

1 Introduction

1.1 Stem cells: definitions and basic theoretical concepts

Every vertebrate organism develops from a single cell: the fertilized egg, also called the zygote. The zygote undergoes rapid mitotic divisions with no significant cell growth, producing a cluster of cells the same size as the original zygote. In mammals this process leads to the formation of the blastocyst, a preimplantation embryo of 30-150 cells. The blastocyst consists of a sphere made up of an outer layer of cells (the trophoctoderm), a fluid-filled cavity (the blastocoel), and a cluster of cells on the interior (the inner cell mass). The next step is called gastrulation, during which cells migrate to the interior of the blastocyst, subsequently forming three germ layers: ectoderm, mesoderm and endoderm. All tissues and organs of the body originate from one of these three layers: The mesoderm will form the muscles, many organs, connective tissue and bones, the ectoderm will form the epidermis and nervous system, while the endoderm will give rise to the gastrointestinal tract, respiratory tract and endocrine glands. The process whereby the unspecialized embryonic cells of these germ layers acquire the features of a specialized cell such as a heart, liver or muscle cell is called differentiation (1, 2).

Cells like the zygote and its early embryonic progeny, which are undifferentiated, have self-renewal capacity and can give rise to specialized cells of several tissues, are called stem cells (3). One distinguishes between three different kinds of stem cells, according to the types of tissues to which they can differentiate. *Multipotent stem cells* (also called unipotent) have the capability of developing cells of multiple but not all germ layers. *Pluripotent stem cells* are able to differentiate into cells derived from any of the three germ layers but cannot grow into a whole organism. Finally *totipotent stem cells* can grow into an entire organism, having the capacity to differentiate into extraembryonic membranes and tissues, the embryo, and all postembryonic tissues and organs. The zygote is an example of a totipotent stem cell (1, 3, 4).

In the recent years, data has emerged that stem cells do not only appear transiently during embryogenesis but also exist in differentiated tissues. Such cells are called adult stem cells (5). According to the official definition of the National Institute of Health of America “an adult stem cell is an undifferentiated cell found in a differentiated tissue that can renew itself and (with certain limitations) differentiate to yield all the specialized cell types of the tissue from which it originated” (6).

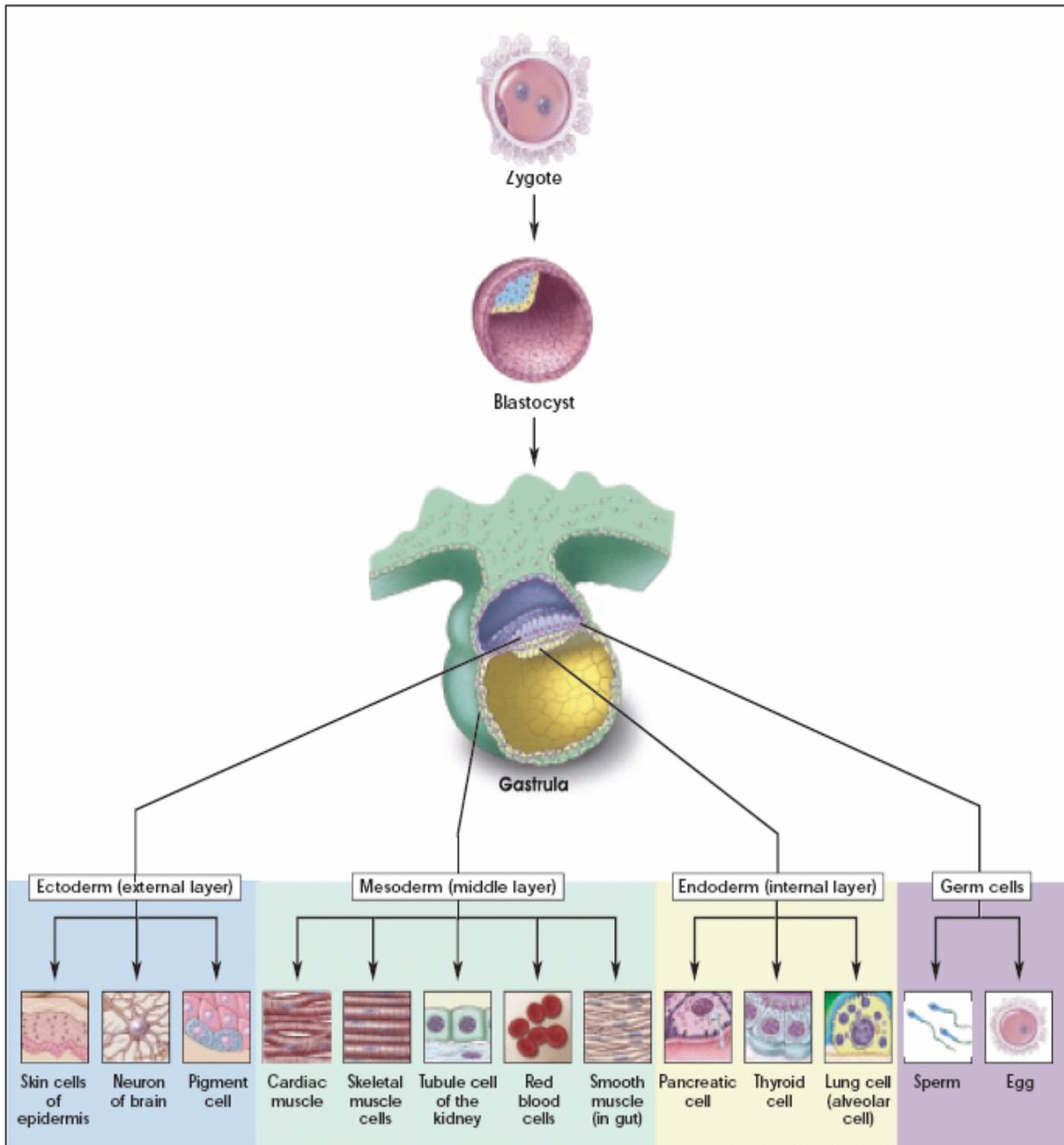


Figure 1.1 : Embryogenesis of human tissues [taken from: Stem Cells: Scientific Progress and future research directions, Department of Health and Human Services. June 2001. <http://stemcells.nih.gov/info/scireport> (6)]

Although adult stem cells have basic similarities to embryonic stem cells (undifferentiated state, ability to produce differentiated cells, self-renewal potential), they also display important differences. The isolation of embryonic stem cells from the inner cell mass of the blastocyst and their subsequent culture is nowadays a standardized procedure. Using different

cell culture techniques (e.g. feeder cell layers) and well-described growth factors, scientists are able to cultivate embryonic stem cells in great numbers and for infinite periods of time (7).

Adult stem cells on the other hand are rather elusive: They are very rare, found in their tissues of origin sparsely dispersed amongst big numbers of differentiated cells (5). When they reside in their normal tissue compartments they produce the cells that are specific to that kind of tissue and they have been found in tissues derived from all three embryonic layers. But when they are taken out of their normal environment, they do not seem to have the same ability to proliferate in culture and at the same time retain the capacity to differentiate into functionally useful cells (8). Very few reports of adult stem cells being propagated in an undifferentiated state for longer periods of time have been published (9). In most cases, adult stem cell-enriched populations undergo senescence in culture (10) or fail to proliferate altogether (11). What is more, no adult stem cell to date has been found which is truly pluripotent, i.e. able to give rise to tissues of all three germ layers (5).

To date, published scientific literature indicates that adult stem cells have been derived from a variety of tissues such as the brain (12), bone marrow (13), dental pulp (14) , spinal cord (15) , skeletal muscle (16), epithelia of the skin (9, 17) and digestive system (18), cornea (19), retina (20), liver (21), and pancreas (22). However, there is no report on isolation of adult stem cells from the thyroid gland.

1.2 Histology and embryology of the thyroid gland

The thyroid gland produces thyroid hormones and calcitonin in two distinct cell types: the thyroid follicular cells and the parafollicular or C cells, respectively (23).

The thyroid follicular cells, which constitute the most numerous cell population in the gland, form the thyroid follicles, spherical structures serving as storage and controlled release of thyroid hormones. Thyroid hormones are produced from follicular cells through iodination of tyrosine and are released to the circulation in their biologically active forms, triiodothyronine (T3) or thyroxine (T4) (24).

The C cells are scattered in the interfollicular space, mostly in a parafollicular position. C cells are responsible for the production of calcitonin, a hormone involved in calcium and phosphate homeostasis (24).

The two diverse cell types, responsible for the dual endocrine function of the gland, originate from two different embryological structures: The thyroid anlage is the site of origin of thyroid follicular cells, whereas the ultimobranchial bodies are the source of C cells. The thyroid anlage is an area enclosing a small group of endodermal cells, and it is located on the midline of the embryonic mouth cavity in its posterior part. The ultimobranchial bodies are a pair of transient embryonic structures derived from the fourth pharyngeal pouch and located symmetrically on the sides of the developing neck. The C cell precursors migrate from the neural crest bilaterally to the fourth pharyngeal pouches and become localized in the ultimobranchial bodies (23). The thyroid follicular cells are therefore of endodermal embryological origin, whereas the C cells originate from the (neuro)ectodermal germ layer (2). A brief summary of the above, including surface markers for the terminally differentiated cells, is shown in the table below.

Cell Type	Embryological Origin	Germ Layer	Characteristics of differentiated cells	Differentiation markers
Thyroid follicular cells	Thyroid Anlage	Endoderm	<ul style="list-style-type: none"> ▪ Follicle formation ▪ Production of Thyroglobulin ▪ Iodination of Tyrosine ▪ Production and storage of thyroid hormones 	<ul style="list-style-type: none"> ▪ Thyroglobulin ▪ Na-I Symporter ▪ Thyroid peroxidase ▪ TSH-Receptor
Parafollicular cells (C cells)	<ul style="list-style-type: none"> ▪ Neural Crest ▪ Migration and localisation in ultimobranchial body 	Neuroectoderm	<ul style="list-style-type: none"> ▪ Scattered in interfollicular space ▪ Calcitonin production 	Calcitonin

Table 1.1: Embryological origin, basic characteristics and differentiation markers of the two main hormonally active cell populations of the thyroid gland: follicular cells and parafollicular cells [all data adapted from: De Felice M, Di Lauro R 2004 Thyroid development and its disorders: genetics and molecular mechanisms. *Endocr Rev* 25:722-746 (23)]

1.3 Stem cell markers

The most widely accepted method for the recognition, characterisation and isolation of stem cells is the detection of stem cell markers (3). Stem cell markers are genes and transcription factors (proteins found within cells that regulate a gene's activity) that are unique in stem cells (6). A list of all available stem cell markers can be found in the Stem Cell Manual of the National Institute of Health of America (25). For the present work, markers were picked which characterise either pluripotent stem cells (Oct4) or multipotent endodermal stem cells (GATA-4, HNF4 α and AFP). The marker p63 is not included in the above list but its expression was analysed in this work because of two published reports claiming a stem cell role for immunohistochemically detected p63-positive stem cells in human thyroid glands (26, 27).

Oct4

Oct4 (also referred to as Oct3/4 or Pou5f1) is a member of the POU (Pit-Oct-Unc) family of transcription factors that are involved in the regulation of cell growth and differentiation in a variety of tissues. POU transcription factors can activate the expression of their target genes through binding of an octameric sequence called the octamer motif (ATGCAAAT) (28).

Oct4 has been proposed as a marker for totipotency or a gene required for totipotency (28, 29). Its expression is evident in pluripotent cells of the developing embryo, including epiblast cells, primordial germ cells, and their in vitro counterparts, embryonic stem (ES) and embryonic germ (EG) cells (30). Differentiation of totipotent cells to somatic lineages occurs at the blastocyst stage and during gastrulation, simultaneously with Oct4 down-regulation. Stem cell lines derived from the inner cell mass and the epiblast of the mouse embryo express Oct4 only if undifferentiated. When embryonic stem cells are triggered to differentiate, Oct4 is down-regulated (31). In addition, Oct4 is transiently expressed in the developing endoderm (32) and neuroectoderm (33) where it may contribute to the specification of cell fate. In mice, a loss-of-function mutation for Oct4 results in early embryonic lethality because of the inappropriate differentiation of pluripotent epiblast cells into trophectoderm (34). Similarly, down-regulation of Oct4 in ES cells induces trophectoderm differentiation, whereas overexpression induces differentiation into extraembryonic mesoderm and endoderm (35), demonstrating that Oct4 is a crucial and dose-dependent determinant of pluripotency in embryonic cells.

Oct4 has been shown to be expressed in some human tumor cells, mainly germ-cell cancers (36-38), but also in different breast cancer cell lines (39, 40) and a colon cancer cell line

(41). Moreover, Oct4 protein could be detected in rare human kidney and lung cancer samples using tumor tissue arrays (42).

Very recently, Tai et al. reported Oct4 gene expression in several human adult stem cells (breast, liver, pancreas, kidney, mesenchyme and gastric stem cells), in the cancer cell lines HeLa and MCF-7 and in human, dog and rat tumors (43). These researchers also investigated normal dog and human skin sections for Oct4 expression but were able to detect only a few cells with positive Oct4 staining. These cells were localized within the basal layer of skin epidermis, where epidermal stem cells reside and therefore probably represent adult stem cells themselves. These results indicate that Oct4 expression characterises adult human stem cells but not normal, terminally differentiated cells (43).

HNF4 α

Hepatocyte nuclear factor 4 (HNF4 α) belongs to the hepatocyte nuclear factor group of transcription factors. Other members of this group include HNF1 α , HNF3 α , β , γ and HNF6. All these transcription factors have been isolated from rat liver using promoter elements of genes specifically expressed in hepatocytes. However, upon cloning of the corresponding cDNAs and using these nucleic acid probes in hybridization experiments, it turned out that these liver transcription factors are not restricted to hepatocytes and thus their names are misleading. Furthermore, the similar names of the HNFs describe transcription factors of most distinct structure and therefore reflect only their common origin of purification. Recently, in humans, mutations in some of these HNFs have been identified that cause an early onset of type II diabetes referred to as MODY (maturity onset diabetes of the young) and/or severe renal defects. These diseases associated with ‘hepatic’ transcription factors document most convincingly the essential function of these transcription factors in non-hepatic cell types (44).

HNF4 α is a member of the nuclear receptor superfamily. The HNF4 transcription factor family has been extensively conserved with members found even in insects (45). Observations in the fly, *Drosophila* (46) and the frog *Xenopus* (47) suggest that HNF4 plays a role early in embryonic development, since this transcription factor is a maternal component of the egg. An early function in embryogenesis seems also to occur in mammals, as HNF4 expression has been reported in murine embryonic stem cells (48) and the knock-out of the gene in the mouse leads to defective gastrulation with embryonic lethality at day 9 (49). This critical phase of early development in the mouse primarily depends on the requirement for HNF4 α for the proper function of the extraembryonic tissue at day 6.5 as the supply of wild-type visceral endoderm rescues the development of the murine embryo through midgestation (50).

Based on *in situ* hybridization analysis in postimplantation mouse embryos, Duncan et al. were able to detect HNF4 α expression in the extraembryonic endoderm, gut and nephrogenic tissue of the developing mouse embryo and suggested therefore that HNF4 α is a marker for primary endoderm in the implanting blastocyst (50). Similarly, HNF3-induced endodermal differentiation of embryonic stem cells led to an increase in HNF4 α signal in the resulting endodermal progenitors (51). All these data suggest that HNF4 α plays an important role in the earliest stages of endoderm formation and is a marker for progenitor cells of endodermal origin.

Interestingly, HNF4 α expression has also been detected in several adult tissues of endodermal origin, such as the liver, intestine, kidney and pancreas (52). It is still unclear how it is possible for a gene expressed in the primary and visceral endoderm of the developing embryo to be present in adult cells. Studies in *Drosophila* have shown that transcription factors acting early in embryogenesis are often expressed later during organogenesis or in adults to serve a function in the differentiated cell (46). Based on this observation, Duncan et al. suggested that there are many parallels in function between the visceral endoderm in the early embryo and the adult organs where HNF4 α is expressed, particularly the liver (50).

GATA-4

GATA-4 belongs to a family of zinc finger transcription factors termed the „GATA-binding proteins“, which regulate gene expression, differentiation and cell proliferation in a variety of tissues by binding to the consensus DNA sequence (A/T)GATA(A/G) (53). Based on sequence divergence and expression pattern, the GATA family members can be divided into two subfamilies: GATA-1, -2 and -3 are expressed in hematopoietic cell lineages and are required for normal hematopoiesis, whereas GATA-4, -5 and -6 are expressed in heart, endoderm and selected other cell lineages (54). Within the endocrine system, GATA-4 is expressed in the hypothalamus, the pituitary, the female and male gonads and the adrenal gland (55).

GATA-4 expression has been reported during the early stages of endoderm formation. Using Northern analysis, Arceci et al found that GATA-4 mRNA was not detectable in undifferentiated F9 embryonal carcinoma cells but was induced during differentiation into parietal or visceral endoderm (56). Mice null for GATA-4 die between embryonic day 8 and 9 because of defects in heart morphogenesis and ventral closure of the foregut. Specifically, GATA-4 null mice present with cardia bifida because of ineffective ventral fusion of the lateral aspects of the embryo and the subsequent formation of the foregut (57). Aberrant heart formation in GATA-4 null mice is probably a secondary effect associated with an intrinsic defect in the definitive endoderm that underlies the splanchnic mesoderm containing the cardiac field (58).

This interpretation is further supported by the observation that GATA-4 null embryonic stem cells can generate cardiac myocytes but are partially defective in their ability to generate visceral endoderm and definitive endoderm of the foregut (54). These data suggest an important role for GATA-4 during early endoderm formation and morphogenesis.

GATA-4 mRNA and protein could be detected in the fetal mouse and human adrenal cortex from embryonic d 14 and gestational week 9, respectively. In the postnatal adrenal, GATA-4 expression is down-regulated (59). GATA-4 protein was evident in 12-week-old human fetal testes and was continuously expressed in the Sertoli and Leydig cells through adulthood (60). GATA-4 expression was found in a majority of germ cells before puberty but its expression was not detected in germ cells after puberty (53). Immunohistochemical analysis and *in situ* hybridisation of adult mouse ovaries showed abundant GATA-4 expression in granulosa cells of primary and antral follicles of mature animals. GATA-4 signal persisted during follicular maturation but no GATA-4 signal was detectable in the corpus luteum after ovulation. Similarly, GATA-4 was down regulated during follicular apoptosis, as granulosa cells within large and small apoptotic follicles lacked GATA-4 message (61). It can therefore be deduced that GATA-4 is expressed in endodermally derived fetal tissue and potentially mitotic cells in adult tissue (granulosa cells, prepubertal germ cells, Sertoli cells), but not in terminally differentiated (i.e. luteal) or apoptotic cells. In accordance with this finding is the expression of GATA-4 in sex-cord derived ovarian tumors (55), pediatric yolk sac tumors (62) and Sertoli and Leydig cell tumors (60).

All these data taken together, suggest that GATA-4 is a marker of early endoderm and is expressed early during differentiation of embryonic stem cells to endodermal progenitor cells. In adult endocrine tissues such as the adrenals, the testis and the ovary, GATA-4 characterises cells which are not terminally differentiated and maintain an increased proliferation potential.

p63

p63 is a homologue of the p53 tumor suppressor gene. It is located on the long arm of chromosome 3 (3q27) and it encodes six isoforms, three transactivating (TA) and three Δ N-isoforms. While the first three isoforms may act as tumor suppressor genes, the Δ N-isoforms may inhibit the p53/TA-p63-driven cell cycle arrest and apoptosis (63).

p63 is consistently expressed by basal/somatic stem cells of stratified epithelia (64), myoepithelial cells of the breast and salivary glands (63), and proliferative compartment of gastric mucosa (65). p63 is strongly expressed in the basal, or progenitor, cells of several epithelial structures present in the epidermis, cervix, urogenital tract, prostate and breast (66).

Experiments with p63 knockout mice indicated that p63 is critical for maintaining the progenitor-cell populations that are necessary to sustain epithelial development and morphogenesis (67, 68).

Based on these observations, Reis-Filho et al proposed a stem cell role for p63 positive main cells of solid cell nests (SCN) of the thyroid (26). Solid cell nests are ultimobranchial body remnants which are located in the middle and upper third of the lateral thyroid lobes (69). They can be found in most thyroid glands if the search is meticulous, as reported by systematic anatomical surveys and autopsy studies (70). It is accepted that SCNs are composed of two distinctive cell types:

- 1) main cells, which are polygonal to elongated or even spindle-shaped cells with centrally located, oval to fusiform nuclei with uneven nuclear envelope showing occasional grooves, and deeply eosinophilic cytoplasm showing squamoid features (including high molecular weight cytokeratins) but lacking intercellular bridges and
- 2) C-cells which account for a minor proportion of solid cell nest population and are characterised by clear cytoplasm and centrally located, small compact nuclei (26). Immunohistochemical analysis showed strong nuclear p63 staining of main cells in all SCNs as opposed to consistent p63 negativity of C-cells and the cuboidal epithelium lining the cystic structures (26). Preto et al supported the hypothesis of a stem cell role of main cells of SCNs using telomerase expression and proliferative activity experiments (71).

AFP

Alpha-fetoprotein is a tumor-associated fetal protein classified as a member of a three-domain albuminoid gene family that currently consists of four members: albumin (ALB), vitamin-D binding protein (DBP), AFP, and alpha-albumin (72). As its name implies, alpha-fetoprotein is mainly expressed in fetal tissues during embryogenesis, such as the fetal liver, the yolk sac, tissues of gastrointestinal origin (73) and the developing brain (74). Although its existence was first described in 1957 (75) and a vast number of reports on the subject have been published since, the exact role of AFP during embryogenesis and development hasn't been fully elucidated (76). Increased levels of AFP in the amniotic fluid and maternal blood during pregnancy have been associated with certain genetic anomalies (mainly neural tube defects), however it is assumed that these AFP levels are coincident events rather than the cause of such changes (76). These assumptions are based on experiments on AFP-null mouse embryos where it was shown that AFP is not essential for embryonic development, although it is required for female fertility (77).

In healthy adult tissues, AFP expression is down regulated (78) so that AFP is only detectable at very low levels in the adult liver (79), kidney and pancreas (80). The detection of AFP in reproductive fluids of mammals (81) and the detectable levels of AFP in maternal blood during pregnancy (which is of fetal origin) (76), pose two exceptions to this rule. Increased AFP levels are found in the serum of patients with hepatocellular carcinoma (82), cholangiocarcinoma (83), germ cell tumors (84) and certain pathologic conditions of the liver such as hepatitis and cirrhosis (82).

The use of AFP as a marker of early endodermal stem cells is based on the fact that it is expressed very early during the formation of embryonic endoderm. In the mouse, AFP has been histochemically detected in the inner cell mass of the blastocyst, in both the outer and the inner layers of the primitive endoderm (85). Bovine AFP has also been detected in the 14-day trophoblast, and by Day 16, AFP is secreted into the amniotic fluid (86). Similar to the AFPs of marmosets and rodents, bovine AFP is detected in both the preimplantation and the postimplantation conceptus (87). Human AFP is expressed in the yolk sac, hindgut/midgut endoderm, and the foregut hepatic diverticulum at 26 days postovulation (76). In summary, all mammalian species studied thus far show histochemical evidence of AFP in the pre- and postimplantation embryos, yolk sac, amnion, embryonic disc, and early primitive streak stages (76).

In embryonic stem cell lines (88, 89) and embryonic germ cell lines (90) derived from human blastocysts, AFP is expressed after differentiation into endoderm has taken place. Based on these AFP expression profiles, a theory about a stem cell origin of hepatocellular carcinoma and cholangiocarcinoma has been proposed (91).

1.4 The theory of asymmetric stem cell kinetics

In vivo, stem cells divide asymmetrically in the sense that each division produces two different cell kinds: an identical copy of the dividing stem cell (self renewal) and a partially differentiated progenitor cell which is committed to giving birth to differentiated progeny (92). The fate of all cells in these transit daughter lineages is terminal division arrest. Only stem cells have division capacity that lasts for the entire mammalian life span (93). It is through the mechanism of asymmetric stem cell kinetics that adult mammalian tissues are able to renew cells while maintaining a constant cell mass (94).

In vitro, however, asymmetric stem cell kinetics poses one of the greatest barriers in expanding adult stem cells in culture. According to James L Sherley (93), when tissue cells are explanted to culture, a given number of adult stem-cell-based tissue turnover units is obtained. Because the overall number of adult stem cells does not increase during asymmetric cell kinetics, the stem cell fraction decreases with time as transit cells accumulate and fill up culture vessels. After the initial transfer of cells to culture, successive dilutions of replete cultures eventually yield a culture dilution in which no stem cells are transferred. This event is soon followed by a complete replicative arrest of the culture when all transit cells complete terminal maturation and division arrest.

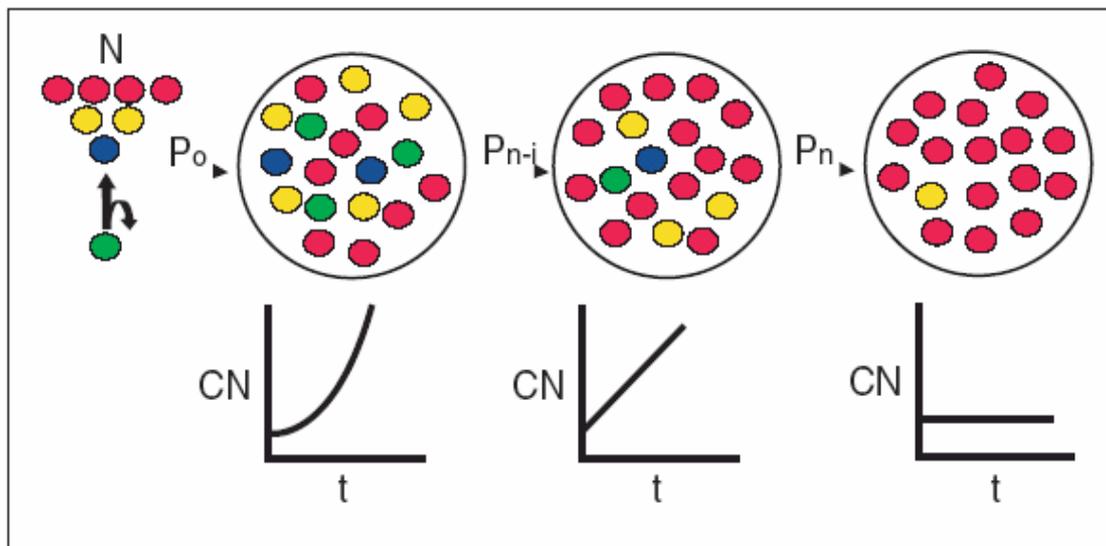


Figure 1.2: Schematic representation of the theory of asymmetric stem cell kinetics in culture. Green dots represent stem cells, blue and yellow dots differentiating transit cells and red dots terminally differentiated cells. Taken from: Sherley JL, “Asymmetric Cell Kinetics Genes: The Key to Expansion of Adult Stem Cells in Culture”, 2002, *Stem Cells*; 20:561-572 (93).

In order to circumvent this problem, Sherley et al have proposed the use of xanthosine, a nucleoside analogue which influences the cell cycle by intervening in p53-dependent regulation of guanine nucleotide biosynthesis (95). In specific, xanthosine has been reported to circumvent IMPDH downregulation by promoting guanine ribonucleotide formation via salvage pathways and thus prevent p53-induced asymmetric cell kinetics (95, 96). This way, xanthosine should induce asymmetrically cycling cells such as adult stem cells to cycle symmetrically with greater frequency and thus, instead of producing differentiated progeny to start propagating themselves (93).

By applying xanthosine to the culture media, Lee et al were able to clonally expand adult rat hepatic stem cell lines (97).

1.5 Aim of the present study

The aim of the present work was to investigate whether cells bearing stem-cell characteristics exist in the fully differentiated human thyroid gland. To address this question, the expression of markers characteristic either for pluripotent stem cells (Oct4) or multipotent endodermal stem cells (GATA-4, HNF4 α , AFP) were analysed in primary thyrocyte cell cultures obtained from nodular goiters after total thyroidectomy. Analysis of the above markers was also performed in two established thyroid carcinoma cell lines, HTh74 anaplastic thyroid carcinoma and HTC human follicular thyroid carcinoma, and in FRTL5 cells, a widely accepted model of differentiated thyroid cells.

Firstly, expression of stem cell marker DNA was analysed by means of reverse-transcription PCR after total mRNA isolation. PCR results were validated using restriction endonuclease experiments.

Secondly, expression of stem cell marker protein was analysed by means of immunocytochemical detection. For immunocytochemistry, thyroid cells were grown in slide flasks, for immunohistochemistry, serial tissue sections of nodular goiters were used.

Thirdly, stem cell marker-positive populations were further quantified using flow cytometry and isolated using fluorescence-activated cell sorting.

To analyse the timely distribution of stem cell marker signal, cells were subcultured for longer periods of time and cells at different passages were tested for the expression of stem cell marker mRNA by means of RT-PCR. In order to selectively increase the number of stem cells in culture according to the theory of asymmetric stem cell kinetics, cells were treated with the nucleoside analogue xanthosine.

To investigate the role of TSH in stem cell growth and differentiation, primary thyrocyte cultures were treated with increased concentrations of TSH for different periods of time and the expression of stem cell marker mRNA was analysed by RT-PCR.