding outer root sheath [17]. The characteristic re-expression of D3 in many tissues in disease states, as well as in tumoral tissue [15] occurs also in skin. Indeed, a striking model of D3-mediated TH regulation in cancer is provided by the presence of D3 in basal cell carcinoma (BCC) [17], which is the most frequent human cancer. This carcinoma is formed upon constitutive activation of the Shh-Gli pathway. The morphogen Shh, through the transcriptional factor Gli2, directly induces D3 mRNA and protein in proliferating keratinocytes and in mouse and human BCCs, whereas it promotes D2 degradation via the E3 ubiquitin ligase adaptor (WSB-1) [71]. Indeed, Gli2-induced D3 reduces intracellular TH activity, thus resulting in increased cyclin D1 and keratinocyte proliferation. Conversely, D3 knockdown in BCC cells causes a drastic reduction in cell proliferation and consequently a five-fold reduction in the growth of BCC xenografts in nude mice in vivo [17]. However, the exact role of D3 in initiation-growth-progression of tumorigenesis is not clear. The restricted range of tumors associated with D3 overexpression suggests that initiation of tumorigenesis does not necessarily require D3, and perhaps modulation of D3 expression is only one of the multiple mechanisms adopted by tumoral cells to escape the differentiative action of T3.

Interestingly, the reactivation of D3 has been also documented in many patho-physiological conditions in which cell proliferation is enhanced, namely in liver and neural regeneration, cardiac hypertrophy [72-74]. Recent studies on the involvement of D3 in tissue injury have shed light on its role in the control of cell proliferation. An explicative model is provided by liver regeneration induced by partial hepatectomy in animal models [72]. In these studies, D3 expression was strongly induced in the liver of mice and rats. Enhanced D3 activity in regenerating liver correlated with increased BrdU incorporation and reduction of local and

systemic T3 and T4 levels [72]. Moreover, our studies offer a new tissue regeneration model in which deiodinases are finely regulated. It is represented by skeletal muscle regeneration. In particular, we demonstrated that TH produced by D2 is essential for normal mouse myogenesis and muscle regeneration. Indeed, D2-mediated increases in T3 were essential for the enhanced transcription of myogenic differentiation 1 (MyoD) and for execution of the myogenic program. Conversely, the expression of T3-dependent genes was reduced and after injury regeneration markedly delayed in muscles of mice null for the gene encoding D2 (Dio2), despite normal circulating T3 concentrations [75].

AIM OF THE STUDY

The goal of this project was to evaluate the role of Type 3 deiodinase (D3) in the normal skin and during epidermal regeneration as in the wound healing process. Although mounting evidence suggests that TH plays an important role in epidermal proliferation, the physiologic role of thyroid hormone in skin is not well understood. In order to analyze D3 function in epidermis regeneration, we generated a conditional mouse model in which D3 is depleted only in the cells of the basal layer in the epidermis and compared regenerative ability of epidermis in the presence or absence of D3. The data obtained from this study might prompt to understand the role of TH in wound repair and to exploit TH regulation as a new efficient strategy to treat cutaneous diseases, in which a proper balance between proliferation and differentiation is required.

RESULTS

Type 3 deiodinase is highly expressed in adult skin

The presence of D3 activity in adult skin has been demonstrated for the first time by Huang TS et al in 1985. Skin is an active site for the inner ring monodeiodination of thyroxine to 3,3',5'-triiodothyronine [67]. Moreover, it has been recently demonstrated that D3 is the primary deiodinase active in murine epidermis [70], but the specific localization of D3 protein in adult skin structures is still unknown. The Fig.4 shows the profile of D3 protein expression in telogen phase of hair follicle cycle of the mouse skin. By immunohystochemical analysis, we observed that D3 protein is highly expressed in the bulge region, in sebaceous gland of hair follicle and is absent in the dermal papilla. In addition, D3 protein is also detectable in infundibulum cells and is extended with the interfollicular epidermis (Fig.4).

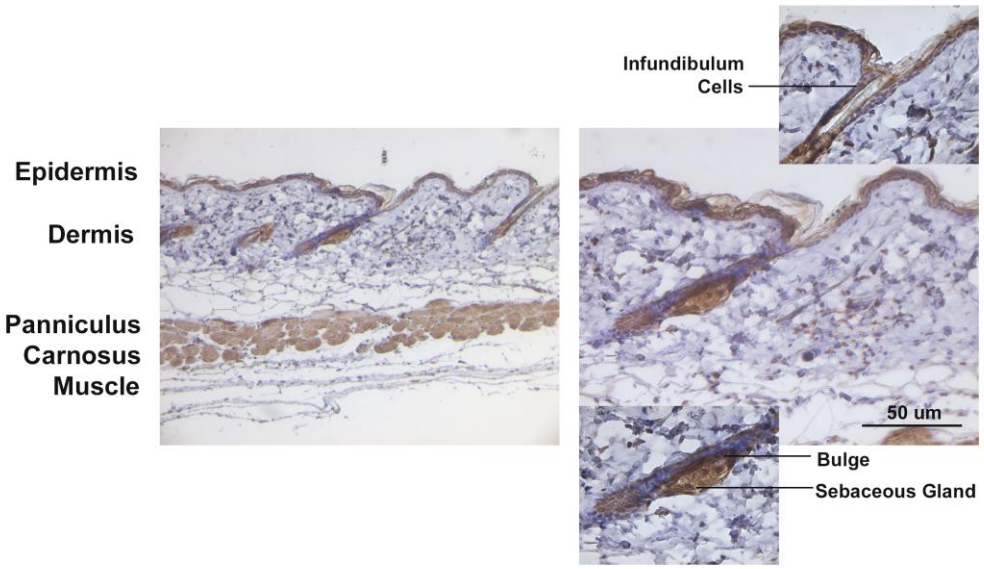

Fig.4: *D3 is specifically expressed in mouse skin. Paraffin sections of mouse skin in telogen phase of the hair follicle cycle were probed with D3-718 antibody. D3 was highly expressed in the bulge, sebaceous gland of hair follicle, but it is absent from the dermal papilla. D3 is evident in infundibulum cells and it is extended in interfollicular epidermis, as well as detectable in panniculus carnosus.*

D3 is induced in the early phase of wound healing

Since D3 is critical for proper cell proliferation in different conditions [17-18, 75], we asked whether D3 plays a role in wound healing process. Notably, regeneration of the epidermis after wounding is a complex and dynamic process consisting of various sequential phases [63-64]. It involves activation, migration and proliferation of keratinocytes from both the surrounding epidermis and the adnexal structures such as the hair follicle and sweat gland [76-77]. Repair of tissue is a self-limiting process and in response to wounding is activated an interplay of different cell types, such as epithelial, mesenchymal and immune cells, recalled to invade the underlying dermis. During wound healing, bulge stem cells mobilize, leave their stem cell niche and contribute to repopulation in the basal layer of the epidermis [77]. In order to investigate the role of D3 in wound repair process, we performed a wound healing experiment in vivo. First, we examined the expression patterns of D3 following wound created in the dorsal skin in WT mice. Three days after full-thickness injury, expression levels of Dio3 mRNA and D3 protein increased significantly in the epidermal keratinocytes and reached a peak at day 6 and then declined at day 10, when the healing process is almost completely terminated (Fig.5). These data indicate that D3 is induced in vivo during regeneration of adult skin, causing tissue-specific reduction of TH signaling in epidermal cells.

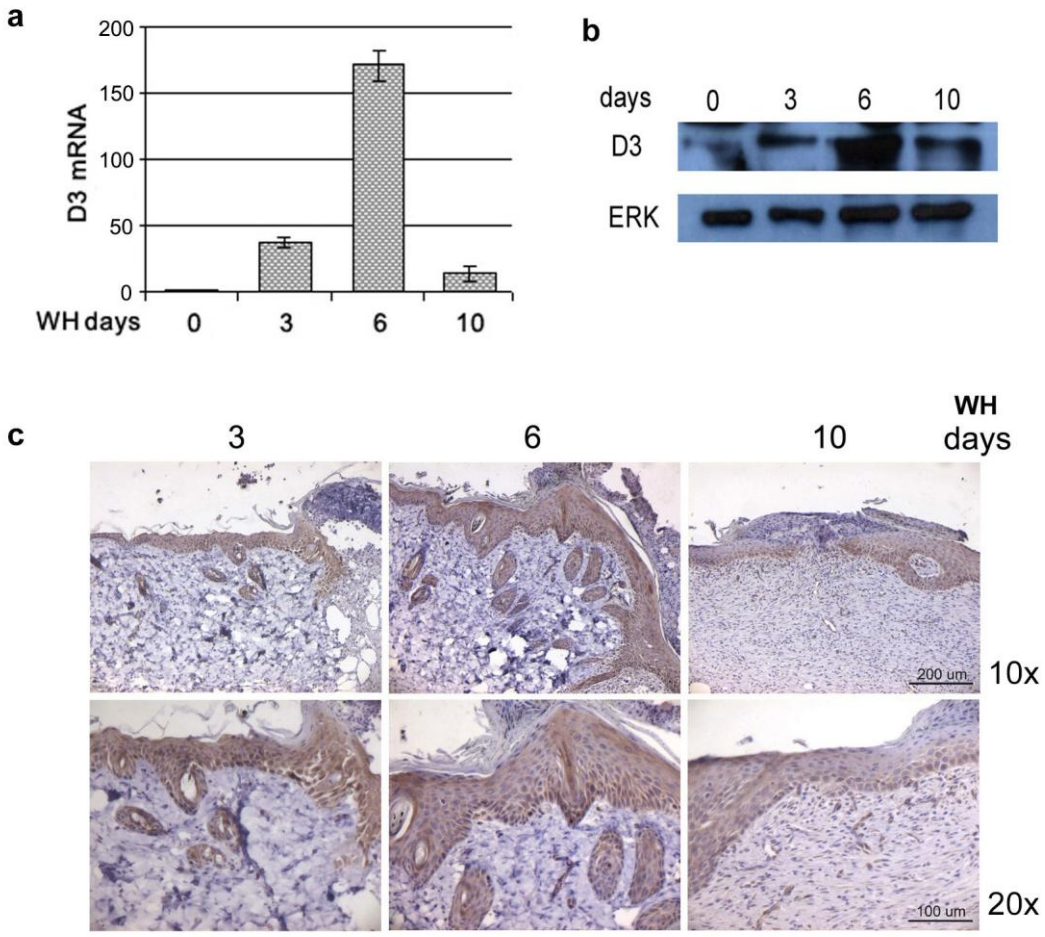

Fig. 5: D3 expression is induced during early phase of wound repair. (a) D3 mRNA expression during wound healing was measured by real time PCR analysis. The wounds were either embedded for sectioning or excised including 2mm of the epidermal margins for RNA isolation, at 3, 6, 10 days after injury. Cyclophilin A was used as internal control. Normalized copies of the target gene in adult healthy skin were set as 1 and it corresponds at day 0. Data are shown as average of 3 separate experiments analyzing 4 mice for each group. (b)Western Blot analysis was performed using our anti-D3 ab(717) and anti-Total ERK ab was used to normalize. (c) Immunohystochemical staining of D3 was executed on 6 um sections of skin with anti-D3 Ab(718).

Generation of a conditional skin-specific knock out mouse

The identification of D3 in epidermal cells prompted us to investigate its specific function in the epidermal context. What is the role of D3 in the skin? To answer to this question, we generated a basal layer cell-specific (K14-CRE) conditional Dio3 knock-out mouse (K14CRE-Dio3$^{Fl/Fl}$) (see Materials and Methods), in which the expression of recombinase

Cre induces loss of D3 activity in epidermal cells. Indeed, we used a Cre expressed under the control of the keratin 14 gene (K14) promoter and responsible for ubiquitous recombination in hair follicles and basal cells of epidermis at E15.5 [78]. We crossed K14CRE mouse with our Dio3 floxed mouse ($Dio3^{Fl/Fl}$, that in this thesis we will name WT mice), in which two Lox sites flank the SECIS sequence, necessary for proper D3 protein translation. The SECIS element is responsible for the incorporation of a selenocysteine residue in the catalytic domain and for the recognition of UGA as a codon for Sec rather than as a stop signal [79]. When the Cre acts, the loss of the SECIS, induces a premature termination of protein translation (Fig. 6a). The epidermis is a dynamic tissue in which keratinocytes proliferate in the basal layer and undergo a tightly controlled differentiation program upon migration into the suprabasal layers. Since the keratinocytes in the basal layer do express the CRE, and will constitute the upper layers, the K14CRE-$Dio3^{Fl/Fl}$ mice will result in the depletion of D3 from the entire epidermis. The Fig.6b shows the reduction in D3 protein following CRE expression. In this context we speculated that K14CRE-$Dio3^{Fl/Fl}$ skin becomes "hyperthyroid" at nuclear level, in contrast with the skin normal TH serum levels that we measured in K14CRE-$Dio3^{Fl/Fl}$ mice.

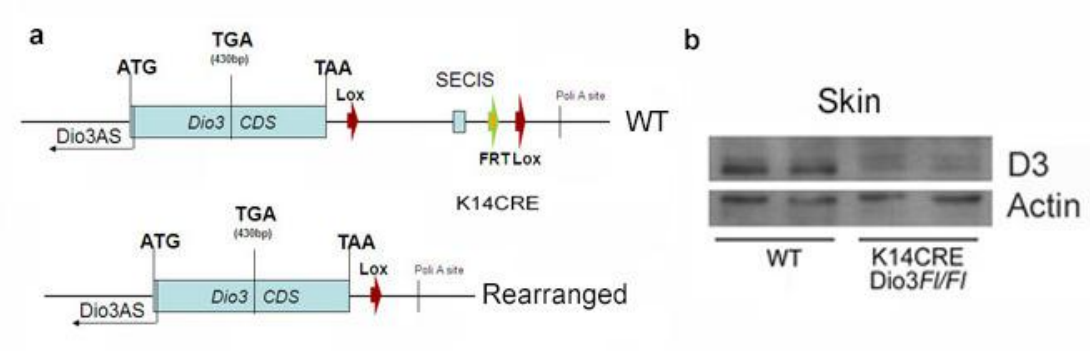

Fig.6: Generation of a conditional skin-specific knock out mouse. (a) Diagram of the Dio3 locus modified with the Lox sites. These sites were introduced surrounding the SECIS element into the 3'-UTR region of the Dio3 locus. The image shows the WT and rearranged D3 allele after K14CRE action. (b)The Western Blot analysis shows the effective D3 reduction in K14CRE-$Dio3^{Fl/Fl}$ skin, caused by an anticipated termination of D3 protein translation.

Characterization of the epidermis in the K14CRE-Dio3Fl/Flmice

The analysis of D3-depleted skin showed a significant reduction of epidermal thickness in K14CRE-Dio3$^{Fl/Fl}$ mice versus WT as demonstrated by Hematoxylin and Eosin staining. K14CRE-Dio3$^{Fl/Fl}$ mice present a thinner epidermis more than 40% thinner than WT epidermis (Fig. 7a). Moreover, to understand if this morphologic alteration derives from a defect in keratinocytes proliferation, we measured expression levels of K14, a specific marker of basal proliferating cells [36, 80]. We found that K14 RNA and protein levels were reduced in K14CRE-Dio3$^{Fl/Fl}$ skin with respect to WT skin (Fig.7b). This data is in agreement with already known T3-mediated repression on K14 promoter [36, 80], and support the concept that T3 signaling is over-active in the K14CRE-Dio3$^{Fl/Fl}$ skin.

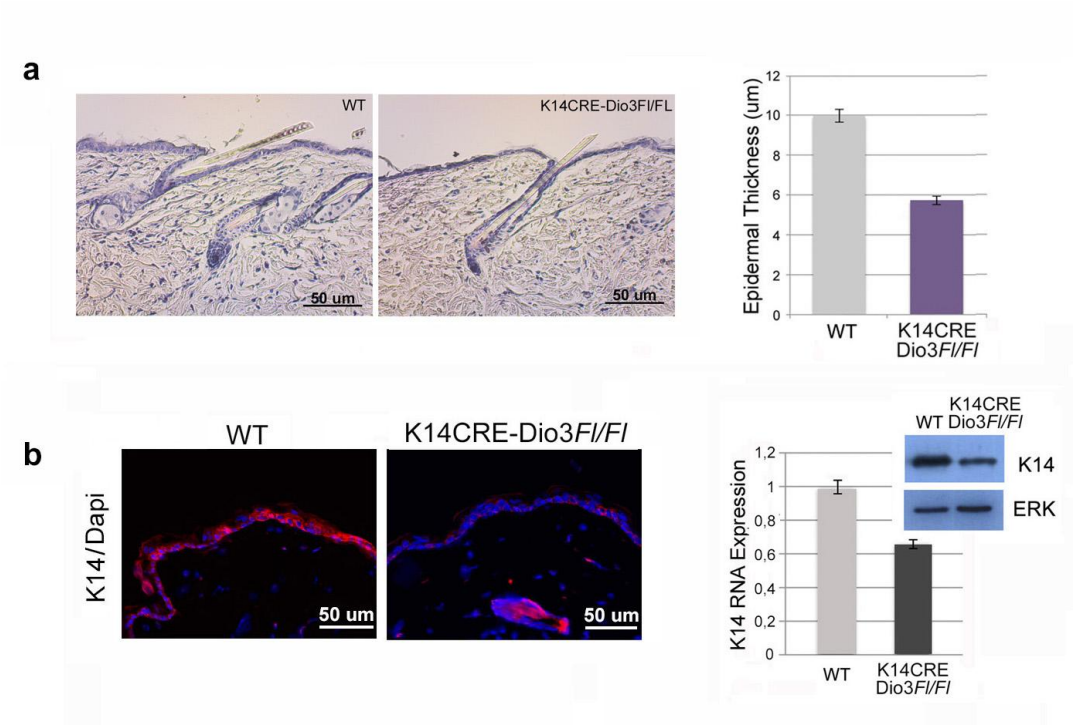

Fig.7: Alterated epidermal phenotype of K14CRE-Dio3$^{Fl/Fl}$ mice (a) Hematoxylin and eosin staining of K14CRE-Dio3$^{Fl/Fl}$ and WT skin and measure of epidermal thickness (um) calculated with LCMicro Software. Scale bar: 50 um. Each bar represents the average ± SEM of determinations in 6 animals for group (P<0,01). (b) mRNA and protein K14 expression in adult K14CRE-Dio3$^{Fl/Fl}$ and WT skin. For WB analysis 4ug of proteins were used and the levels were normalized with ERK-Tot. mRNA levels were normalized to Cyclophilin A. Each bar represents the average ± SD of determinations in 4 animals for group.

Type 3 deiodinase is critical in the control of the proliferation in primary mouse keratinocytes

T3 often acts as a differentiating agent, controlling the balance between proliferation and differentiation. This occurs in many cellular systems [18, 60] and we have previously shown that proliferation is reduced by T3 treatment in basal cell carcinomas, one of the most common tumors in humans [17]. Using the same rationale, we hypothesized that D3-depletion would impact on the proliferation of primary keratinocyte. To demonstrate this, we grew colture primary keratinocytes from Dio3$^{Fl/Fl}$ mice. Adeno-CRE (Ad-CRE) infection of these keratinocytes resulted in effective D3 depletion, with a genomic rearrangement of about 90% of the total genomic DNA (Fig.8a), in agreement with the genetic strategy used. In keratinocytes infected with Ad-CRE versus Ad-GFP, we observed that the cellular proliferation was reduced. This was demonstrated by measurement of BrdU incorporation and by the decreased Cyclin D1 mRNA levels (Fig.8b).

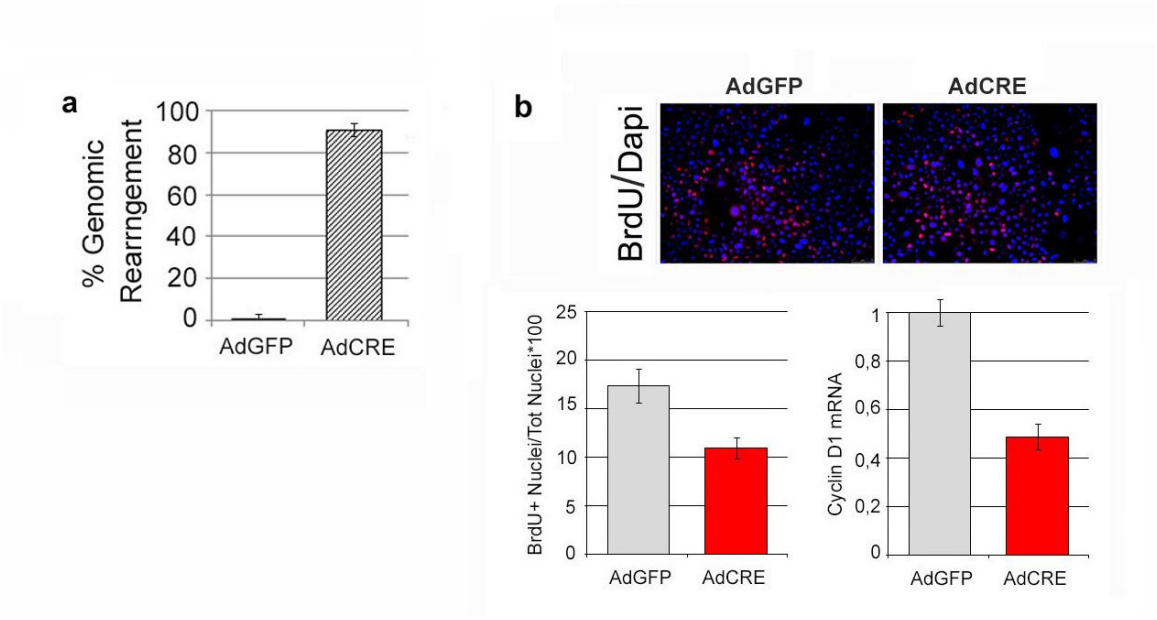

Fig.8: D3 is critical in the control of the proliferation in primary mouse keratinocytes. (a) % D3 genomic rearrangement was measured in primary mouse keratinocyte (Dio3Fl/Fl)and infected with AdCRE and AdGFP. (b) Immunofluorescent staining with anti-BrdU antibody was performed at 48h from the infection. Percentage of BrdU-labeled nuclei wa evaluated as BrdU+ nuclei/Tot nuclei. n = 50 fields, P < 0.01. Cyclin D1 mRNA levels were measured from AdCRE and AdGFP keratinocytes. Data are shown as average ± SD.

Altered morphology was observed in Ad-CRE infected keratinocytes, which showed smaller and rounded form, which suggested an early differentiation (Fig.9a). Keratinocytes are cultured in medium containing low concentration of calcium ions. In this condition desmosome assembly is inhibited and the cells fail to stratify, with differentiated cells detaching into the culture medium. The morphologic aspect of Ad-cre infected keratinocytes seemed typical of cells which are closed to detaching from the plate. Therefore, we measured specific differentiation markers: K1, specifically expressed in the spinous and granular layers; Loricrin, characteristic of granulosum stratum; Pvrl4, a cell adhesion molecule mainly implicated in the formation of cadherin-based adherens junctions [81]; desmoglein 1 (Dsg1), an epidermal adhesion molecule [82]. All those markers were induced different fold in D3-depleted keratinocytes (Fig.9b), suggesting that local excessive TH drives the cell towards a functional differentiation.

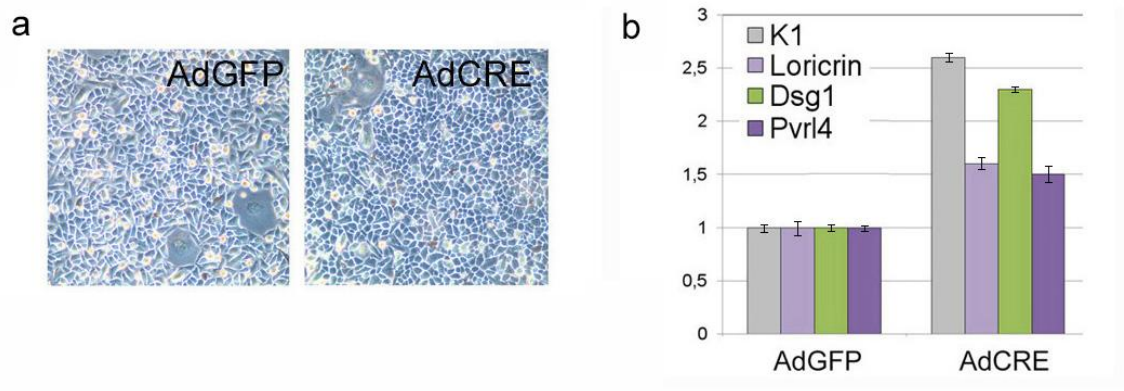

Fig.9: (a) Morphology of AdCRE and AdGFP primary mouse keratinocytes cultured in low calcium conditions (Scale bar: 100um). (b) K1, Loricrin, Dsg1 and Pvlr4 mRNA expression levels were measured from AdCRE and AdGFP keratinocytes. Data are shown as average ± SD.

This was similar to what observed in WT keratinocytes growing with exogenous different TH concentrations (data not shown), while this did not occur in $D2^{Fl/Fl}$ keratinocytes (derived by the $Dio2^{Fl/Fl}$ mice) infected with Ad-CRE. In these latter cells, the absence of D2 in primary keratinocytes did not affect the proliferative or differentiative capacity.

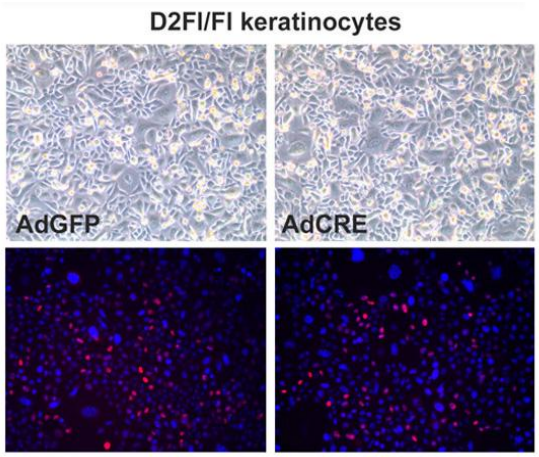

Fig.10: Dio2Fl/Fl primary mouse keratinocytes. Morphology and BrdU incorporation analysis. Scale bar:100um

These *in vitro* data indicate that D3, by inactivating TH, is a critical modulator of cellular proliferation and that hyperthyroid conditions act as a differentiating agent in primary keratinocytes. In addition, the results obtained in $D2^{Fl/Fl}$ keratinocytes underline the specificity of the cellular effects obtained with D3-depletion and support the concept that D3 is the primary active deiodinase in murine epidermis [70].

Defective epidermal regeneration in K14CRE-$Dio3^{Fl/Fl}$ mice following epidermal wound

Since D3 is highly induced during the wound process, we speculated that D3 might play a critical role in the regulation of epidermal regeneration. To test this hypothesis, we compared the regeneration potential of K14CRE-$Dio3^{Fl/Fl}$ mice versus WT animals during the wound healing process. We first evaluated changes in wound area as a measure of wound closure in WT and K14CRE-$Dio3^{Fl/Fl}$ mice. In WT mice, wound areas were reduced with linear kinetics throughout the 10-day period of observation, at the end of which ~90% closure was observed. In contrast, wound closure was significantly delayed in K14CRE-$Dio3^{Fl/Fl}$ mice, which showed a 60% wound closure at 10 days vs 6 days in WT mice, respectively (Fig.11a). Notably, the maximal difference was observed at day 3 and 6 after wound, when

proliferation in the basal layer cell is highly active and D3 expression is induced. Histological examination revealed an effective delay in cutaneous regeneration, as showed by the absence of epidermic patch in K14CRE-Dio3$^{Fl/Fl}$ skin at 6 day after wound, in contrast to the formation of the normal epidermal layer under the scar in WT skin (Fig.11b).

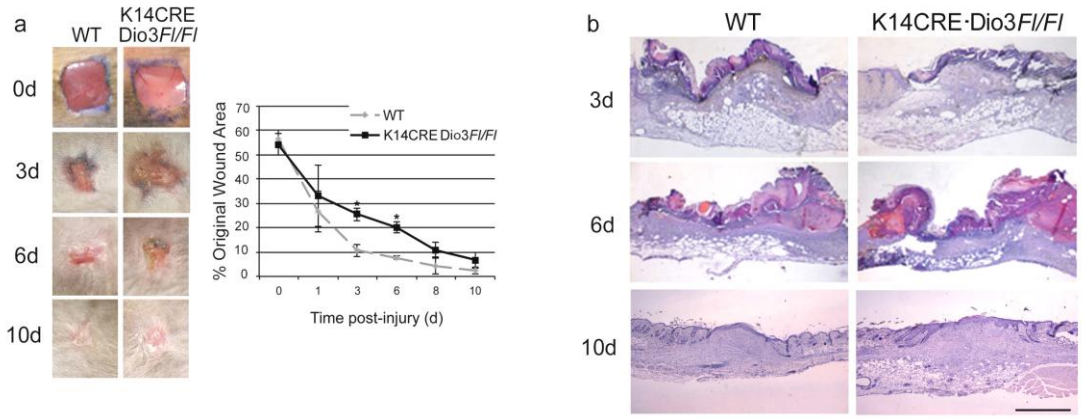

*Fig.11: Defective epidermal regeneration in K14CRE-Dio3$^{Fl/Fl}$ mice following epidermal wound. (a) Kinetic analysis of skin excisional wound healing. Wound sites were photographed at the time indicated. Representative results from three independent experiments with four animals in each group are shown. Quantization of data of changes in percentage of wound area at each time point in comparison to the original wound area. Values represent mean ± SEM. *, p< 0.01, WT vs K14CRE-Dio3$^{Fl/Fl}$ mice. (b) H&E staining of the healing process at the time indicated. Scale bar:500um.*

We speculated that this phenotype could be due to a proliferative defect. Therefore, we analyzed cell proliferation at 3 and 6 days after damage, by measurement of BrdU incorporation in actively regenerating K14CRE-Dio3$^{Fl/Fl}$ versus WT skin. We observed a decrease in keratinocyte proliferation, as demonstrated by reduced number of BrdU^{+} and Ki67^{+} (data not shown) nuclei in the zone surrounding the wound in D3-depleted skin. To obtain a quantitative assessment of proliferation in the two groups, we measured the percentage of the BrdU^{+} nuclei versus the total nuclei. At day 3 and at day 6 after injury the BrdU^{+} nuclei present in K14CRE-Dio3$^{Fl/Fl}$ skin were less than ~50% of what present in normal skin ($p<0.01$) (Fig.12).

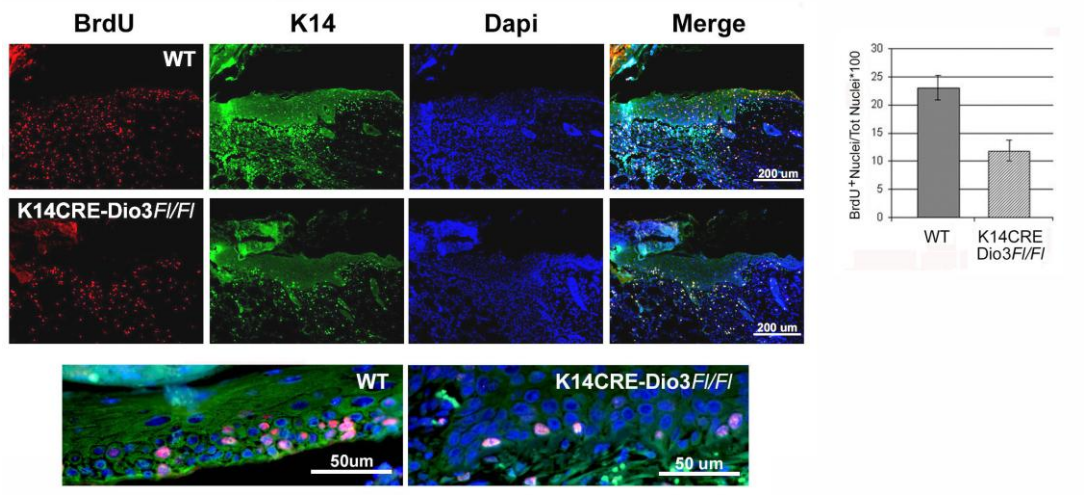

*Fig.12: Reduced proliferation capability in wound area in K14CRE-Dio3$^{Fl/Fl}$ mice. Immunofluorescent staining for BrdU positive nuclei and K14 protein (used as control of analyzed epidermal area) in sections of skin at 6 days from injury in WT vs K14CRE-Dio3$^{Fl/Fl}$mice. The quantization of the proliferation rate is showed in graph as measure of BrdU+ nuclei/Total nuclei and was repeated in three independent experiments (4 animals for group). Values represent mean ± SEM. *, p< 0.01, WT vs K14CRE- Dio3$^{Fl/Fl}$ mice.*

To confirm with a different approach the presence of a proliferation deficit, we measured proliferation markers such as Cyclin D1 and K14. Cyclin D1 and K14 mRNA and protein levels were strongly reduced in D3-depleted skin (Fig.13), which indicates that normal proliferation after wound is impaired by the absence of D3.

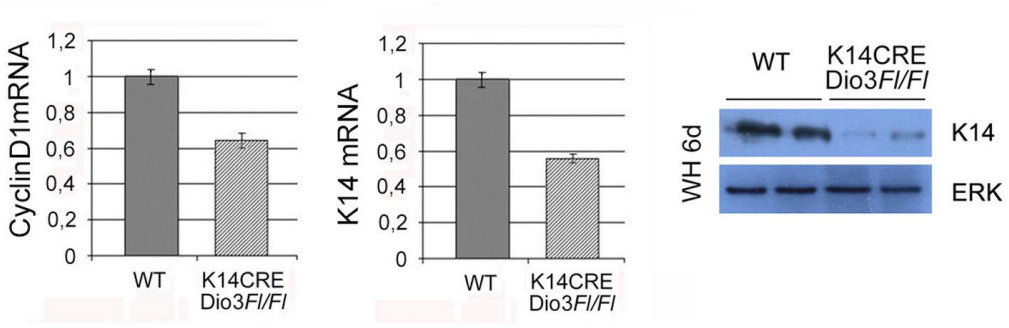

Fig.13: K14CRE-Dio3$^{Fl/Fl}$ mice have a proliferative deficit. Cyclin D1 and K14 mRNA expression levels were measured in wounded skin at day 6 from damage in WT vs K14CRE-Dio3$^{Fl/Fl}$ mice. The values are shown as average ± SD. Western blot analysis shows protein levels of K14 at day 6 of the healing process. The protein levels were normalized with ERK-Tot.

In order to assess whether the proliferation impairment corresponded to a premature differentiation we measured the expression levels of early and terminal differentiation markers at different days after wound. Interestingly, mRNA levels of involucrin, loricrin and filaggrin are increased in K14CRE- Dio3$^{Fl/Fl}$ after wound (Fig. 14). In addition, RNA and protein levels of K10 are also induced in D3-depleted epidermis at day 6 from wound (Fig. 6b). These data suggest that an hyperthyroid environment in D3-depleted skin drives towards an earlier epidermal differentiation. This was also supported by the induction of E-cadherin levels (a TH-responsive gene) in K14CRE- Dio3$^{Fl/Fl}$ versus WT wound (Fig.6c).

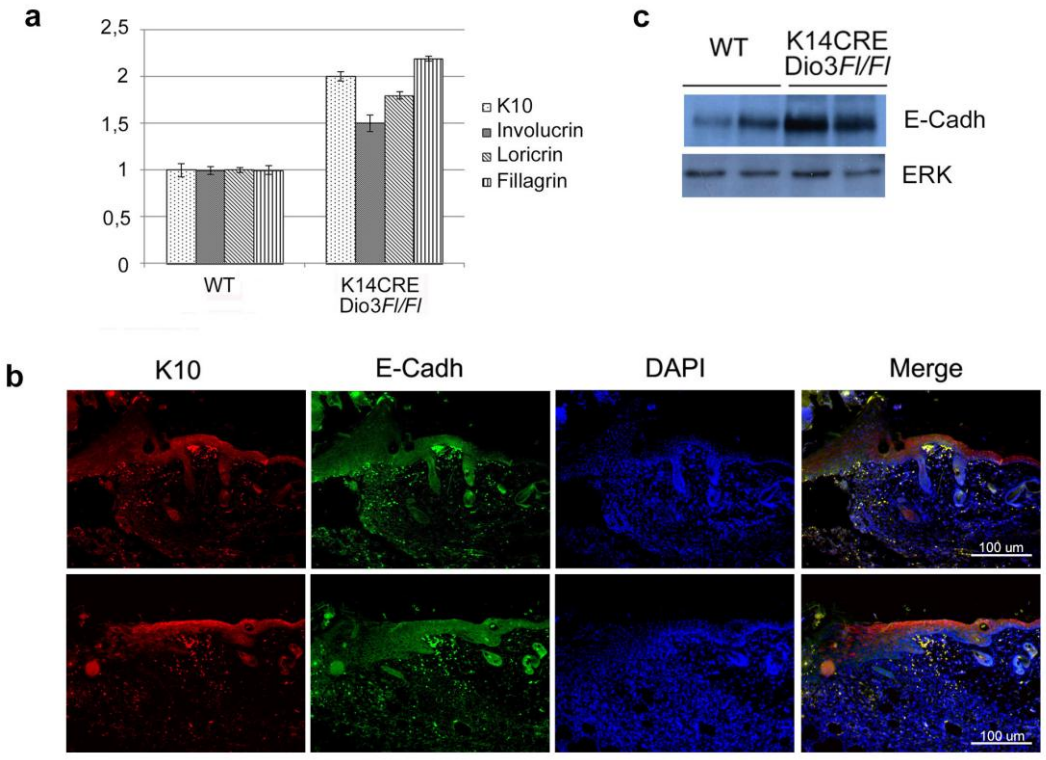

Fig.14: Premature differentiation in K14CRE-Dio3$^{Fl/Fl}$ mice during epidermal regeneration. (a) K10, Involucrin, Loricrin and Fillagrin mRNA expression were measured in RNA samples extracted from wounded region of skin. Data are expressed as mean ± SD. (b) Immunofluorescent staining for K10, E-Cadherin, DAPI or merged images in wounded skin of K14CRE-Dio3$^{Fl/Fl}$ and WT mice at day 6 from injury. (c) Western blot analysis of E-Cadherin in proteic extracts of the wounds.

These wound healing experiments revealed a distinct delay in epidermal regeneration in K14CRE- Dio3$^{Fl/Fl}$ mice compared with WT animals. This delay was caused by the defective proliferation in the absence of active D3 in the face of an enhanced cellular differentiation of keratinocytes. In summary, our data indicate that increased nuclear levels of TH in K14CRE-Dio3$^{Fl/Fl}$ keratinocytes compromise the normal epidermal regeneration of the skin.

D3 in the skin plays a central role in thyroxine clearance

Since D3 is expressed in adult mice only in brain and skin, and given the large mass of skin, we speculated that D3 present in the skin may play a role in thyroxine clearance. Indeed, when we intraperitoneally injected supraphysiological doses of T4, the ability to metabolize T4 by K14CRE-Dio3$^{Fl/Fl}$ mice was reduced of the 40% at 24 hours and of about 33% at 48 hours from T4 injection (Fig.15). This result led us to uncover a new specific role of epidermal D3 in the regulation of plasma TH concentrations by clearance.

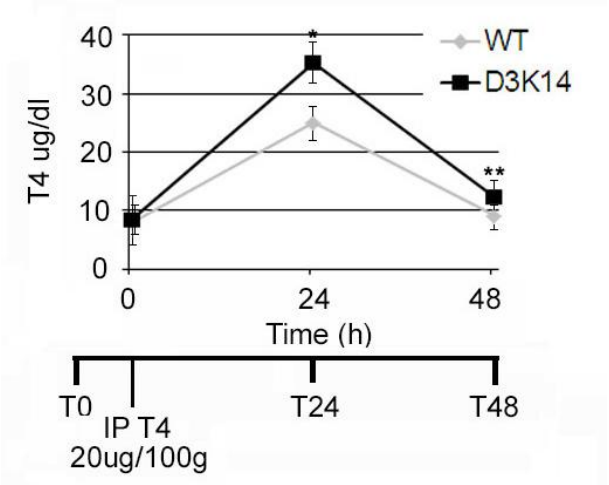

Fig.15: K14CRE-Dio3$^{Fl/Fl}$ mice show reduced T4 clearance.(Top) Serum T4 concentrations at different times after the intraperitoneal administration of supraphysiological doses of T4 (20 ug/100g BW). T4 serum levels were represented in relation to the first point of curve (T0, before the T4 injection). (Bottom) Scheme of experimental design with the times of collection.

DISCUSSION

Although mounting evidence suggests that TH signaling strongly influences epidermal homeostasis, the physiologic role of TH in normal and pathological skin is not well understood. The importance of TH action in the skin is already evident in lower vertebrates. Indeed, in Amphibian metamorphosis, the skin is transformed from a bilayered non-keratinized epidermis into a stratified, keratinized epithelium. This transformation is controlled by T3 and correlates with the expression of adult keratins and the loss of embryonic keratins [83]. In this study, we show that D3, by degrading TH in epidermal cells, is necessary for keratinocyte proliferation and proper differentiation. This is reflected in the essential requirement for an active D3 in wound repair.

Consequences of D3 depletion in keratinocytes *in vitro* and *in vivo*

Here, we showed that an hyperthyroid environment produced by D3 depletion reduces keratinocyte proliferation and promotes epidermal differentiation, as demonstrated by reduction of Cyclin D1 levels and BrdU incorporation and the increase of specific differentiation markers K1, loricrin, Pvrl4 and Dsg1. This is in agreement with previous studies [60], which provided the first evidence that THs can inhibit clonal expansion and promote differentiation of epithelial progenitors [60]. The reduction of keratinocyte proliferation observed in K14CRE-Dio3$^{Fl/Fl}$ mice could be explained by their continuous exposition to an excess of TH, and is in agreement with the evidence that T3 and T4 treatment significantly reduce the colony-forming efficiency, proliferation, cell number viability of primary human ORS-keratinocytes and stimulate their apoptosis [60].

The effect of elevated TH signaling on proliferation and differentiation of keratinocytes is reflected on K14CRE-Dio3$^{Fl/Fl}$ skin phenotype, which is characterized by thinner epidermis and by a decreased level of K14, a prototypic marker of dividing basal keratinocytes. Tomic-Canic et al. in 1995 demonstrated that, in the presence of T3, the constitutive activation of

T3R on K14 promoter is not only reversed, but the extent of transcriptional activity is further inhibited approximately 5-fold below the level of basal expression [36]. This evidence is in agreement with the reduction of the K14 mRNA and protein observed in K14CRE-Dio3$^{Fl/Fl}$ epidermis, in which the loss of D3 activity (the major TH inactivator) leads to an hyperthyroid environment.

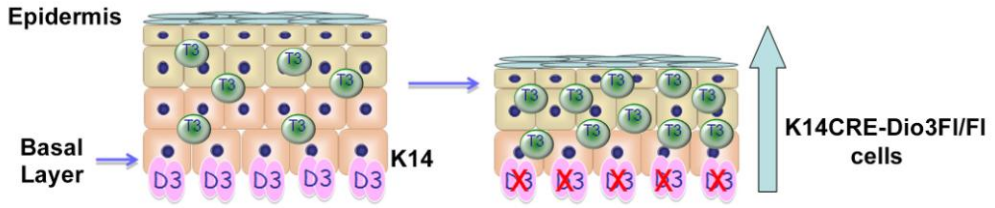

Fig. : Cartoon of K14CRE-Dio3$^{Fl/Fl}$ epidermis. Thinner epidermis and reduction in K14 levels in hyperthyroid tissue, when D3is absent.

Impact of D3 in the skin on the plasma TH concentrations

T3 is a circulating hormone whose levels are fairly constant and stable in the plasma concentration in healthy. Accumulating evidences indicate that the pleiotropic effects on growth, metabolism and differentiation are essentially executed through the capacity to modulate TH signaling at the single tissue or cell level, without affecting the hormonal concentrations in the plasma [14]. Indeed, at plasma level, a potent hypothalamic-pituitary-thyroid axis preserves systemic T3 circulation by dangerous oscillations with a fine homeostatic mechanism controlling hormone secretion by the thyroid gland. In association with this particular control, the family of deiodinases constitutes a further potent mechanism to control thyroid hormone signaling, allowing cells to customize their own T3 footprint in a spatial- and temporal-dependent fashion. Thus, tissues expressing D3 have lower T3 concentrations than what would be expected from plasma contribution, and virtually D3-

expressing tissues have a gene expression profile typical of hypothyroid cells [14]. Santini et al. showed that D3 plays an important role in the clearance of TH by inactivating T4 to rT3. They observed that plasma rT3 concentrations increased significantly 6 and 12 h after topical application of a T4-containing cream, which demonstrates the presence of D3 activity in human skin and indicates that normal human skin serves as a substantial, but incomplete, barrier to T(4) passage [84]. In our system, we observed that D3 depletion from the skin does not change the plasma TH concentrations in basal conditions. Probably homeostatic mechanisms acting at the hypothalamic-pituitary level preserve plasma from changes in TH concentrations. However, in response to injection of supraphysiological doses of T4, the ability to metabolize T4 from the plasma is strongly reduced with no D3 in the skin. Our results show for the first time that D3 in the skin is necessary for proper T4 clearance, revealing a new specific role for D3 in epidermal context.

D3-mediated control of TH signaling is necessary for proper wound healing

T3 is a well-known differentiating agent. Recent studies have shown a close relationship between TH metabolism, via deiodinases expression, and the regulatory networks that control tissue repair. This leads to the concept that finely tuned TH concentration is essential in the control of the proliferation and differentiation balance necessary in the regeneration processes. In this scenario, massive induction of D3 expression in the early phases of regeneration may well correlate with a requirement of increased cellular proliferation. Presumably the induction of D3 leads to a localized region of hypothyroidism within the tissue, and this could influence healing or regenerative processes. In this regard, considerable interest has been generated by recent observations that D3 is induced in vivo at sites of tissue injury and inflammation and in cell culture systems by conditions of hypoxic or oxidative stress [15-16]. Moreover, D3 induction has been observed in animal models at sites of local inflammation [85] in peripheral nerves in response to cryolesion-induced injury [73], and in the heart in the setting of myocardial infarction [74] and cardiac hypertrophy [86]. D3

expression was strongly induced in the liver of mice and rats in a model of liver regeneration induced by partial hepatectomy [72]. With the present study, we propose for the first time that D3 is induced during epidermal wound healing. There are many evidence that confirm the importance of T3 during wound healing, and indeed it is known that hypothyroid states result in poorer wound healing [87]. Despite this the reported data about requirement for local control of TH signaling during epidermal proliferation and wound healing is still very contradictory [87-88-89-90-91]. Lennox and Johnston reported that exogenous T4 improved the rate of wound healing in rats as well as the strength of the scars. They further reported that wound-healing speed was diminished in hypothyroid rats [92]. Mehregan and Zamick observed that additional TH stimulated the rate and quality of wound healing in euthyroid rats. Scars were smoother in animals receiving T3 in drinking water [88-89]. Pirk et al. noted no change in wound healing with euthyroid hamsters receiving ip T4 [90]. Cannon reported that hypothyroidism did not diminish wound strength in pigs [93], and Ladenson et al. found no wound healing deficits in hypothyroid humans [94]. The hormone replacement therapy in hypothyroidism cases was beneficial with regard to wound healing [65]. Finally, the same study of Safer JD in 2005 highlights a conflicting data regard to T3 treatment of wounds. Mice treated with 3.8 ug of topical T3 daily did not have wound healing acceleration beyond that of the mice treated with 150 ng T3 daily. Mice treated with ip T3 did not have wound healing acceleration beyond that of control mice [58]. In this work the authors speculate that a lower dose of T3 could be administered and accelerate wound healing with even less systemic consequence than seen with the 150-ng T3 dose. Moreover, they affirm that the topical treatment would be useful for situations requiring rapid healing with little concern for the scar quality, as the authors do not evaluate the wound quality. In another their work of 2003, Safer et al. affirm that TH mediates inhibition of keratinocyte growth when themselves are co-coltured with dermal fibroblasts [57]. Thus, in vivo, skin proliferation directly stimulated by T3 may be offset by inhibiting factors dependent on the systemic T3 [95]. In the present study, we determined that D3 strongly increases in the early phases of regeneration after injury and decreases in the late phases of tissue repair (Fig.5). Importantly,

our data show that the epidermis of K14CRE-Dio3$^{Fl/Fl}$ mice exhibits a regenerative defect after injury with a delayed closure of wound area (Fig.11). Indeed, D3-depleted epidermis displays a fewer number of proliferating cells around damage site with a reduced BrdU incorporation (Fig.12). We do not still know if this proliferating defect is due to a reduced migration of wound invading cells or to an increased cellular apoptosis triggered by hyperthyroid environment. Our preliminary data indicated that D3-depleted keratinocytes have an inability to migrate respect to WT cells. On the other hand, a link between excessive thyroid hormone and apoptosis has been previously demonstrated in amphibian metamorphosis [96]. All metamorphic changes that take place during body remodeling are controlled by T3, which causes developmental programmed cell death through apoptosis in tadpole tails to reshape them to acquire the adult form [97-98-99]. Moreover in human and mouse basal cell carcinoma cells where excessive T3, consequent to D3 depletion, induces apoptosis of tumoral cells [17]. Further studies are necessary to better understand the underlying mechanism of delayed wound healing in D3-depleted skin.

Our data demonstrate for the first time that the presence of D3 in the skin is necessary for proper epidermal homeostasis and for regular wound healing and that then the tissue thyrotoxicosis compromises a normal epidermal regeneration, owing to slowed proliferation and accelerated differentiation, specifically.

Finally, these discoveries extend insight into TH action on skin, providing new knowledge about the role of deiodinases, and in particular of D3 on skin biology and during skin regeneration. Unfortunately, our knowledge about molecular causes and effects of TH action remains largely incomplete, especially regarding molecular pathway governing D3 in the normal skin and during regeneration processes. Putative upstream regulators of D3 in keratinocytes are not known at the present. Multiple growth factors and hormones regulate D3 activity by inducing D3 mRNA expression [34-100]. Among them, the TGFβ signaling is one of these [101]. Many recent evidence associate TGFβ signaling with wound healing and epidermal regeneration processes [102-103-104], besides the fact that the TGFβ promotes

the basal phenotype of epidermal keratinocytes by transcriptional induction of K14 and K5 keratin genes [105], both target of TH. Thus, further studies on the interplay between TGF-β and TH signaling pathways could help to test new specific pharmacological and molecular genetic approaches targeting the D3 gene in this scenario.

CONCLUSIONS

The skin exhibits typical abnormalities in hyperthyroidism and hypothyroidism and it is well known that thyroid dysfunction is associated with skin pathologies. Smooth, thin and warm skin are epidermal changes in thyrotoxicosis states, in association with hair and nail changes as fine hair (loses wave), alopecia, shiny, soft and friable nails.

Recent studies have shown a close relationship between TH metabolism, via deiodinases expression, and the regulatory networks that control the single specific requirements of the cell. This leads to the concept that finely tuned TH concentration is essential in the control of the proliferation and differentiation balance necessary in many tissue processes in which the deiodinases are expressed.

This study provides a new specific role for type 3 deiodinase, the principal terminator of TH action, in the skin. The data in this thesis define that D3 is essential in epidermal regeneration processes. In this scenario, massive induction of D3 expression in the early phases of regeneration may well correlate with a requirement of increased cellular proliferation in these circumstances. Our data demonstrate for the first time that the presence of D3 in the skin is necessary for proper epidermal homeostasis and for regular wound healing. Tissue thyrotoxicosis compromises a normal epidermal regeneration, especially acting through a proliferation slowness and differentiative acceleration.

The possibility to target therapeutically one specific deiodinase, in a defined cellular context, opens new potential approaches to the treatment of selective and somehow ‘T3-sensitive’ cutaneous diseases. Further studies are necessary to a better understanding the molecular pathways at the basis of TH action in the skin and to allow a clinical approach in hypo- and hyperthyroidism to treat specific skin symptoms.

MATERIALS AND METHODS

Generation of K14CRE-Dio3$^{Fl/Fl}$ mice. We generated a plasmid harboring floxed sites in the dio3 locus, and specifically flanking the SECIS mRNA structure located at nt 933 and 1609 in the dio3 mRNA. This region is necessary for the proper recognition of the TGA coding as a selenocysteine recognition codon rather than a conventional STOP codon. In absence of the SECIS, the TGA codon, within the D3 catalytic domain, is recognized as stop codon, and protein translation terminated. Once obtained the homozygous Dio3$^{Flox/Flox}$ mice, we crossed these mice with heterozygous ACTB-FLP1 mice, expressing the FLPe recombinase variant transgene Flp that catalyze the recombination between the frt sites, flanking the neomycin cassette. Progressively, we crossed the Dio3$^{flox/flox}$ mice without FLP with K14CRE mouse, and we inbreeded the animals up to obtain the K14CRE-Dio3$^{Fl/Fl}$ mouse line. Since D3 is parentally imprinted, we used only male mice K14CRE-Dio3$^{Fl/Fl}$ for crossing and we used as control the Dio3$^{Fl/Fl}$ mouse.

T4 Clearance experiment. 8 week old mice were intraperitoneally (i.p.) injected with 20ug/100g of T4. Starting one day before injection, the blood was collected by retro-orbital withdrawal at 24 and 48 hours after a single T4 injection. The serums were separated and the determination of T4 was measured by Rodent T4 Elisa Test Kit (Endocrine Technologies Inc. USA). Experiments were performed with 6 mice per group and repeated two times in these conditions. The difference were statistically significant. All mouse experiments were approved by the Italian Ministry of Health.

Excisional wound preparation and analysis. 2 Full-thickness wounds were created in the dorsal skin under sterile conditions. Briefly, mice were anesthetized with i.p. administration of avertin. After shaving and cleaning with betadine and 70% ethanol, the dorsal skin was picked up at the midline and punched through two layers of skin with a sterile disposable scalpel generating one wound of 8mm x 8mm on each side of the midline. Each wound site

was digitally photographed at the indicated time intervals, and wound areas were determined on photographs using Adobe PhotoShop (version 7.0; Adobe Systems). Changes in wound areas over time were expressed as the percentage of the initial wound areas. The mice were killed at different time points after injury and wounds were either embedded for sectioning or excised including 2 mm of the epidermal margins for RNA and protein isolation. Three animals were analyzed per genotype and time point. All mouse experiments were approved by the Italian Ministry of Health.

Histology, Immunostaining and Immunoblotting. Mouse tissue was fixed in 4% PFA and embedded in paraffin wax. For epidermal thickness analysis, 6 um serial paraffin sections of uninjured and wounded skin were cut and stained with hematoxylin & eosin (H&E) and the measurements were executed by CellF Software. For assessment of BrdU incorporation, mice were injected ip with 1 ml/100 g body weight of BrdU (Zymed, Invitrogen) 3 hours before harvesting the skins, according to the manufacturer's protocol. Immunofluorescence was performed using anti-BrdU, -K14, -K10, or -E Cadh Abs. Deparaffinized sections were permeabilized with 0,5% Triton, unmasked in Citrate Sodium 0,01M ph 6.0 and blocked with Avidin/Biotin Blocking kit to eliminate endogenous biotin. The sections were further incubated with PBS containing 5% goat serum and 6% BSA to reduce nonspecific reactions. The sections were incubated with primary Abs at 4°C overnight. Normal histoblock reagent was used as negative control. After incubation with biotinylated mouse, rabbit or goat secondary Abs, immune complexes were cross interacted with Streptavidin-Cy3 reagent (Sigma) and visualized using the OLYMPUS IX51 microscope, according to the manufacturer's instructions.

Cell cultures and reagents. Wild-type, $D3^{Fl/Fl}$ and K14CRE-$Dio3^{Fl/Fl}$ mutant primary mouse keratinocytes were isolated from newborn mice. Their skins were placed in dispase o/n at 4°C by dispase treatment (Dispase I, Roche) and then keratinocytes were isolated from epidermis by enzymatic dissociation in trypsin, and cultured under low Ca^{2+} conditions (0.02 mM). The infection experiments were performed with 75 MOI of AdCRE-GFP and AdGFP

virus. To evaluate proliferation, cells were incubated with BrdU labelling reagent (B-5002 Sigma) for 2h before of the harvest. For immunofluorescence staining, cells were fixed with 4% formaldehyde and permeabilized with 50mM NaOH, then blocked with 5% goat serum and incubated with monoclonal Ab G3G4 (Developmental Studies Hybridoma Bank) and DNase. Alexa Fluor 595–conjugated secondary antibody was used. The cell DNA was stained with 300 nM DAPI (Molecular Probes, Invitrogen).

Real-Time RT-PCR. Skin and keratinocytes RNA were extracted in TRIzol reagent (Invitrogen). Nuclear RNA was treated with DNase I (Roche). Complementary DNA (cDNA) synthesis was obtained using SuperScript III (Invitrogen). Real-time PCR was performed using the SYBR Green PCR master mix (Biorad) in an iQ5 Cycler (Biorad). Expression of target genes was normalized for cyclophilin A. For primer sequences see Table below.

Gene	**Primer sequences (5'- 3')**
Cyclofillin A	F - CGCCACTGTCGCTTTTCG R - AACTTTGTCTGCAAACAGCTC
Cyclin D1	F - GCTCCTGTGCTGCGAAGTGGA R - TCATGGCCAGCGGGAAGACCT
Desmosglein1	F - CCACCTGATAACGCAAATATAATTG R - TTGCATTTCTCTGCCACCATAC
Fillagrin	F - GTCCAAAGTGCAGCAGCAAG R - TTAAAATCAAGGTGCTTTGCTGTAA
Involucrin	F - AAAGCTTCAAGGGAACAGCA R - CGGTTCTTCAATTCGTGTTT
Keratin 1	F - TCGTGACCATCAAGAAGGAT R - ACAACATTGGTTTCGCTGAT
Keratin 10	F- CGTTGGCATTGTCAGTTGTC R - CGAGCTGGAGGGTAAAATCA
Keratin 14	F - GATGTGACCTCCACCAACCG R - CCATCGTGCACATCCATGAC
Loricrin	F - CCTTCACATTTTAAGCGCCC R - TGAGCGACTCAATGGCTTCTT
Poliovirus receptor-related 4	F - GGCATCGTTTACAGGCCAAT R – AGCACCACTGTCACTACGTCAGA

Statistics. Differences between samples were assessed by Student's 2-tailed t test for independent samples. P values less than 0.05 were considered significant. Relative mRNA levels (in which the first sample was arbitrarily set as 1) are reported as results of real-time PCR in which expression of cyclophilin A was used as a housekeeping gene.

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There is a time to study, one to suppose, one to experiment, one to discover, one to demonstrate, one to write.... and finally the time to thank comes.

First of all, my gratitude goes to my tutor Prof. Domenico Salvatore, because I owe this goal to him, for the possibility that he gave me to profitably work in his research group and to captivate in me the devotion to this stimulating work.

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Finally, I want to thank my friends mice, if they had not been, this work would not have realized!

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PUBBLICATIONS

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