REVIEWS
AND THEORETICAL ARTICLES

The Role of Genetic Factors in Endocrine Tissues Development and Its Regulation In Vivo and In Vitro

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Abstract—Endocrine cells can be fully functional after heterotopic transplantation into mammals, which makes them a promising tool for regenerative medicine. However, to date, cellular therapy for endocrine diseases is limited by the inability to efficiently and stably obtain endocrine cells in vitro. The review focuses on current knowledge of the molecular genetic mechanisms involved in embryonic development of mouse and human endocrine tissues, as well as the key genetic factors regulating the differentiation of pluripotent stem cells (PSCs) into the endocrine lineage in vitro, using the example of parathyroid cells. The data presented make it possible to suggest an alternative approach to the PSCs differentiation toward parathyroid cells based on genetic engineering by inducing endogenous expression of key differentiation factors.

INTRODUCTION

Every year the prevalence of patients with endocrine disorders caused by impaired secretion and metabolism of different hormones increases worldwide. One of the promising approaches to the treatment of hormonal diseases is the use of regenerative medicine. This is determined by a number of features of secretory cells of the endocrine glands, providing their use as controlled bioreactors of hormones in vivo. Primarily, owing to the fact that in the body there are both glandular and diffuse endocrine systems, the excretion of hormones in mature endocrine cells is largely independent of their location in the body. In addition, the endocrine graft does not require innervation for its functioning. For these reasons, endocrine cells can be transplanted heterotopically to easily accessible sites in the human body, for example, subcutaneously or into muscles, regardless of the location of the gland itself. This approach has been successfully used for several decades to study endocrine disorders in mice, when hormone-producing cells were transplanted into animals under the kidney capsule. In the case of other types of cells (cardiomyocytes, hepatocytes, neurons, etc.), heterotopic transplantation is difficult or impossible.

At present, development of the methods for in vitro differentiation of human pluripotent stem cells (PSCs), such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), into hormone-producing endocrine cells for cell replacement therapy of different endocrine diseases and long-term correction of hormonal imbalance in patients with endocrine disorders attracts much attention. The use of autologous endocrine cells obtained from patient PSCs in vitro is a promising approach, since transplantation of such cells, on the one hand, frees patients from constant injections of expensive drugs, especially taking into account the long duration of endocrine diseases and considerable decrease in the overall quality of life, and, on the other hand, the procedure is safe because of the absence of host reaction against the graft and tissue rejection. However, there are still no effective and well-reproducible protocols for differentiation of human PSCs toward endocrine cells, in particular, to mature pancreatic islet cells and parathyroid cells. This is largely because of the limitations of empirical selection of differentiation conditions using growth factors and/or chemical molecules that activate and/or inhibit the required signaling pathways in accordance with data on the endocrine gland formation in vivo. The action of the selected factors in vitro in most cases is pleiotropic, and the use of reagents from different manufacturers or of animal origin leads to poor reproducibility of results. Since the main task of cell differentiation in vitro is the recapitulation and realization of the genetic program of embryonic devel-

opment, an alternative approach to differentiation is also possible through direct genetic programming of PSCs based on the current knowledge on embryology of the endocrine glands. Genetic programming of PSCs allows for specific activation of certain genes, rather than the entire regulatory networks. Target genes can be induced using the methods of overexpression or CRISPR/Cas9 Synergistic Activation Mediator (SAM). The CRISPR/Cas9 SAM technology allows for specific activation of the promoter of the gene of interest, thus initiating its transcription [1]. In 2018, by inducing expression of pancreatic transcription factors, it was possible to trans-differentiate α-cells to β-cells in vivo [2]. The development of an approach that will allow for programming PSCs differentiation in the required direction involves the use of knowledge on transcription factors that regulate embryonic development of each tissue. In this regard, understanding the genetic aspects of the organization and regulation of early development of endocrine tissues is necessary for the development of protocols for genetically programmed differentiation of PSCs into endocrine cells.

MORPHOGENESIS OF ENDOCRINE GLANDS OF ENDODERMAL ORIGIN

Endocrine glands are formed in the first months of embryonic development from the cells of definitive (embryonic) endoderm (DE). The definitive endoderm is formed from epiblast cells at the third week of development and gives rise to the embryonic gut tube, from which all organs of endodermal origin bud off [3]. At the beginning of the third week of development, in the posterior part of the embryo on the epiblast surface, appears the so-called primitive streak, or groove, along which epiblast cells start to migrate. Moving forward, the cells undergo epithelial-mesenchymal transition, lose pluripotency, acquire an elongated shape, and slip under the primitive streak. Such cell movements are called ingression, and cell migration into the underlying layers is called invagination. Some invaginating cells replace the cells of the underlying layer (hypoblast) and give rise to the embryonic DE. Cells migrating to the space between the epiblast and the formed DE form the mesoderm. The cells remaining in the epiblast form the ectoderm. By the end of the third week of development, in the trilaminar germ disc, simultaneously begin the processes of neural tube formation from the ectoderm and the gut tube formation from DE by folding of cell layers along the anterior-posterior axis of the embryo. By the end of the fourth week, the gut tube is a closed primitive gut, divided by gradients of morphogens into four parts along the anterior-posterior axis: (1) anterior foregut, (2) posterior foregut, (3) midgut, and (4) hindgut. The endoderm of the primitive gut forms the epithelial lining of the gastrointestinal tract and gives rise to the secretory cells of different glands. The cells of the mid-gut and hindgut form precursor cells for all parts of the gastrointestinal tract. The cells of the posterior foregut give rise to the pancreas and liver. The cells of the anterior foregut form the pharyngeal apparatus, consisting of four pairs of pharyngeal pouches, separated by four pairs of pharyngeal arches. As a result of segmentation and further development of pharyngeal apparatus, head and neck structures are formed, in particular, the thyroid gland, parathyroid glands, and thymus [4]. Ectodermal cells of the neural crest actively migrate to the pharyngeal arches and participate in the formation of mesenchyme and segmentation of the pharyngeal pouch endoderm, as well as in the development of the thymus and parathyroid glands and in differentiation of the thyroid parafollicular cells (C cells) [5].

The thyroid is the first among human endocrine glands to develop. Its development starts approximately at the 24th day of gestation in the form of a protrusion of endodermal and mesodermal cells at the pharyngeal floor [6]. The cells begin to migrate posteriorly, and by the end of the 7th week, the thyroid primordium reaches its final position in the pharynx. The cells of the ventral part of the fourth pair of pharyngeal pouches give rise to the precursors of parafollicular C cells of the thyroid gland, secreting calcitonin. By the end of the third month, the first follicles containing colloid, a source of thyroxine and triiodothyronine, synthesized by follicular cells, are found in the thyroid gland [7]. The parathyroid glands are formed during the period from the fifth to the 15th week of embryonic development from the epithelium of the third and fourth pairs of pharyngeal pouches. The inferior pair of parathyroid glands is formed from a common primordium with the thymus; the superior pair of glands is formed from a common primordium with the thyroid gland. At the seventh week of development, both primordia lose their connection to the pharyngeal wall and migrate downward in the posterior direction, herewith the primordia of the parathyroid glands take their final position on the dorsal surface of the thyroid gland [8]. The pancreas begins to develop at the end of the first month of gestation on about the 26th day as two outgrowths of the duodenal primordium formed by the cells of the posterior foregut [9]. The endocrine part of the pancreas is formed in the third month of development from parenchymal pancreatic tissue scattered throughout the gland. Insulin secretion starts around the fifth month of development [10].

MOLECULAR GENETIC MECHANISMS OF DEFINITIVE ENDODERM FORMATION IN VIVO

The formation and differentiation of DE cells are provided by the interaction of the components of the regulatory network that controls spatial and temporal expression of key endodermal genes in the developing

embryo. With the beginning of gastrulation the expression of evolutionarily conserved transcription factors BRACHYURY, SOX17, and FOXA2 is detected in epiblast cells. Among them, BRACHYURY plays an important role in the specification of mesodermal cells, while SOX17 and FOXA2 are necessary for the specification of DE cells [11, 12]. Formation of endoderm and mesoderm is linked evolutionarily, however, the question on the existence of bipotent "mesendodermal" precursors in the mammalian embryonic development is still controversial [13, 14]. The expression of BRACHYURY, SOX17, and FOXA2 factors is regulated by the concentration of NODAL cytokine, which is a key participant of the TGFβ/Activin/Nodal-mediated signaling pathway that determines the fate of endodermal and mesodermal precursors [15, 16]. NODAL is secreted by parietal extraembryonic endoderm cells and provides the induction of "mesendodermal" genes through the phosphorylation of cytoplasmic proteins of the SMAD family (SMAD2, SMAD3, SMAD4), which activate different transcription factors, in particular, FOXH1 [17, 18]. The fate of "mesendodermal" precursors is determined by the NODAL concentration gradient formed by mutual antagonism of the NODAL and FGF/BMP regulatory networks [19, 20]. A high NODAL level induces SOX17 expression and cell differentiation toward endodermal cells, while inhibition of NODAL by factors of the FGF and BMP families leads to the induction of mesodermal differentiation [21]. The specification and differentiation of endodermal precursors is also supported by the autoregulatory NODAL expression loop [22, 23]. It is currently believed that the dominant role in the DE cells induction belongs to SOX17. Using knockout mouse models, it was demonstrated that DE does not form in embryos with impaired *Sox17* gene, since epiblast cells loose their ability to migrate during gastrulation. The resulting defects lead to the developmental arrest and fetal death at E10.5 [24]. The *Foxa2* abnormalities do not lead to embryonic lethality; however, defects in the foregut, overall growth retardation, and inability to form midline structures are observed [25, 26].

Following gastrulation, a volumetric blind-ended gut tube is formed from the flat cellular layer of DE, in which the anterior-posterior and dorso-ventral polarities are established. At this stage, DE cells have not yet been committed and are characterized by high plasticity [27]. A key role in the endoderm specialization is played by reciprocal epithelial-mesenchymal interactions between DE and mesodermal cells along the entire gut tube. The DE specification along the anterior-posterior axis of the embryo is provided by the concentration gradient of morphogens of the WNT, BMP, and FGF families, as well as their antagonists, SFRP-1, SFRP-2, SFRP-3, SFRP-5, CRESCENT, DKK1 (WNT antagonists) and CHORDIN, NOGGIN (BMP antagonists). In the region of high WNT, BMP, and FGF concentrations, the posterior embryonic gut is formed, while the ante-rior embryonic gut is formed in the region of their low concentrations [28, 29]. Expression of the SOX2 transcription factor is the main foregut marker, and expression of the CDX2 transcription factor is the hindgut marker [30]. Retinoic acid (RA), a vitamin A derivative synthesized by mesodermal cells, playes an important role in regionalization of expression of different transcription factors along the primitive gut [31]. The concentration gradient of RA is distributed along the anterior-posterior axis of the entire embryo, from the prospective pharynx, where RA is practically or completely absent, to the midgut and hindgut region, where a high concentration of RA is detected [32]. In mice with impaired RA synthesis, no foregut compartmentalization occurs [33]. At later stages of development during organs primordia formation in the pharyngeal region, the RA concentration controls the boundary positions of the forming organs. For instance, an increase in the RA concentration in the anterior part of pharyngeal arches induces the formation of more distant structures [34]. An important role in reciprocal epithelial-mesenchymal interactions is played by highly conserved SHH morphogen, secreted by endodermal cells along the gut tube [35]. In mesenchymal cells, SHH activates expression of the BMP-4 factor and homeobox-containing transcription factors encoded by conserved *Hox* genes, which control the regionalization of the primitive gut. In particular, *Hoxa3* and *Hoxb4* are specifically expressed in the foregut, while *Hoxc5* and *Hoxa13* are expressed in the midgut and hindgut [36].

The establishment of dorso-ventral (back–belly) polarity of the foregut precedes the formation of the pharyngeal apparatus and segregation of the lung buds, esophagus, and trachea from the cells of ventral DE of the primitive gut. Dorso-ventral polarity of the foregut is established in response to WNT, BMP, and FGF signals from cells of the underlying mesoderm. WNT2 and WNT2B induce the expression of NKX2.1, NKX2.5, and PAX1 transcription factors in the ventral foregut [37]. *Nkx2.1* expression in the ventral region is maintained by BMP4-mediated downregulation of the SOX2 transcription factor, which marks dorsal foregut structures. Mesodermal cells also express the NOGGIN factor, a BMP4 antagonist, so that *Sox2* expression is not inhibited in the underlying endodermal cells and further division of structures into dorsal and ventral regions takes place. In addition, WNT secretion by dorsal mesodermal cells inhibits *Nkx2.1* expression in the dorsal foregut. Differential expression and reciprocal inhibition of NKX2.1 and SOX2 are also involved in the formation of dorso-ventral polarity of the gut [38–40].

MOLECULAR GENETIC MECHANISMS OF THE PARATHYROID GLANDS DEVELOPMENT IN VIVO

Parathyroid glands originate from pharyngeal pouch endoderm of an embryo. The most important

factor in the formation of the whole pharyngeal apparatus is the conserved transcription factor TBX1. Disruption of the *Tbx1* gene can lead to the development of 22q11.2 deletion syndrome (DiGeorge syndrome) [41]. The *Tbx1* knockout mice lack thymus and parathyroid glands and die after birth due to developmental defects of heart, skeleton, and facial muscles [42, 43]. It was demonstrated that *Tbx1* expression in the pharyngeal apparatus is regulated by the SHH factor. However, the mechanism of this regulation remains unknown [44]. *Tbx1* expression in the endoderm activates expression of the WNT11R and FGF8A factors in the underlying mesodermal cells, which, in turn, promotes morphogenesis of the pharyngeal pouch endoderm [45]. Formation of the parathyroid gland primordia is controlled by factors of the FGF (FGF8, FRS2α), BMP (BMP4), and SHH families, as well as by *Hox* genes [46, 47]. Cells of the anterior dorsal epithelium of the third pharyngeal pouch, expressing FGF8 and PAX1 factors, give rise to the common primordium of the thymus and the inferior pair of parathyroid glands, which is subdivided to parathyroid and thymic domains. This separation is provided by reciprocal interaction of the SHH, BMP2/4 and FGF8/10 signaling pathways, which induce the formation of the parathyroid glands and thymus [48]. The main marker of thymic domain is the FOXN1 transcription factor. The parathyroid domain expresses the GCM2 transcription factor, which is an exclusive marker of both precursors and mature parathyroid cells and is necessary for their differentiation and survival [49, 50]. The loss of GCM2 leads to apoptosis of parathyroid precursor cells [51]. *Gcm2* mutations are one of the reasons for the development of familial forms of isolated hypoparathyroidism and hyperparathyroidism [52, 53]. Some studies have demonstrated a change in the level of *Gcm2* expression in parathyroid adenomas [54].

Early differentiation of parathyroid cells is directed by SHH. In *Shh⁻/Shh⁻* mice, the parathyroid gland primordium is not formed and *Gcm2* expression is completely absent [55]. TBX1 is a candidate factor mediating the effect of SHH on organogenesis of the third and fourth pharyngeal pouches [56, 57]. It is suggested that the *Shh-Tbx1-Gcm2* regulatory pathway is responsible for initial determination of parathyroid cell fate. In addition, TBX1 interacts with the transcription factor FOXI3, which is involved in the development of the pharyngeal apparatus and the formation of the thymus and parathyroid glands from the endoderm of the third pair of pharyngeal pouches. *Foxi3* is expressed in the pharyngeal pouch endoderm and the ectoderm of the pharyngeal apparatus in mice at approximately the same time as *Tbx1* [58]. Inactivation of *Foxi3* in the TBX1-domain of pharyngeal pouches leads to thymus and parathyroid glands aplasia [59]. *Foxi3⁻/Foxi3⁻* mice are not viable and die in utero or shortly after birth due to abnormalities of the pharyngeal apparatus formation and segmentation and, as a consequence, development of severe defects in skull and face structure [60]. Formation of the common thymus and inferior pair of parathyroid glands primordium is controlled by the regulatory network of conservative transcription factors HOXA3, PBX1, EYA1, SIX1/SIX4, PAX1/PAX9, the defect of which leads to hypoplasia or aplasia of the thymus and parathyroid glands. In *Hoxa3⁻/Hoxa3⁻* mice, the parathyroid gland and thymus primordium is completely absent [61]. In *Pbx1⁻/Pbx1⁻* mice, hypoplasia of parathyroid glands and thymus and decreased *Tbx1* and *Gcm2* expression are observed [62]. *Eya1⁻/Eya1⁻* mice lack thymus and parathyroid glands. In homozygous mice with knockouted *Six1* gene only thymus is absent, since *Gcm2* expression is initiated but not maintained further during development [63]. Expression of PAX1 transcription factor in the third pharyngeal pouch depends on *Eya1* and *Six1* expression. In *Pax1⁻/Pax1⁻* mice, the level of *Gcm2* expression is decreased and hypoplasia of the parathyroid glands is observed [61]. The separation of a common parathyroid glands and thymus primordium from the walls of the pharyngeal pouch occurs as a result of cell apoptosis controlled by the action of FGF, BMP4 and other signals from the underlying mesenchyme [64−66].

The transcription factor MAFB plays an important role in the segregation and migration of parathyroid precursors. In *MafB* heterozygous mice, the parathyroid glands are mislocalized between thymus and thyroid gland, while in *MafB* homozygotes, parathyroid cells do not migrate and remain connected with thymus [67]. *MafB* expression and further differentiation and maturation of parathyroid cells are regulated by the linked cascade of GATA3 and GCM2 factors [68]. The most upstream factor is GATA3, followed by GCM2 and then MAFB. *Gata3* knockout in mice results in impaired parathyroid formation and considerable developmental defects; in *Gata3* heterozygous mice, the parathyroid glands are formed, but their partial dysfunction is observed [69]. GCM2 initiates expression of the main markers of mature parathyroid cells, such as the parathyroid hormone (PTH) and the calcium-sensing receptor (CASR) [70]. It is shown that *Gcm2*, *Gata3*, and *Tbx1* mutations in mice and humans lead to the development of hypoparathyroidism [71, 72]. Expression of *Gata3*, *Gcm2*, and *MafB* in parathyroid cells is observed also upon morphogenesis completion [67, 73]. In the postnatal period, these factors physically interact with each other, causing synergistic activation of the *Pth* gene promoter in mature parathyroid cells [74].

DIFFERENTIATION OF PSCs INTO PARATHYROID CELLS IN VITRO

Differentiation of PSCs into parathyroid cells in vitro, according to the in vivo development, involves a stepwise generation of DE cells and their differentiation into anterior foregut cells, then into cells of the
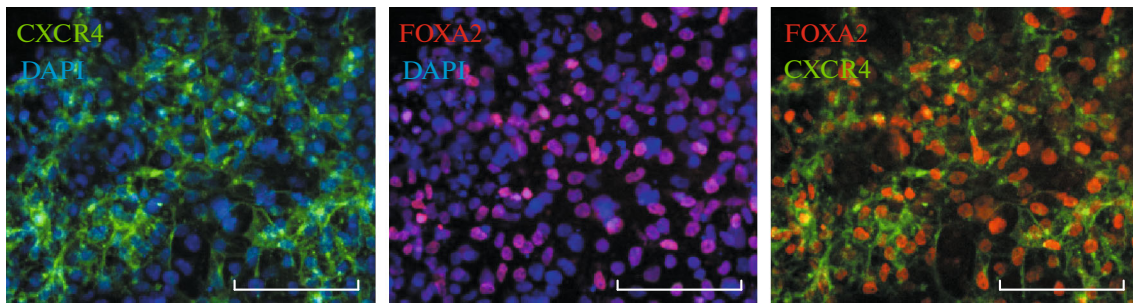
Fig. 1. Example of a successful differentiation of human iPSCs into DE cells. Immunocytochemical staining of DE cells for endodermal markers CXCR4 (green channel) and FOXA2 (red channel). Nuclei are visualized with DAPI staining (blue channel). Scale bar, 100 μm.

ventral foregut region, and then into parathyroid cell precursors (pre-PTC) with subsequent expansion and maturation.

Today protocols of DE cells generation are well established. Most of them are based on the activation of the WNT and TGFβ signaling pathways by Activin A, which acts as an embryonic factor NODAL, and GSK3β inhibitors (CHIR99021 and others), respectively [75]. Commercial kits for efficient differentiation of PSCs into DE cells are already available, such as STEMdiff Definitive Endoderm Kit (StemCell Technologies), PSC Definitive Endoderm Induction Kit (Thermo Fisher Scientific), and StemXVivo Endoderm Kit (R&D Systems) and other. Analysis of *Sox17*, *Foxa2*, *Cxcr4*, *c-Kit*, *EpCAM*, and *Mixl1* expression is used as the main marker of differentiation in the generated DE cells (Fig. 1). Currently there are strategies for selection of iPSCs with the highest endodermal differentiation potential due to the clonal differences of human iPSCs in their predisposition to the in vitro differentiation [76, 77]. In addition, recently, using genome-wide CRISPR screening, it was demonstrated that inhibition of the JNK-JUN signaling pathway, which indirectly affects the exit of PSCs from the pluripotent state, considerably increased the efficiency of DE and its derivatives generation [78]. To date, a number of protocols have also been developed for obtaining cells of the ventral foregut endoderm [79–81]. However, the efficiency of such differentiation varies among iPSC lines depending on their genetic and epigenetic characteristics, as well as the reprogramming method [82].

As for the further differentiation of committed endodermal cells towards parathyroid cells, there are currently no effective and reproducible methods for obtaining parathyroid hormone-producing cells from human PSCs. A number of protocols for generation of parathyroid cells based on the induction of *Gcm2*, *Casr*, and *Pth* expression in committed endodermal cells using the Activin A, FGF8, and SHH factors have been reported. The first published protocol for parathyroid differentiation is based on the production of PTH-secreting cells from human ESCs through the DE stage using only the Activin A factor in the presence of low fetal calf serum concentrations [83]. Subsequently, the protocol was modified by the same group of authors by adding the SHH factor, and the induction of *Pth* expression in differentiated cells was evaluated [84]. The main disadvantages of the suggested approach are poor reproducibility of the protocol and the use of ESCs cultured on feeder cells. In 2011, another group showed that inhibition of the BMP and TGF-β signaling pathways upon PSCs differentiation into endodermal lineage increases the efficiency of differentiation into cells of the anterior foregut endoderm, which can serve as a source of parathyroid cells. However, the proposed scheme with the use of additional SHH and/or FGF8 factors for the induction of parathyroid differentiation provided only an insignificant increase in *Gcm2* expression compared to the control [85].

Thus, at present, the development of reproducible and effective methods for the production of PTH-secreting cells from PSCs in vitro is a crucial task. An approach using the CRISPR/Cas9 SAM technology which implies specific controlled activation of key regulatory genes of parathyroid differentiation in committed endodermal progenitor cells (Fig. 2) can be applied for generation of parathyroid cells. Since the production of DE cells, as well as those from later stages of the foregut development, is not much challenging, the main targets of genetically programmed parathyroid differentiation can be the *Gata3*, *Shh*, *Tbx1*, and *Gcm2* genes, which are key inducers of parathyroid differentiation in vivo.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflicts of interest.
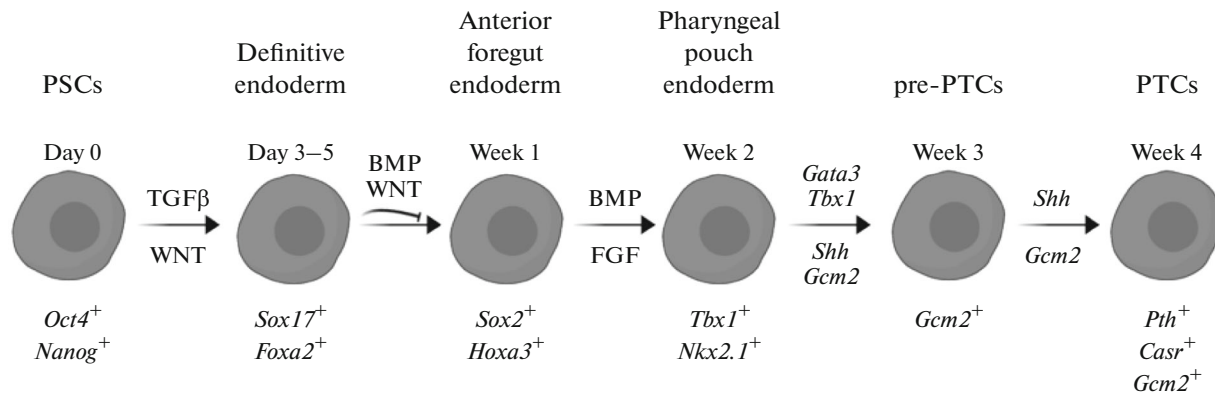
Fig. 2. The main stages of human PSCs differentiation into parathyroid cells by induction of *Gata3*, *Tbx1*, *Shh*, and *Gcm2* expression in committed endodermal cells. The main marker genes of the corresponding differentiation stages are shown below. PSCs, pluripotent stem cells; pre-PTCs, precursors of parathyroid cells; PTCs, parathyroid cells.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Statement of compliance with standards of research involving humans as subjects. This article does not contain any research involving humans as a subject.

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