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19.1 Introduction

The thyroid is an elegant butterfly-shaped gland with endocrine function located in front of the trachea. From the histological point of view, thyroid is mainly composed of endothelial cells, C cells (or parafollicular cells) and thyroid follicular cells (TFCs). Endothelial cells make up the vascular network important for the correct TSH-mediated hormonal stimulation of the gland to produce and release thyroid hormones (THs) (T4 and T3) from thyroid follicles. C cells are involved in calcitonin secretion, important for the correct calcium homeostasis and phosphate metabolism. Thyroid hormones are involved in the regulation of many metabolic and biological processes such as skeletal and brain development, and they are considered very crucial already immediately after birth. During the last two decades, several mammalian as well as non-mammalian animal models (i.e. zebrafish [1]) have been intensively used for studying the embryonic development of thyroid gland. Thyroid is an endoderm-derived organ. Its organogenesis begins with the formation of the thyroid anlage at the level of the pharyngeal arches. During this phase (E8.5 in mice and 4th gestational week in humans), so-called specification stage, a specific subpopulation of endodermal cells in the pharyngeal floor becomes committed to thyroid fate and distinguishable due to the co-expression of four specific genes: *Nkx2-1* [2], *Pax8* [3], *Foxe1* [4, 5] and *Hhex* [6, 7]. Afterwards, the entire process of thyroid organogenesis requires, after a first specification of thyroid progenitors, thyroid bud formation and evagination from the endodermal layer, relocalization of the

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thyroid primordium to a position distant from its initial site, then functional differentiation of thyroid follicular cells and formation of functional mature thyroid follicles [4]. During the final morphological and terminal or functional differentiation, thyroid follicular cells organize into polarized monolayer epithelial structures, the follicles, and at the same time, all the genes, such as *Tg*, *Tpo* [2], *Slc5a5* (encoding for NIS) [8] and *Tshr* [2], known to be involved in the complex machinery for thyroid hormone synthesis start to be expressed. Afterwards, the first follicles producing T4 can be detected highlighting the end of thyroid organogenesis [9].

Most of the knowledge on thyroid embryogenesis, the morphogenetic features or the genes controlling such a sophisticated and elegant process is derived from studies performed on mouse embryos. The first studies performed using null mutant mice for the aforementioned transcription factors *Nkx2-1* [10–12], *Pax8* [10, 13], *Hhex* [10, 14] and *Foxe1* [15] have demonstrated their importance for the correct organogenesis of the gland. Indeed, mice lacking one of these displayed an abnormal embryonic development of the thyroid gland characterized by agenesis. A more detailed analysis of the mutant embryos has demonstrated the function of the aforementioned transcription factors is necessary for the survival of thyroid precursors [10]. Indeed, the specification stage is not affected, but immediately after budding the primordium degenerates, leading to a complete absence of thyroid tissue at birth [10]. Moreover, using *in vivo* and *in vitro* systems, the additional role of *Nkx2-1* and *Pax8* has been clearly defined in the control of the expression of the functional thyroid-related genes important for thyroid hormone synthesis (such as *Tshr*, *Tg*, *Tpo*, *Nis* and *Duox 1* and *2*) [16]. Moreover, other genes have been described to lead to thyroid dysgenesis when knocked out, i.e. *Tshr* [17], *Shh* [18], *Fgf10* [19] and *Fgfr2* [20]. A defective embryonic development of the gland leads to a hypothyroidism state characterized by mice displaying low levels of thyroid hormones associated with most of the typical clinical features such as defective skeletal development. In humans, low plasma levels of thyroid hormones can lead to a physical and mental retardation condition, called also cretinism, if it is not properly diagnosed after birth. Indeed, a rapid diagnosis and a prompt supply of thyroid hormones just after birth can normalize cognitive development [21]. Nevertheless, in some cases IQ deficits are detected in adolescents with congenital hypothyroidism despite a correct substitution therapy [21]. Congenital hypothyroidism (CH) is the most common endocrine disorder affecting 1 in 1,000–2,000 newborns [22]. Two are the major causes of CH: defective embryonic development of the gland or a defective hormone synthesis. A very representative case is the mis-regulation of the hypothalamic-pituitary-thyroid axis due to *Trhr* loss of function [23, 24]. Thyroid dysgenesis (TD), representing roughly 85 % of CH cases, is a condition that appears with different scenarios: the gland can be absent (agenesis or athyroid), hypoplastic (hypoplasia), located in a different place (ectopy) or with only one lobe absent (hemiagenesis). In the rest of the cases where the synthesis of thyroid hormones is negatively affected, CH has been associated with mutations in genes encoding for *TSHR*, *NIS*, *TPO*, *DUOX2*, *DUOXA2* and *DEHAL1* (iodotyrosine dehalogenase 1) [25].

Taking together all the knowledge from studies performed using animal models (such as null mutant mice) and from genetic screening in patients affected by CH,

we can conclude that very little is known about the morphogenetic events occurring during thyroid organogenesis, the genes and epigenetic mechanisms controlling such processes.

Uncovering new genes implicated in pathogenesis represents a big step in improving the pre-natal diagnosis of congenital defect in the structure and function of the gland. In addition, the only known and used therapy to treat CH patients is based on the hormone substitution, so it would be very rational to hypothesize about an alternative approach to cure CH. Nevertheless, even if promptly treated after birth in some cases, the classical hormone substitution does not avoid development of mental retardation or low IQ.

19.2 Embryonic Stem Cells as System Model to Study Embryogenesis

During the last 20 years, embryonic stem cells (ESCs) have emerged as a very promising tool to investigate the molecular and morphological events occurring during the organogenesis of several organs. Embryonic stem cells are pluripotent cells derived from an early embryo that can be used as a cell line in tissue culture. Life begins with the fertilized egg that has the incredible capacity to give rise to the entire body with its whole complexity. From zygote to eight-cell stage (known as morula), an embryo has an extraordinary capacity called totipotency [26] consisting in the potentiality of the cells to become extra-embryonic or embryonic tissue. During this period, the pre-implantation phase, an embryo goes through additional and rapid cell division (cleavage) leading to the formation of the blastocyst, a hollow structure composed of an inner cell mass (ICM) surrounded by an outside cell layer (so-called trophectoderm – TE). The external cells, the trophoblasts (TE), are responsible for the connection to the uterus, formation of large parts of the placenta and also to provide nutrients to the embryo after implantation [27]. Whereas ICM is composed of undifferentiated cells that are no longer totipotent but pluripotent and able to give rise to the embryo proper and additional extra-embryonic tissues. After implantation, the pluripotent cells of the ICM will form the epiblast that afterwards through the gastrulation gives rise to the three different primary germ layers (ectoderm, mesoderm and endoderm) and primordial germ cells (PGCs). Then during the progression of the embryogenesis, each layer represents the starting point for the differentiation of specific lineages, tissues and organs. Indeed, the nervous system, epidermis and neural crest derive from ectoderm; somites (which form muscles), the cartilage of the ribs and vertebrate, the notochord, blood heart, blood vessels and connective tissue derive from mesoderm; gut, lungs, liver pancreas and thyroid are endoderm-derived organs [28].

The concept of the existence of a population of cells that during the normal embryogenesis proliferate and differentiate into many different lineages is clear. How can this capacity be translated *in vitro*? In 1981, two famous groups started to derive and culture pluripotent cells from mouse blastocyst [29, 30]. Even if these cells (called ESCs), derived from ICM, did not show any loss of differentiation

potential, it was necessary to wait a few more years for the final experimental evidence that ESC were really pluripotent. The final demonstration arrived in 1984 when Bradley and colleagues injected ES cells (showing a normal karyotype in contrast to ECCs) into blastocysts and demonstrated their contribution to the development of all cell lineages including germ lines in the generated chimeric mice [31]. There is ample evidence now that ESCs can be differentiated *in vitro* into many cell types, and many approaches and protocols have been proposed to drive the differentiation of ESCs into specific lineages. One of the typical approaches, somehow recapitulating gastrulation *in vitro*, is the formation of aggregates, called embryonic bodies (EBs). It was shown that ESC-derived EBs show molecular and morphological signatures of endodermal, mesodermal and ectodermal lineages. EBs derived by culturing ESCs in suspension can be re-plated and cultured in 3D or adherent conditions for long periods of time [26]. During the *in vitro* differentiation, they can spontaneously differentiate into many of the three germ layer-derived tissues, such as cardiomyocytes, skeletal muscles, smooth muscles, neurons, epithelial cells, pancreatic cells, hepatocytes and glial cells [26]. Many extrinsic factors can both positively and negatively influence the differentiation of EB-forming cells into specific lineages (i.e. cell density, media formulation, amino acids, extracellular matrix proteins, growth factors, morphogens, concentration and quality of the foetal serum). A second common approach for the differentiation of ESC is the adherent monolayer culture. Contrary to EBs method, where a mixture of germ layers is present, it is possible to differentiate a specific cell type derived from a specific germ layer [26]. Many groups in the endocrine field have proposed protocols for the *in vitro* differentiation of endodermal cells, in particular pancreatic beta cells, starting from human or mouse pluripotent stem cells. They generally use a multi-step approach that recapitulates the different embryonic phases that occur during normal development *in vivo*. Briefly, during the first phase, ESCs have to be differentiated into definitive endoderm, and then subsequential exposure of ESC-derived definitive endodermal cells to morphogens (such as FGF10, inhibitors of Notch and Sonic hedgehog pathways, retinoic acid and other growth factors) leads the differentiation into pancreatic hormone-expressing endocrine cells [32, 33]. Recapitulating pancreas development *in vitro* is simply an elegant example of how embryonic stem cells can be used for studying endoderm-derived organ development. Concerning the differentiation of endoderm-derived tissues from pluripotent cells, many studies have focused on the middle and posterior part of the foregut – midgut with pancreatic cells and posterior gut with hepatocytes [34]. Concerning anterior endoderm, Green and colleagues described how to generate anterior foregut endoderm (AFE) starting from human ES and induced pluripotent stem cells (iPS cells) [35]. Concerning the possibility to further differentiate AFE-derived tissues from ESC, two interesting and elegant studies have shown the potential of mouse ESCs to be differentiated into endodermal progenitors positive for the lung and thyroid marker NKX2-1 and additionally expanded and differentiated into thyroid and lung lineage [36, 37].

ESCs can be also efficiently differentiated into mesoderm-derived lineages such as hematopoietic cells, endothelial cells and cardiomyocytes [38], chondrocytes and cartilage-like tissue [39]. ESCs have been also successfully used in the

neurobiology field for differentiating ectoderm-derived lineages such as neural cells for uncovering and characterizing molecular events occurring during neurogenesis [40]. In 2002, Wichterle and colleagues were the first to propose an efficient protocol to differentiate ESCs into a specific neural cell type, spinal motor neurons, using retinoic acid and sonic hedgehog [41]. After this, many independent works have proposed several protocols to differentiate ESCs into different neuronal cell types such as progenitors, retinal photoreceptors, cerebellar neurons and cerebral neurons [38].

Moreover, Sasai and colleagues have recently illustrated the possibility to use both human and mouse ESCs to successfully recapitulate the 3D organization of complex ectoderm-derived tissues such as optic cup [42], retina [43] and adenohypophysis [44]. 3D stem cell culture has been shown to be successfully used to differentiate endoderm-derived tissues such as liver organoids, islet organoids, gut organoids and pulmonary progenitors that in presence of specific scaffolds was shown to reconstitute airway epithelium [45]. Those findings have opened a new avenue in the possibility to use ESCs as *in vitro* model for studying tissue morphogenesis. Moreover, generating 3D organoids resembling a tissue from the morphological and functional point of view represents an additional tool to perform drug screening and physiological studies.

During the last years, many studies have described the possibility to differentiate specific cell lineages acting on the expression of genes known to play a role in the correct embryonic development of several tissues. In 2004, Kyba and colleagues have shown how the ectopic induction of *HoxB4* – known to be involved in haematopoietic stem cell differentiation – in ESC-derived EBs promoted the differentiation into lymphoid-myeloid progenitor cells [46]. Similarly, Ahfeldt and colleagues found that the induction of the expression of *PPARG2* or its co-expression with *CEBPB* and/or *PRDM16* in hEBs-derived mesenchymal progenitor cells committed the cell fate into white and brown adipocytes [47].

During the past, several teams have proposed different protocols of differentiation of pluripotent stem cells into thyroid-like cells. Indeed, all the published works have shown an incomplete analysis of differentiated cells, such as morphological and functional [48, 49]. In 2012, Sabine Costagliola's group published the first protocol of differentiation of pluripotent stem cells into thyroid follicular cells [50].

19.3 Generation of Functional Thyroid from Embryonic Stem Cells

As it has been previously described, two are the major approaches to differentiate embryonic stem cells into specific lineages: (1) defined media approach – differentiation of ESCs into a specific cell type is achieved modulating signalling pathways, known to be important for the development of the specific tissue/organ, by supplementing the medium with the antagonists or agonists of the defined pathway; (2) the second approach is the ectopic expression of defined transcription factors known to be important for the *in vivo* development of the specific tissue.

Concerning thyroid development, the external signalling responsible for the commitment to thyroid fate of a subpopulation of endoderm cells at the level of the pharynx is unknown yet.

The lack of fundamental information made to approach the differentiation of pluripotent stem cells into TFCs by inducing the two genes proposed to be the master genes controlling thyroid embryogenesis, *Nkx2-1* and *Pax8*. In the first instance, embryonic stem cells have been genetically engineered to make possible the induction of both transcription factors [50, 51]. A doxycycline-responsive promoter can make possible to temporally control the co-expression of *Nkx2-1* and *Pax8*. The protocol developed by Costagliola and colleagues can be summarized in three important and key steps: (1) generation of EBs and embedding of EBs into a 3D-Matrigel support; (2) induction of *Nkx2-1* and *Pax8* co-expression by doxycycline-TetOn system; (3) stimulation of folliculogenesis by rhTSH treatment (Fig. 19.1a). During the first step, ESCs are cultured in hanging drops, and this makes possible the initial differentiation of the pluripotent stem cell into the three different germ layers including endoderm. Because thyroid cells have to be organized in a three-dimensional structure to make possible thyroid hormone synthesis, Costagliola and colleagues have taken advantage of growing cells into an extracellular matrix-based environment. This has been aimed at embedding and growing ESC-derived EBs in a gelatinous mixture of proteins called Matrigel (mix of different extracellular matrix proteins) that resembles the extracellular environment that can be found in several tissues including thyroid. The second step that has been described crucial for the *in vitro* thyroid differentiation is the inducible co-expression of both *Nkx2-1* and *Pax8*. As expected from several studies performed using different cell lines, the expression of both transcription factors is sufficient to induce the expression of other thyroid-related genes such as *Tg*, *Tpo*, *Slc5a5* and *Foxe1* [16]. Moreover, other studies have demonstrated the capacity of both transcription factors to regulate their expression in a feedback loop manner [52–55]. Indeed, when *Nkx2-1* and *Pax8* are ectopically induced by doxycycline for only 3 days in ESC-derived EBs, the up-regulation of the endogenous forms of both transcription factors as well as *Foxe1*, *Hhex*, *Tg*, *Slc5a5* and *Tshr* can be detected [50]. Only when both *Nkx2-1* and *Pax8* are ectopically induced, cells become committed to thyroid fate as suggested from the activation of the endogenous forms of both transcription factors [50]. Those observations have led to the last point of the protocol of differentiation where doxycycline is removed and substituted by rhTSH for the remaining differentiation period (Fig. 19.1a).

At the end of the differentiation, cells differentiated after induction of both transcription factors and subsequent rhTSH treatment show at transcriptional level the up-regulation of the four transcription factors (*Nkx2-1*, *Pax8*, *Hhex* and *Foxe1*), known to play a pivotal role during thyroid development, and of the genes encoding for the protein important for thyroid hormone synthesis such as TG, TPO, TSHR and NIS (Fig. 19.1b). Immunofluorescence analysis of the cells shows clusters of cells co-expressing thyroid markers NKX2-1, PAX8, FOXE1, TG and NIS (Fig. 19.1c–f). Detailed morphological characterization showed that

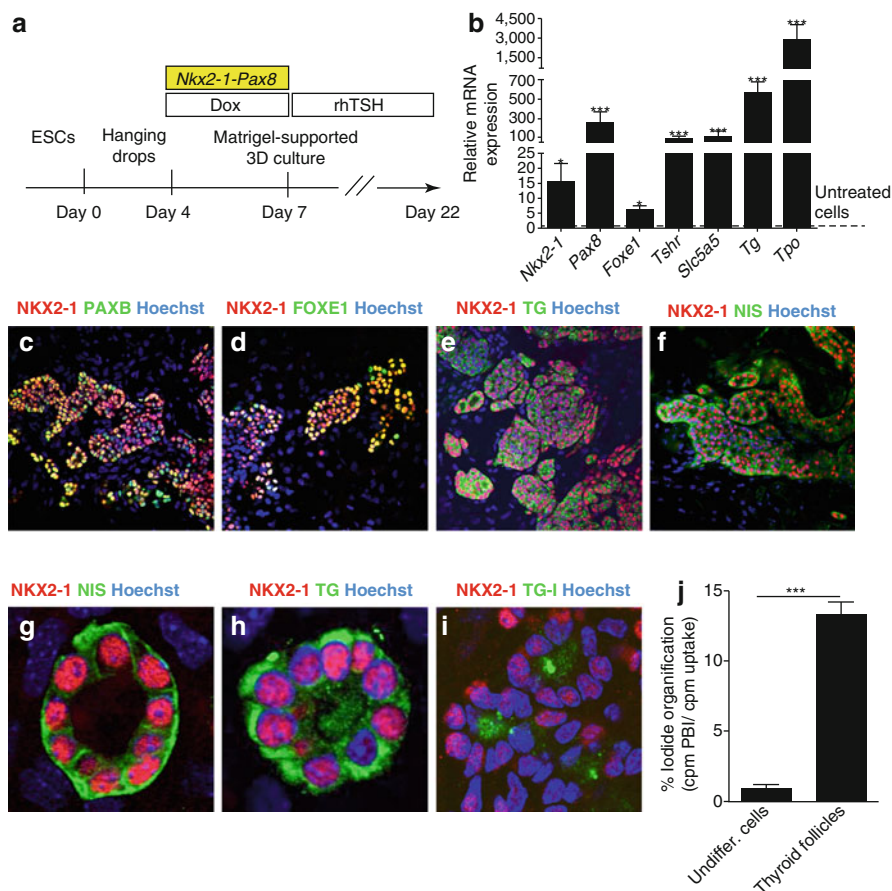


Fig. 19.1 Generation of functional thyroid follicles from pluripotent stem cells. **(a)** Schematic diagram of the thyroid follicle differentiation protocol from ESCs. **(b)** Expression of endogenous *Nkx2-1* and *Pax8*, *Foxe1*, *Tshr*, *Slc5a5*, *Tg* and *Tpo* at day 22 in cells differentiated after Doxycycline-mediated induction of *Nkx2-1-Pax8* and subsequent treatment with rhTSH. Relative expression of each transcript is shown as fold change compared to untreated cells (*baseline*) at day 22 as mean \pm s.e.m. ($n=6$). Unpaired *t*-test was used for statistical analysis. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. **(c–i)** Immunostaining at the end of the differentiation (day 22) for NKX2-1 and PAX8 **(c)**, NKX2-1 and FOXE1 **(d)**, NKX2-1 and TG **(e, h)**, NKX2-1 and NIS **(f, g)**, NKX2-1 and iodinated TG (TG-I) **(i)**. **(j)** Histogram showing the organification percentage of iodine-125 at day 22 in ESC-derived thyroid follicles; undifferentiated cells column refers to cells at day 22 differentiated without Doxycycline and rhTSH (Modified from Antonica et al. [50])

ESC-derived thyroid cells were capable to organize into follicular structures, confirmed by polarization of NIS at the basolateral membrane (Fig. 19.1g) and accumulation of TG in the luminal compartment (Fig. 19.1h), while functional analysis highlighted the capacity of these cells to metabolize iodide (Fig. 19.1i, j) [50]. More interestingly, confirming their functionality, ESC-derived thyroid follicles can be transplanted into hypothyroid mice (Fig. 19.2a). The exogenous tissue,

transplanted under the renal capsule, can perfectly integrate into the host, as shown by the presence of follicular epithelium (Fig. 19.2b, c) histologically positive to the transcription factors (NKX2-1, PAX8 and FOXE1) [50], functional thyroid markers NIS (Fig. 19.2d) and TG (Fig. 19.2e) and finally T4 (Fig. 19.2f) and more interestingly, the formation of angiofollicular units shown as NKX2-1-forming follicles surrounded by endothelial cells forming vessels (Fig. 19.2g). The correct integration, functionality and formation of a vascular network around

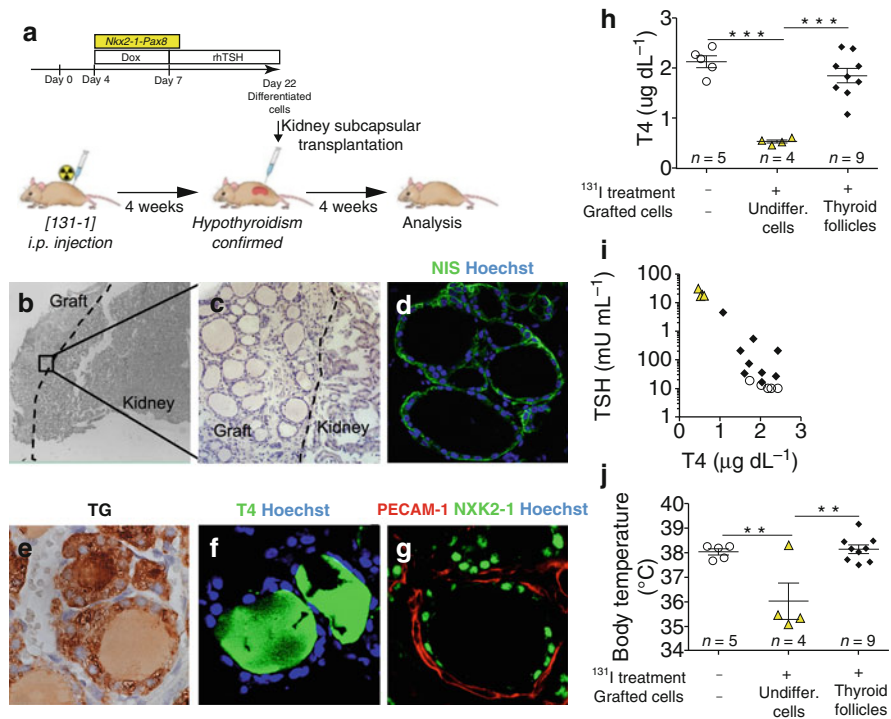


Fig. 19.2 Rescue of hypothyroidism in mice by transplantation of ESC-derived thyroid follicles. (a) Schematic diagram of protocol for ESC-derived thyroid follicles transplantation under the renal capsule of mice after radio-iodine ablation of thyroid. (b–g) Histological examination of kidney sections 4 weeks after grafting. Hematoxylin-eosin staining shows the integration of the transplanted tissue in the cortical area of the host kidney (b) characterized by single cuboidal epithelium organization of transplanted tissue (c). Immunofluorescence analysis confirms the expression of Nis (d) and Tg (e), production of T4 (f) and formation of vessels (PECAM-1) surrounding NKX2-1-forming follicles (g). (h) Total plasma T4 levels 4 weeks after transplantation of ESC-derived thyroid follicles in iodine-131-treated mice. (i) Relationship between plasma TSH and T4 levels 4 weeks after grafting. (j) Body temperature measurements 4 weeks after grafting. In (h–j), open circles show iodine-131-untreated and ungrafted mice; yellow triangles show mice treated with iodine-131 and grafted with undifferentiated cells (cells cultured without Dox and rhTSH) and black diamonds show mice treated with iodine-131 and grafted with ESC-derived thyroid follicles. The values are shown as a dot plot (h, j) or scatter plot (i) and data are mean \pm s.e.m. Tukey's Multiple Comparison Test (h, j) was used for statistical analysis. ** $P < 0.01$, *** $P < 0.001$ (Modified from Antonica et al. [50])

the transplanted ESC-derived thyroid follicles lead to the restoring of the normal serum level of thyroid hormones (Fig. 19.2h) together with the normalization of thyroid homeostasis, shown as a decreased serum level of TSH (Fig. 19.2i) and a symptomatic recovery from the hypothyroidism state shown as a normalization of the body temperature (Fig. 19.2j) [50].

19.4 Embryonic Stem Cell as Model to Study Thyroid Embryogenesis and Modelling Congenital Hypothyroidism

The establishment of the first protocol of differentiation of pluripotent stem cells into functional thyroid cells has provided a novel and complementary system to study thyroid organogenesis. To date, very few studies have been performed *in vivo* in order to obtain a genome-wide expression profile of the thyroid cells at different developmental stages [56]. The *in vitro* model of thyroid organogenesis would be an alternative system to study and uncover new genes playing a role during the embryonic development of the gland. The unlimited source of biological material makes this model very useful to get access to the complete transcriptional profile. Moreover, the model represents also a valid system to study the function of genes (generate ESC mutant lines for specific genes and evaluate the effect during the *in vitro* differentiation) or signalling pathway hypothetically important for the commitment to thyroid fate or for the survival of thyroid cells and even their organization into follicles. Obviously, the system doesn't seem to be suitable to study migration of thyroid cells, another aspect important for the correct development of the gland.

This approach would be interestingly translated from mice to humans in order to uncover new genetic causes of congenital hypothyroidism. During the last years, human embryonic stem cells and more interestingly induced pluripotent stem cells have been emerging as a very interesting tool for the *in vitro* modelling of human diseases.

In 2006, Shinya Yamanaka – Nobel Prize for medicine and physiology 2012 – showed that mouse fibroblasts could be reprogrammed into embryonic stem cell-like cells by the simultaneous ectopic expression of four genes [57]. These cells were called induced pluripotent stem cells (iPSCs). More interestingly, 1 year later they reported that a similar experimental approach was used to reprogram adult human fibroblasts into iPSCs [58]. Briefly, the co-expression of the four key transcription factors *KLF4*, *SOX2*, *c-MYC* and *POU5F1* (also known as *OCT4*) successfully reprograms differentiated somatic cells back to a pluripotent state [58]. During the last 5 years, it has been shown how iPSCs can be differentiated into many different cell types, in similar ways as ESCs.

As it has been discussed, only few mutations in genes already known to be important for thyroid development have been found in CH patients with thyroid dysgenesis [59]. Several studies have been published during the last year demonstrating how iPSCs can be used *in vitro* for modelling genetic diseases [60].

The most reasonable approach would be to take advantage of the iPSC technology to derive pluripotent stem cells from fibroblasts of CH patients with thyroid dysgenesis. Applying the protocol of differentiation to CH patient-derived iPSCs might reveal differences at the transcriptional and morphological levels compared with healthy control-derived iPSC. This approach would lead to identify new genes playing an important role in normal and pathological thyroid development. Nevertheless, the selection of CH cases will be important as well as the good controls essential for the validation of the approach. Due to the fact that the *in vitro* model cannot be used for studying the migration of the gland during the foetal life, CH patients with thyroid ectopy should be excluded. Discordant monozygotic twins represent an interesting category of CH cases, and our model could help us to understand the genetic causes of a different thyroid phenotype in two ‘identical’ individuals. Our model might represent a good approach to investigate the causes of the differential thyroid organogenesis occurring in discordant monozygotic twins when mutations in coding or regulatory regions (exome sequencing and whole genome sequencing provide an additional tool to discover new mutations) lead to a differential expression of genes important for the development of the gland. Nevertheless, the model might not be a suitable model when epigenetic events occurring during early embryogenesis of the monozygotic twins are the cause of a differential development of the gland. Another interesting study would be the analysis of familial cases of CH. Exome sequencing of an entire family with CHTD (congenital hypothyroidism with thyroid dysgenesis)-affected members in combination with full transcriptional profiling of cells differentiated from healthy and CHTD-derived iPSCs would represent an interesting and valid approach to uncover new genes involved during thyroid embryogenesis. Identifying new genes will have a clinical impact increasing the list of genes that can be screened during pre-natal diagnosis.

Finally, it has been shown that ESC-derived thyroid follicles can be transplanted *in vivo* and restore thyroid homeostasis in mice affected by hypothyroidism. Those findings open new avenues towards conception of stem cell technology for the treatment of hypothyroidism. Nevertheless, I strongly believe we are still far from applying this approach for regenerative medicine. For example, genetic instability, due to culture conditions or the genetic engineering, together with the difficulty to obtain always homogeneous populations of differentiated cells are important pitfalls for regenerative medicine.

19.5 Conclusions

In this chapter, I discussed the first *in vitro* protocol of differentiation of pluripotent stem cells into functional thyroid follicles. This model enters in the large and steadily growing list of tissues that can be differentiated in 3D stem cell culture. This model would represent an additional and complementary model to study and characterize molecular and morphological events occurring in thyroid organogenesis. More interestingly, this ESC-based model can be used to uncover new genetic causes of thyroid dysgenesis in CH patients. In the far future, we are facing the idea to use the approach of curing severe hypothyroidism using transplantation of autologous ESC-derived thyroid tissue.

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