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Lecture

The bioartificial thyroid: a biotechnological perspective in endocrine organ engineering for transplantation replacement*

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Abstract. A new concept for *ex situ* endocrine organ bioengineering is presented, focused on the realization of a human bioartificial thyroid gland. It is based on the theoretical assumption and experimental evidence that symmetries in geometrical coordinates of the thyroid tissue remain invariant with respect to developmental, physiological or pathophysiological transformations occuring in the gland architecture. This *topological arrangement* is dependent upon physical connections established between cells, cell adhesion molecules and extracellular matrix, leading to the view that the thyroid parenchyma behaves like a deformable "putty", moulded onto an elastic stromal/vascular scaffold (SVS) dictating the final morphology of the gland. In particular, we have raised the idea that the geometry of the SVS *per se* provides pivotal epigenetic information to address the genetically-programmed, thyrocyte and endothelial/vascular proliferation and differentiation towards a functionally mature gland, *making organ form a pre-requirement for organ function*. A number of experimental approaches are explored to obtain a reliable replica of a human thyroid SVS, and an informatic simulation is designed based on fractal growth of the thyroid intraparenchymal arterial tree. Various tissue-compatible and degradable synthetic or biomimetic polymers are discussed to act as a template of the thyroid SVS, onto which to co-seed autologous human thyrocyte (TPC) and endothelial/vascular (EVPC) progenitor cells. Harvest and expansion of both TPC and EVPC in primary culture are considered, with specific attention to the selection of normal thyrocytes growing at a satisfactory rate to colonize the synthetic matrix. In addition, both *in vitro* and *in vivo* techniques to authenticate TPC and EVPC lineage differentiation are reviewed, including immunocytochemistry, reverse trascriptase-polymerase chain reaction, flow cytomery and proteomics. Finally, analysis of viability of the thyroid construct following implantation in animal hosts is proposed, with the intent to obtain a bioartificial thyroid gland morphologically and functionally adequate for transplantation. We believe that the biotechnological scenario proposed herein may provide a template to construct other, more complex and clinically-relevant bioartificial endocrine organs *ex situ*, such as human pancreatic islets and the liver, and perhaps a new approach to brain bioengineering. (www.actabiomedica.it)

Key words: Bioengineering, biomaterial, biotechnology, scaffold, thyroid, endothelial cell, stroma, thyroid vessel, transplantation, thyroid papillary cancer

* This work is dedicated to the memory of Giorgio Toni and Angelo Bairati, two pioneers in the study of cellular mechanisms regulating the interplay between morphology and physiology during embryonic development and adult life

A piece of silk veil […] was placed on a cover slip and moistened with a drop of plasma. A fragment of tissue was deposited in the center […]. As the silk veil acted as a skeleton for the plasmatic jelly, the original fragment and the new tissue cells, the culture could he handled […] without deformation of the cells […], the growth was contimuous , and […] the cells could use the silk threads as a support.

Alexis Carrel

Journal of Experimental Medicine 15, 516-528, 1912

Introduction

More than 50 years ago Angelo Bairati and Giorgio Toni, studying the stromal scaffold and related vascular tree of the liver, adrenals, gonads and spinal cord during pre-natal development (1, 2) and adult life in lower vertebrates and man (3, 4) stated: *"The fibrillar structure of the stroma acts as a scaffolding to favor harmonic development of an organ during morphogenesis"* (1, 3) and *"in organs that quickly grow in volume without differentiation, a small amount of stomal fibrils are formed; in contrast, organs that grow slowly but simultaneously differentiate have a much richer and differentiated stroma"* (2). A substantial amount of data was reported to support these conclusions, among which the most important was the observation that a rich fibrillary layer tangential to the primitive caudal neural tube can be found in the early human fetus (Figure 1). This finding was confirmed by Lofberg and Ahlfors twenty-eigth years later in the amphibian embryo (5).

The foundation for these observations derived from earlier studies performed by Angelo Bairati's and Giorgio Toni's mentor, Oliviero Mario Olivo, pupil of the "Nobel in memoriam" proposed, Italian morphologist, Giuseppe Levi (6), a pioneer in tissue culture methods (7). Levi's technique was perfected by Olivo in 1928-29, while working in the laboratory of the Nobel Laureate, Alexis Carrel, at the Rockefeller Institute for Medical Research in New York, to study chick embryonic heart (8, 9), thus providing Levi's group with innovative principles to grow cells outside of their native environment. These principles will be later exploited by Levis's three most renown pupils, the Italian Nobel Laureates Rita Levi-Montalcini, Renato Dulbecco and Salvador Luria, to conduct their research on neural growth factors, viruses and bacteria (10).

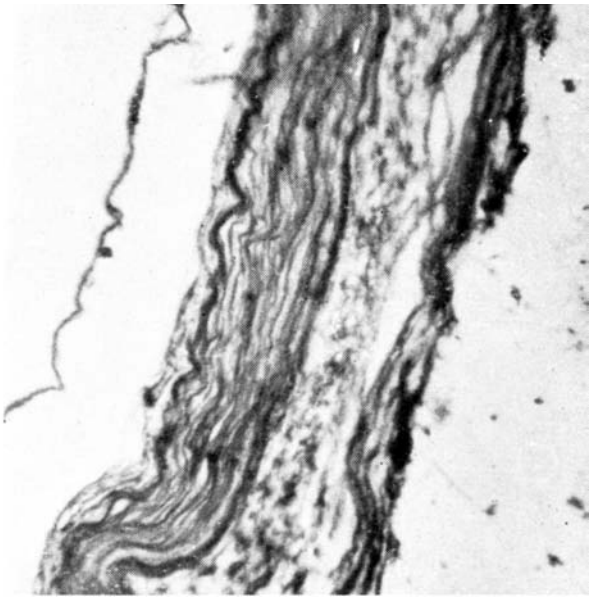

Figure 1. Histologic section (Urechia-Nagy method) showing the perineural (central part of the image) and subepidermal (right side of the image) fibrillary lamina covering the dorsal aspect of the spinal cord in an approximately 9 weeks human fetus (CR length = 32 mm, original magnification x 145), as reported in the 1950's work of Giorgio Toni on stromal scaffolds during human development (from ref. 2, with permission). This arrangement of perineural fibers is surprisingly similar to that observed during the same developmental stage for predecessor neurons and their processes in the basal and dorsal human telencephalon (24), raising the possibility that extracellular fiber scaffolds may guide and orient neuroblast trajectories

Although the investigations on stromal and vascular scaffolds conducted by Angelo Bairati and Giorgio Toni were never given due recognition by the International scientific community, they have recently received posthumous attention (11). Nevertheless, these investigators anticipated one of the most debated concepts in modern molecular embryology, namely the epigenetic role of the three-dimensional (3D) arrangement of stroma and vessels to dictate the shape and differentiation stage of prenatal and adult organs (12, 13). This concept is to become of paramount importance for endocrine tissue engineering (14, 15).

The last three decades have witnessed an exponential growth in the importance of the role played by the extracellular matrix (EMC) and different classes of adhesion molecules in the regulation of developing, differentiating and regenerating tissues and organs (16-21). In particular, evidence has accumulated that homeobox gene expression in the developing vertebrate brain is involved in local polyclonal lineage restriction induced by the 3D arrangement of extraneuronal components, including reticular fibers and extrinsic axon bundles (22, 23). Similarly, topographic arrangement of primordial cortical neurons in man and differentiation of neural crest precursors in lower vertebrates have been shown to depend upon 3D features of their trailing processess, namely neural cell-adhesion molecules and ECM fibrillary proteins (12, 24) This has brought further momentum to the concept that extracellular scaffolds may act as templates from which organ morphology takes place, even for structures like the brain (25, 26). Furthermore, it has been shown that growing blood vessels orchestrate 3D development of parenchyma in rodent pancreas (27) and thyroid (28), emphasizing the role of vascular architecture in vertebrate endocrine organ shape and forcasting a more general theory in glandular organogenesis (29) Finally, the role played by the 3D position reached by each cell during embryonic growth (positional information) to achieve a specific differentiation lineage (30, 31) has been extended to the epigenetic influence of environmental geometry on cell activity (32-34), including the role of gravity (35), by acting as a deformating influence on the architecture of cell growth trajectories (36). By the end of the 20th century, a number of new theories were proposed on the role played by interactions between cell surface, ECM and extracellular spaces to bring about 3D morphology as a pre-requirement for organ physiological activity (34, 37). Among others, cell tensegrity (38), fluid mosaic interactions (39), topobiology (40) and wiring versus volume neural transmission (41, 42) have gained acceptance and experimental support, including our own observations on tensegrital structure of the human cornea (43), topobiological organization of the vertebrate neuroendocrine system (44), and computational power of transmission lines in the mammalian and human hypothalamus depending on their 3D architecture (see R. Toni et al., this Issue, and 45).

The culmination of these data has led to the belief that the shape of an organ may be the primary cause of its physiological activity, and not the consequence (46, 47), at least in multicellular organisms set into a Darwinian perspective of life evolution (48). Thus, new light can now be shed over a long-standing debate and a partially misleading concept in biomedicine, namely that organ shape is the consequence of its global, physiological performance (49). Indeed, current theoretical and experimental evidence, including the ability of stem cells to acquire different phenotypes depending on the geometry of their host environment (50-54), argue that it is the 3D architecture of the organ that dictates its future function (37, 55).

Current and new concepts in endocrine organ bioengennering for human transplantation with special reference to the thyroid gland

In recent years, experimental approaches in the field of endocrine tissue engineering for transplantation have been focused on assumptions different from those mentioned above. Studies have been conducted in animal models and man to restore abnormal endocrine function by using isolated, autologous and either immunocompetent or immunoprotected, allogenic endocrine cells (56). In humans, pancreatic islets have been both autotransplanted following pancreatectomy for chronic pancreatitis or benign pancreatic tumors (57), and allotransplanted into the liver, directly or after microincapsulation as bioartificial organ grafts to treat diabetes type I and selected type 2 diabetics (58, 59). Unfortuntaly, results have been modest with only few patients achieving insulin independence due to exhaustion of the transplanted cells, despite long-term immunosuppresion and a number of other treatments (59, 60). Of note, all described studies were performed following specific isolation, purification and harvest of apparently large quantities of islet cells from different donors (> 6000 islets equivalents / kg recipient body weight). In contrast, semipurified islets derived from a single donor achieved a long survival time (61).

While improved survival has been attributed to reduced antigenicity of the implanted islet cells, we belive that the partial retention of the original 3D structure of the semipurified islets played a critical role. Indeed, we have shown that anterior pituitary cells, isolated from adult male rats and seeded in primary monolayer culture have limited life span (62), partly due to apoptotic involution from disruption of the original 3D tissue arrangement (63, 64). This possibility is supported by the evidence that, in monolayer culture, elements that are undergoing active growth attempt to restore the original 3D tissue geometry by restricting the number of quiescent cells able to enter the cell cycle via intercellular contacts (62) (Figure 2).

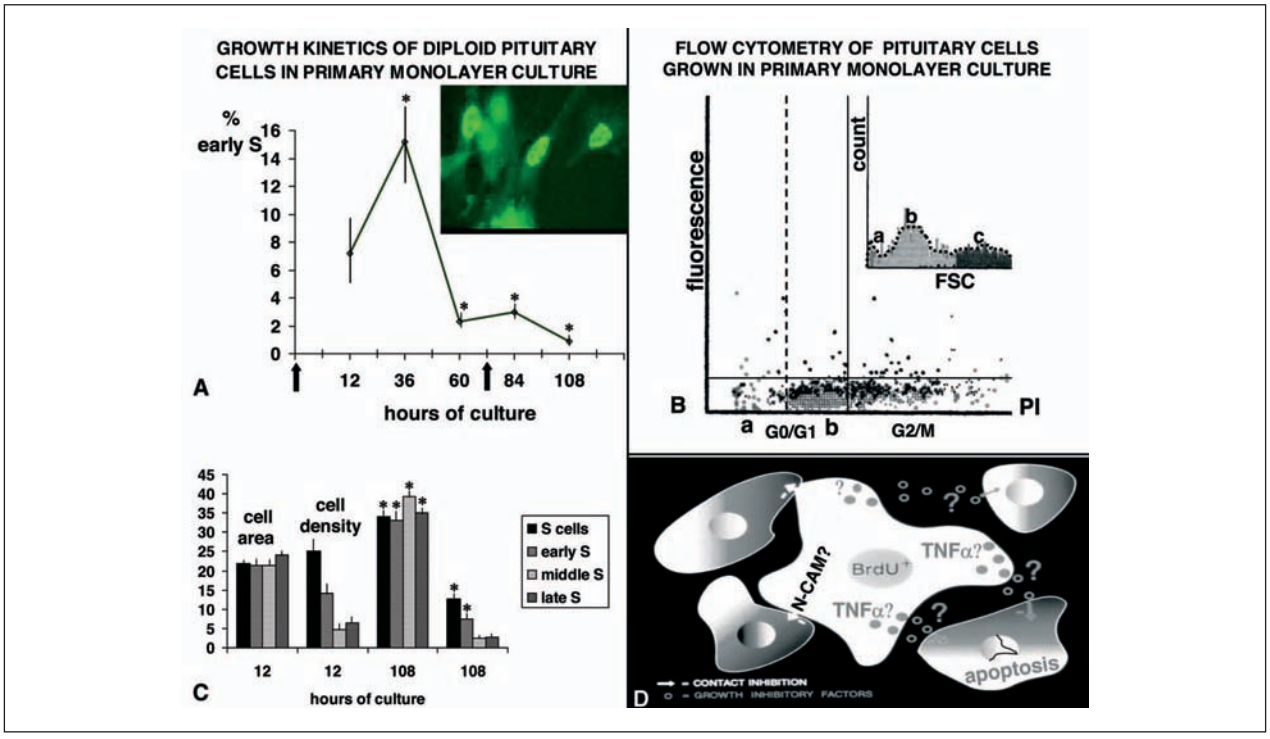

Figure 2. Studies on primary monolayer cultures of two-month old male, rat anterior pituitary cells. A) Viable cells were seeded at 40 000/cm^2; after 72 hours (resting period), a first change of fresh culture medium, containing 10% rat serum, was made and cells incubated for additional 2 hours with 15 μM bromodeoxyuridine (BrdU) / 1.5 μM 5-fluoro-2-deoxyuridine, to label replication sites in the interphase cell nucleus (end of incubation time between 10.00 – 11.00 a.m.). For longer periods of growth a second medium change occurred 72 hours later; both changes are indicated on the graph absissa by vertical arrows. After fixation, cells were procesed using an indirect immunofluorescence method (inset shows BrdU-immunoreactive [IR], pituitary cells in culture), and studied by bivariate DNA/BrdU analysis using flow cytomery, as previously described (62). Note that after an intial growth spurt, a statistically significant reduction occurs to early S-phase cells for the duration of the culture, despite addition of rat serum, suggesting that mechanisms other than growth factor deficiency are involved in regulating long-term, pituitary cell replication in culture. Values represent the mean ± SD of 3-6 different cultures for each time point. Statistical calculations were performed after arcsin transformation of percentage values; B) Typical cytometric profile of pitutary cells in primary monolayer culture, 108 hours after the resting period. Note that besides diploid (G0/G1) and tetraploid (G2/M) cells, an hypodiploid pool (a) is present, showing a Forward Scatter profile lower than that of diploid (b) and tetraploid elements (c) as expected to occur in apoptotis (196); C) Distribution of fractional area (10^{-2} μm^2) and density (cells/mm^2) of BrdU-IR pituitary cells in monolayer culture, as determined by morphometric analysis. Values represent the mean ± SD of 2 different cultures for each time point. Note that a statistically significant, progressive increase occurs in the size of all proliferating elements, accompanied by a reduction in density of early S-phase cells. This suggests that replication rate is regulated by BrdU-IR elements, possibly acting on committed cells in the quiescent pool G0/G1 via inhibitory influences, including the TNFα / TNFα receptor system (197) and the cell-adhesion molecules / integrins signalling (64, 198), as schematically depicted in D). PI = propidium iodide, TNF α = tumor necrosis factor α; N-CAM = Neural-Cell adhesion Molecule, * = $p < 0.05$ (from Mosca S, Zamai L, Martelli AM, Vitale M, Toni R, 1991-1994, unpublished results)

This mechanism also occurs during embryonic development of the neural tube (65) and pituitary (66).

To summarize, loss of the mature 3D pattern in endocrine epithelial tissues in culture, like the anterior pituitary and pancreatic islets, may ultimately lead to an autodestructive process, i.e. apoptosis (67). Thus, it is possible that the explanation for the overall lack of success in islet cell transplantation is that pancreatic endocrine cells cannot survive and function for long as randomly dispersed elements, particularly when injected into structures that have a 3D structure completely different from its natural organ. In addition, current procedures for islet delivery, using venous routes, result in unnatural "clumps" of endocrine elements without specific spatial arragement in the hosting parenchyma, and are deprived of any associations with their original vessels (59, 68). It is possible, therefore, that ECM-islet interaction may be a critical factor for graft survival (69).

In contrast to pancreatic cells, other endocrine glands have received much less attention. In man, only anecdotal reports supports the ability to transplant allogenic viable, adrenal tissue (70**).** Similarly, only few succesful allotransplants of parathyroid glands have been reported (71), although parathyroid gland autotransplantation is a well established procedure to avoid surgically-acquired hypoparathyroidism (72). Finally, some reports of even long-term, succesful autotransplants of thyroid gland fragments in human subjects have been published (73, 74). However, the ready availability of synthetic thyroid hormone has hindered further development of clinical investigations on the feasibility of either thyroid auto- or allotransplants in man.

Undoubtly, endocrine tissues offer a particularly challenging scenario for any attempt at biotechnological reproduction due to their anatomical complexity, cellular heterogeneity and integrated network of extrinsic and intrinsic communication signals (44). Very recently, however, entire organs including the urethra, urinary bladder, penile cavernous bodies, uterus, vagina and kidney have been engineered and reconstructed *ex situ*, based *on the dependence of organ function from organ shape* (75) to repair congenital and acquired defects of the human genitourinary tract (76). These achievements have encouraged us to explore the possibility of developing a bioartificial, implantable construct made with human autologous, thyrocyte and endothelial progenitor cells grown onto a scaffold of degradabale biomaterial, for potential clinical application in thyroidectomized individuals. Although we are aware that in contrast to genitourinary organs, such a biotechnological realization might not represent a priority for the current health market due to the availability of thyroid hormone therapy at a relatively low cost nevertheless we have good reasons to pursue it. We believe undertaking this approach may provide the basis from which to realize other more structurally complex, bioartificial endocrine and parenchymal organs of greater clinical importance such as pancreatic islets to cure diabetes mellitus, the liver to treat intractable hepatic insufficieny or perphas parts of the brain (for the concept of bionic structures in the brain, see R. Toni et al, this Issue).

Historical and theoretical bases for bioengineering of the bioartificial thyroid

By accepting the idea that *form makes function* a long scientific tradition is reincarnated. At the end of the 15th century, Leonardo da Vinci was an authoritative advocate of this concept when in his 1505 AD. *Codice sul Volo degli Uccelli* (Codex on Bird's Flight), he attempted to reproduce bird flight by designing a flying apparatus shaped as natural wings, including points of reduced weight and air resistance corresponding to pneumatic bones and joints (77). Modern aerospace engineering has relied more upon the physical laws of air flow, object movement, propeller technology and material science than on the mere external morphology of wings, fuselage and tail. Nevertheless, beyond an apparent theoretical and philosophical ingenuity in adhering to the general principle that form makes function (78), the establishment of specific geometry in the scaffold of any aircraft is a fundamental concept for its flight performance (79).

Similarly, we believe that a specific geometry and architecture of the stromal and vascular scaffold may be an inescapable requirement to reproduce the mature morphology of a human thyroid gland, and hence to replicate its physiological activity. The concept is consistent with studies on the invertebrate and verte-

brate morphogenesis in which *pattern controls growth* and not vice versa (80, 81). In fact we expect that the scaffold geometry *per se*, primarily the connective stroma, will provide critical epigenetic inputs (34), corresponding to positional information of a topobiological nature (30, 40) and capable of promoting the growth and differentiation of seeded autologous cells (likely thyroid and endothelial progenitors) towards a parenchymal and vascular morphology (and related differentiation state) equivalent to that of a native thyroid gland. As a result, a functionally mature organ should be obtained.

To this purpose, it is instructive to note that during human embryonic development, when the thyroid bud is starting to form, folliculogenesis prevails over stromal formation. In contrast, during fetal development, when thyroid macroscopic morphology is well established, a direct relationship is found between functional follicular differentiation, but not thyroid epithelial mass, and stromal expansion (82) (Figure 3). This supports the conclusion, admirably put forth by Giorgio Toni in his 1950 paper on developing endocrine tissues in humans (2), that as long as endocrine epithelial cells (like thyrocytes) proliferate, the connective and fibrillary scaffold acts as a guidance for growth trajectories. In summary, all these observations suggest the existence of a topobiological interplay where stromal bundles act as lines of force of a "morphogenetic field" (83) or "topobiological state space" (47) to permit the 3D arrangement of nascent vessels, follicles, and their pertinent function via epigenetic inputs to homeobox and histodifferentiation genes.

A number of recent observations in the adult thyroid seem to support this assumption. In preliminary studies of the thyroid intraparenchymal arterial (and related nervous) distribution we have suggested that the vascular 3D architecture dictates the macroscopic shape of the thyroid lobe (Figure 4). Specifically, va-

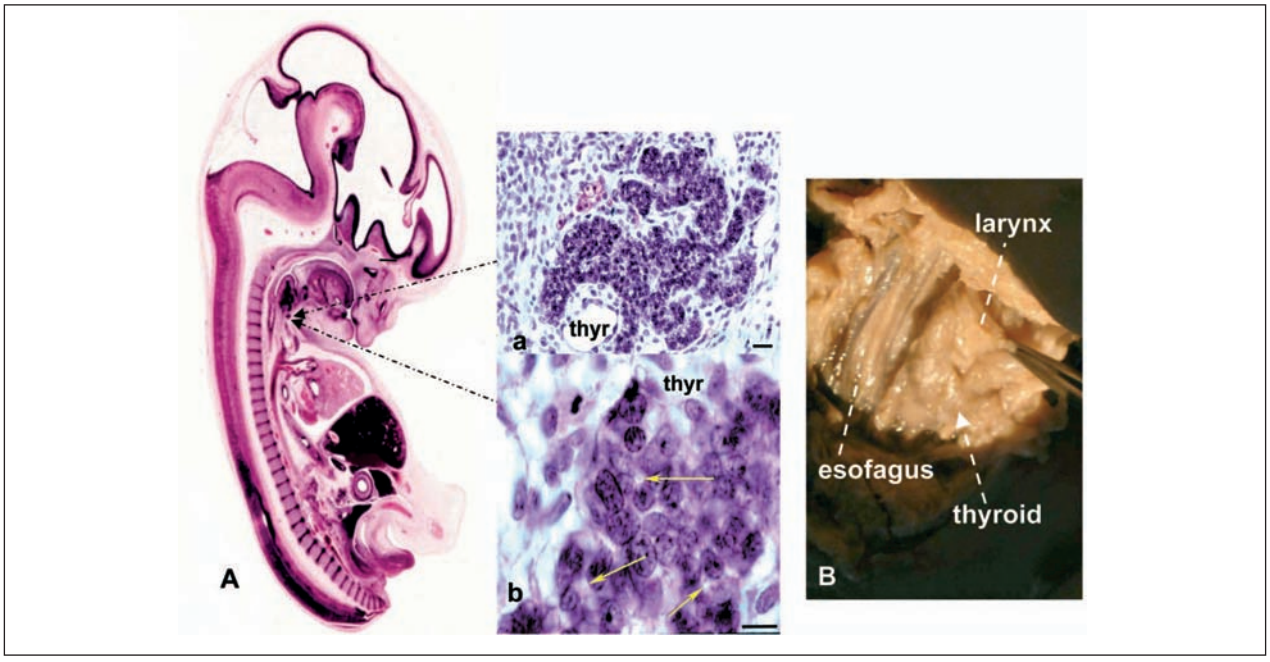

Figure 3. A) Light microscopic view of a sagittal section of a human embryo at 7 weeks of development (crown-rump length = 20 mm; hematoxylin/eosin staining). At this prefollicular stage the thyroid gland is deprived of its mature morphology and the thyroid bud (thyr) exhibits only a minimal amount of stromal tissue (a), although actively-replicating thyrocytes presents several intracellular canaliculi (arrows in b), later fusing to convey secretory material into a *de novo*-formed follicular cavity. Bars: a = 20 μm; b = 5 μm (from ref. 44, with permission, partly modified); B) Macroscopic dissection of the neck region in a 5 month-old human fetus (from the collection of the Museum of Human Anatomy at the University of Parma, Italy). At this developmental stage when the mature morphlogy of the gland is established, a relationship can be found between functional follicular differentiation and an increase in stromal tissue (82)

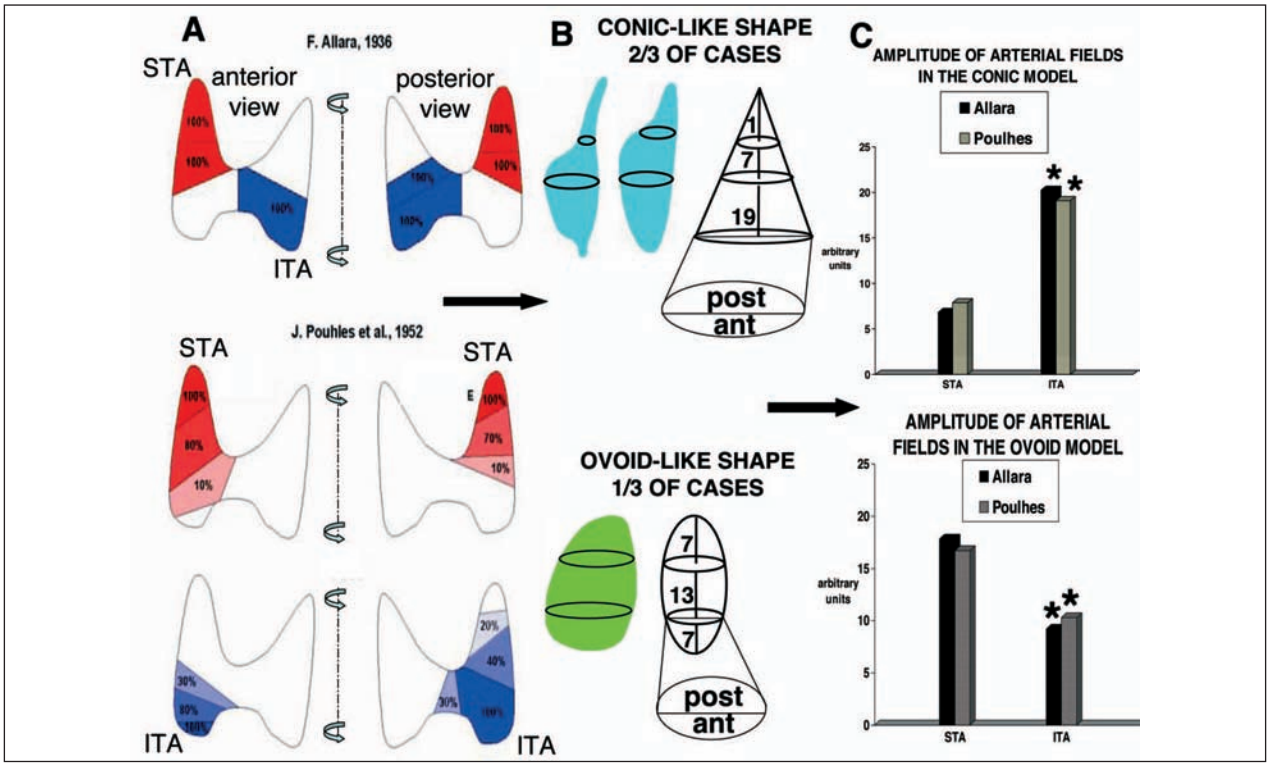

Figure 4. Relationship between thyroid lobe geometry (macroscopic shape) and intraparenchymal vascular extension in humans. A) Graphic representation of data on the intralobar distribution of the superior (STA) and inferior (ITA) thyroid arteries, as determined from descriptions reported in two independent studies (see ref. 199, 200); B) Since in a normal, euthyroid population of adults the thyroid lobe morphology can vary from a conic-like to an ovoid-like shape (201), we have conducted a theoretical study to determine whether differences occur in the extension of both the STA and ITA territories, following approximation of lobe morphology to that of a cone or ellipsoidal ovoid. In a first step, the thyroid lobe (histmus excluded) has been divided into thirds having equal cranial-caudal length by a transversal plane. Each third has then been futher divided in half by a frontal plane of equal anterior-posterior diameter and, when necessary, each half in smaller, irregular volumetric fractions each encompassing the distribution of STA and ITA territories, as shown in A). In a second step, we have expressed the extension of STA and ITA territories in any portion (halfs and/or smaller irregular fractions) of each third in terms of percentage of the data in A), considered as pure numbers. In a third step, we have transformed these percentages as values proportional to those indicating the ratio between volumes in each third of a cone or ellipsoidal ovoid. In particular, the volumes of thirds remain in a 1:7:19 and 7:13:7 proportions when referred to a cone or ellipsoidal ovoid, respectively (courtesy of Dr. Michele Caffo, INFN, Dept. of Physics, University of Bologna, Italy). Finally, we have obtained the extension of STA and ITA territories in an entire, conic-like or ovoid-like lobe by summing all values referred to any portion (halfs and/or smaller irregular fractions) of each third. Statistical differences between STA and ITA lobar extensions in each regular geometric model were evaluated by χ^2 test, and considered significant if $p < 0.05$. Note that in the conic model ITA territory prevails over that of STA, whereas in the ovoid model the opposite is true. Post = posterior; ant = anterior, * = $p < 0.05$

scular, nervous, and parenchymal structures maintain constant reciprocal and geometrical relationships even if transformations occur to bring about loss of their metrical and/or projective properties (84) such as in 3D deformations in the vascular scaffold (for a review on the symmetries in human organs see AE Ermolenko and EA Perepada, this Issue). Confirmation of this concept is indirectly given by the result of the histometric analysis of different tissue components in normal adult, human thyroids, showing that the stroma, vessels and thyrocytes maitain constant quantitative relationships throughout lifespan, despite changes in thyroid volume (85). The consequence is a *topological arrangement* of the thyroid tissue. This topological arrangement suggests that the arterial variability (e.g. lack of a specific artery) may modify both thyroid morphology and its functional regulation. Vascular modifications, in fact, are expected to bring about mo-

difications in both parenchymal architecture and autonomic nerve supply accompaning each vascular branch and in some instance resulting in thyroid dysfuntion of clinical relevance (86-88).

Similarly, we believe that the above mentioned topological relationships also apply to hyperfunctioning thyroid nodules whose growth within the gland may be dictated by changes in the volume of the glandular portions where they develop (Figure 5). Changes in thyroid tissue volume, in fact, imply changes in its architecture, leading to the speculation that the "adenomatous potentiality" of a tissue area may also be linked to the geometry of the thyroid lobe (89). In summary, we think that the growth characteristics of the different histologic components of the thyroid tissue (epithelial, mesenchymal, endothelial and nervous elements) are much more conditioned by their 3D architecure than that commonly believed (90, 91). Therefore implications can be made for a dependence of thyrocyte growth on its architectural and geometrical relationships within the stromal scaffold and related vascular and nervous elements.

Based on all this, it would be of paramount importance to know the macroscopic, 3D architecture and geometry of the stromal scaffold in normal adult, human thyroids to design a functional bioartificial

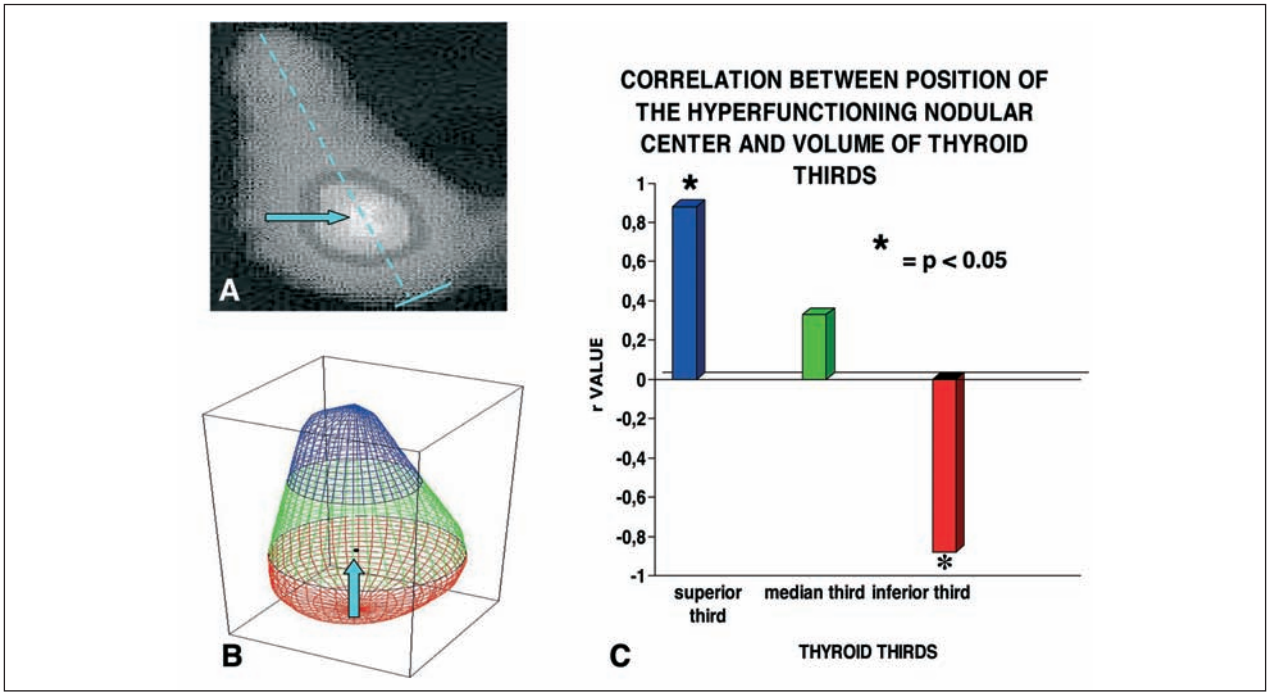

Figure 5. The relationship between geometry of the thyroid lobe and functional activity of the thyroid tissue. A) We have analyzed the Tc ^{99} scintigraphic profiles of 10 hyperthyroid subjects (TSH < 0.05 μU/ml, 3 males, 7 females, mean age = 59 ± 2 years) with hyperfunctioning, solitary thyroid adenoma. In a first step, the position of the hot nodule has been assimilated to that of its region of highest radionuclide captation or "functional center" (arrow), and this identified as the geometrical center of gravity of the thyroid lobe shape, obtained by choosing image points with pixel intensity higher than a *ad hoc* selected, captation threshold. In a second step, the position within the thyroid of the functional center (and thus of the entire hot nodule) has been determined as its projection onto the maximal longitudinal diameter of the lobe, here depicted as a dotted line; B) After transformation of the scintigraphic image into a pixel matrix of grey levels, a recently developed algorithm for 3 D reconstruction of thyroid volume from a bidimensional image has been applied (see Spaletta G, this volume), and both the thyroid lobe morphology and volume have been obtained. Each lobe, then, has been divided in thirds, here depicted with different colors, following the procedure described in the legend of Figure 4, the volume of each third computed, and the center of gravity of the hot nodule represented as a dense point (arrow) within a given third; C) A statistically significant correlation (non-parametric r values) has been found between position of the nodular functional center and volume of the superior and inferior thyroid thirds. This suggests that the hyperfunctional core keeps a constant, reciprocal association with the sorrounding thyroid parenchyma, raising the possibility for the existence of a field of adenomatous potentiality linked to the lobe geometry (macroscopic shape)

thyroid gland. Unfortunatly, since the majority of studies on human thyroid stroma have been focused on its developmental, ultrastructural, biochemical and pathological features (82, 92-95), we are not aware of clear data on either its macroscopic or microscopic 3D arrangement. However, the anatomical tradition of vascular casts seems to offer an unexpected resource to resolve this problem. Beginning in the 2nd half of the 17th century, the Dutch anatomista Jan Swammerdam and Fredrik Ruysch developed the now widely known technique of injecting blood vessels with solidifying insoluble susbtances, often coloured, to depict the 3D vasculare tree of body organs (96). Glands, and in particular the thyroid, were of great interest to Ruysch who attributed the function of these organs to the pattern of arrangement of their blood vessels (97, 98), anticipating modern concepts on the importance of the vascular supply for thyroid hormone delivery rate (99, 100). We believe that vascular casts of intraparenchymal thyroid arteries and veins represent a sort of "photographic negative" of the 3D thyroid stromal scaffold, acting as a physical support for the vessels coursing throughout the parenchyma. Thus, vascular casts can be considered as a natural replica of the stromal scaffold, otherwise remaning elusive in its basic 3D features, and might be used to infer its geometrical properties, necessary for the realization of a biocompatible synthetic replica. Additional information could be obtained from angiographic techniques, as detailed below.

Vascular casts, digital multilayer angiotomography and conventional angiography to realize the template of a biocompatible stromal scaffold of the adult human thyroid

In collaboration with Giovanni Mazzotti at the University of Bologna, and Vincent Delmas, at the Department of Human Anatomy of the 5th University of Paris, we have initiated a prospective study of the anatomical organization of the vasculature of endocrine glands, on caucasian cadavers of the different age, sex and body mass index (BMI). The intent is to obtain 3D casts of the arterial and venous supply of the adult, human thyroid gland. As a result, it should be possible to identify common features of the thyroid, vascular, 3D, macroscopic architecture, and prepare a template of the corresponding stromal scaffold (here coined as stromal/vascular scaffold or SVS).

A preliminary ultrasound screening of the cadaveric thyroid texture has been considered to exclude both nodular growth (101) and abnormal thyroid volumes (102) that could alter the intraglandular vascular architecture (103). In fact, we have shown that ultrasound features of both nodular lesions and the vascular anatomy may be succesfully detected in post-mortem, human parenchymal organs like the liver (Figure 6), kidney and adrenals (104-107). In addition, a number of technical variables have been taken into account to achieve optimal resin injections as a result of previous experience on vascular casts of the liver (Figure 7), pancreas and adrenals (108, 109). All these factors may modify the natural 3D assembly of the intragladular vasculature when compared to *in vivo* angiographic images (110).

In a second prospective study, developed at the Radiology Department of the Scientific Foundation and Clinic "G.B. Morgagni" in Catania, Italy, we have begun to exploit the imaging power of digital multilayer angiotomography to visualize *in vivo* the finest vascular arrangement of the human thyroid in European caucasian patients, referred for study of neck vessels (Figure 8). We believe that the analysis of digital matrixes leading to image formation will provide data to build an *in vivo* model of the microstructure of the SVS that can be compared with post-mortem models obtained by corrosion casts studies.

Finally, we are considering the possibility of using conventional thyroid angiograms of European caucasians (Italians), studied for suspected vascular problems in the subclavian and/or carotid arteries, as in previous investigations on the anatomy of thyroid arteries (87, 88). An estimate of thyroid volume of each subject (89, see also G. Spaletta this Issue) may provide a further criterion to select normal individuals based on reference values in a caucasian sample of euthyroid cases (102). Evaluation of parenchymal phases of each angiogram might offer data to create an additional *in vivo* model of SVS, to be compared with those obtained by injection casts and digital angiotomographic studies.

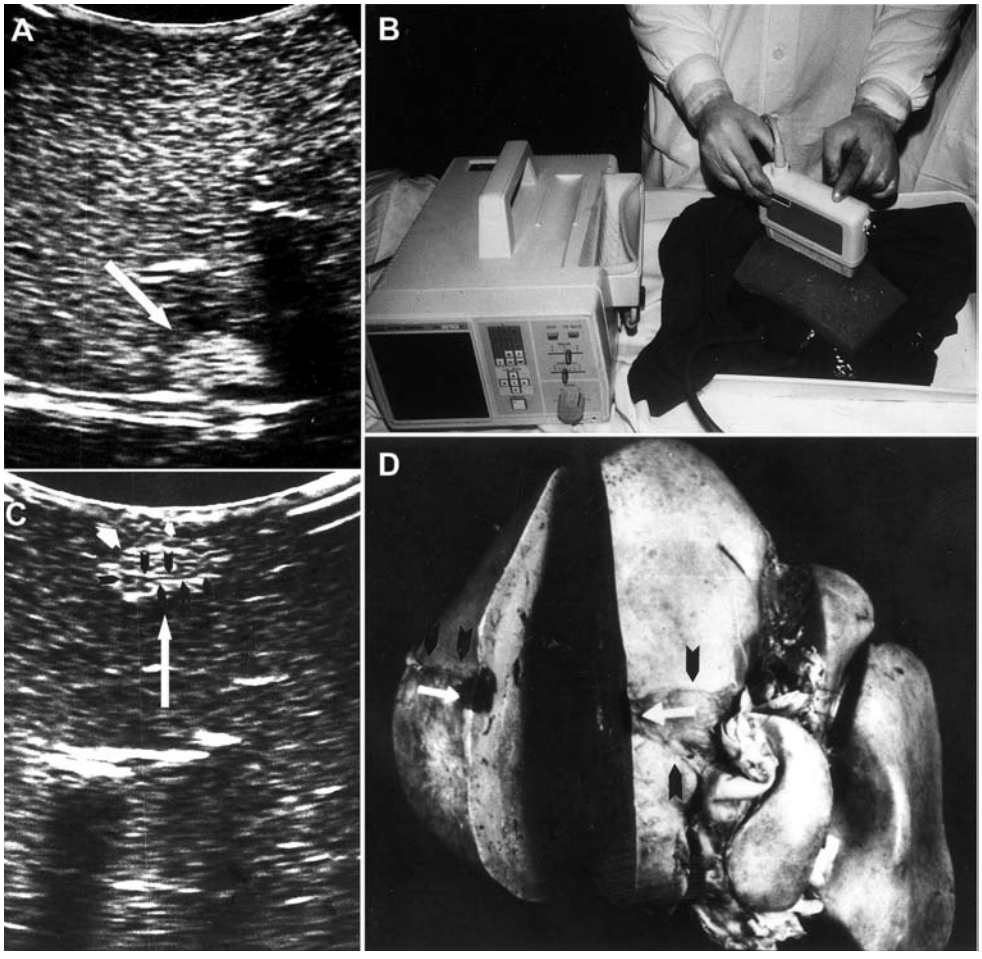

Figure 6. Evidence that ultrasound features of nodular lesions may be successfully detected in post-mortem, human parenchymal organs. A) Subcostal scan obtained during a routine ultrasonographic examination of the liver using a 5 MHz convex probe (Ansaldo AUC 940 real-time scanner). A small hyperechoic nodule (white arrow) is visible in the right lobe adjacent to the visceral surface of the gland. Note fine parallel echoes arranged horizontally and absence of a peripheral hypoechoic halo; B) Sudden death of the patient occurred few months later. The liver, removed from the cadaver, was subjected to ultrasonographic examination, using either an Aloka 210 real-time scanner with a 3.5 MHz linear array-type probe (depicted in the image) or an Ansaldo scanner with 5 MHz convex probe. A layer of *kitekò* material was placed between the transducer and the liver to improve image resolution; C) Parasagittal scan with a 5 MHz convex probe of the isolated liver obtained from the visceral surface of the right lobe. Note a hyperechoic lesion (long white arrow) made up of fine parallel echoes arranged horizontally and forming the wall of internal anechoic, vascular channels (small black arrows). The ultrasonographic features of the lesions are identical to those in A). A hyperechoic bundle (white arrowheads) connects the lesion to the visceral surface of the liver; D) After perfusion fixation of the liver, a parasagittal cut was made on the visceral surface of its right lobe. An hypervascular lesion or hemanagioma was found within the gland parenchyma (white arrows), superficially covered by and in anatomical connection with the gastroduodenal legament (black arrows) (From Toni R, Mosca S, Favero L, Grigioni W, Bolondi L, 1988, unpublished results)

Principles for a fractal model of stromal/vascular scaffold of the human thyroid

While acquiring a suitable archive of the vascular distribution of the thyroid gland, we have also initiated a feasibility study utilizing informatic simulation of an average SVS of the normal adult thyroid gland (111) to construct a synthetic replica. In particular, we are trying to establish whether a fractal nature exists for the intraparenchymal, thyroid arterial branching in humans (for a review on fractal systems see A. Strumia, this Issue). Only one or few fractal rules may be

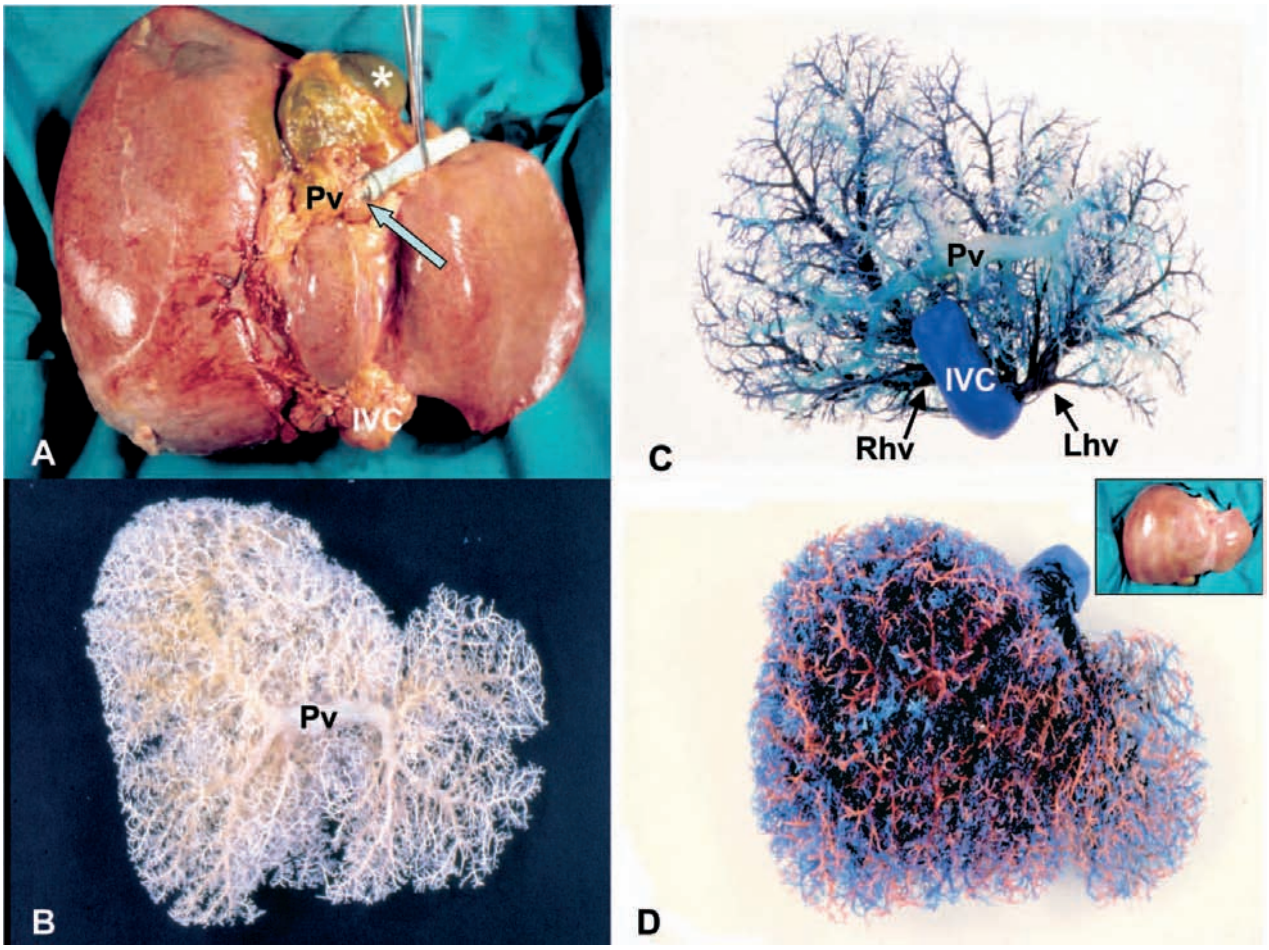

Figure 7. Results of injection techniques to obtain casts of the vascular scaffold in human, post-mortem parenchymal organs, like the liver. A) After removal from the cadaver, the liver is injected with coloured, synthetic resins through a cannula inserted either into the portal (arrow) or hepatic veins. A description of the technique is given in ref. 108; B) Vascular cast of the portal vein distribution, seen from the visceral surface of the organ, as depicted in A). Note that use of highly diluted resin allows for injection of the finest venous collaterals; C) Vascular cast of both the portal (light blue) and hepatic (dark blue) veins, seen from the visceral surface of the organ, as depicted in A). Note that use of less diluted resin than in B) allows for detection of only main venous branches; D) Vascular cast of both the portal (red) and hepatic (blu) veins, seen from the diaphragmatic surface of the organ, as depicted in the inset. Note that use of a more diluted resin than in C) allows for simultaneous identification of the finest branches for both venous systems. RL = right lobe, LL = left lobe; Pv = portal vein; IVC = inferior vena cava; Rhv = right hepatic vein: Lhv = left hepatic vein; * = gall bladder (from Favero L, Mosca S, Toni R, 1985-1987, unpublished results)

sufficient to describe even complex cell behaviors, like the epithelium-stroma interaction (112), suggesting that a fractal branching model of SVS might reasonably reflect our hypothesis that SVS acts as basic driving force for epithelial cell growth trajectories. Furthermore, vascular trees exhibit some constant geometrical relations, typical of fractals. These were explored at the end of the 19th century by the German embryologists Wilhelm Roux, who studied the relation between size of vessels and their bifurcation angle. His results layed groundwork for calculation of the stem / branch diameter relation, and development of the diameter exponent Δ, that indicates the scale factor for vessel caliber in a branching vascular tree (113). Finally, recent studies have shown that many blood vessels in the human body display a branching geometry that has fractal structure (114), including coronary, pulmonary, renal and retinal vascular trees

(115, 116). Although these trees do not extend over an infinite range as pure mathematical fractals do nevetheless a fractal element can be detected between the scales of the different branches (117). For these vascular systems, optimal ratios of a number of morphological parameters including calibers, vessel length, and branching angles have to be computed. After optimization of these measures, topologically appropriate reconstructions of the vascular tree can be obtained retaining, at least on a statistical basis, some physiological features of the original arrangement, like the regional blood flow distribution (115). It is, therefore, important for fractal simulation of the thyroid SVS to determine optimal measures for these parameters.

Using a recently developed algorithm for 3D reconstruction of the thyroid lobe from a bidimensional scintigraphic image (see G. Spaletta G., this Issue), the origin of 2^{nd} order thyroid arteries was superimposed on the surface of the 3D reconstructed lobe. Distribution of arterial origins on the lobe profile and knowledge of arterial calibers from 2^{nd} to 4^{th} order arteries were obtained from thyroid anatomical diagrams of Major RH (118) and Papadatos D (119). Then, a fractal growth inside the empty volume of the lobe was imposed, up to the 4^{th} order arteries. To achieve this result, basic relations of a fractal tree were computed, including mean length of 2^{nd}, 3^{rd} and 4^{th} order arteries. Assumptions were then made for a non-binary growth, such as a current branch is *s* times shorter than the previous branch (scale factor) and θ^1, θ^2, θ^3 = angles between 2^{nd}, 3^{rd} and 4^{th} order arteries, respectively.

Specifically, a 3D fractal growth was realized following the approach of *Diffusion Limited Aggregation*. The idea was to start with a choosen target point, immobile on the surface lobe. A source point can then be choosen at a random position inside the lobe, and allowed to diffuse. When it touches the target, it also becomes immobile and part of an aggregate. Similar sources can be created, one-by-one, and each stops upon hitting the cluster. In this way, an aggregate with intricate branch structures results, stemming from the target and totally included within tissue limits. We are currently studying the feasibility of imposing constraints, so the current branch avoids previous branches. The result is a virtual 3D space limited by the profile of a real adult thyroid lobe subdivided into chambers. Each chamber corresponds to a thyroid lobule, where the chamber contour equates that of one of more SVS branches penetrating the thyroid parenchyma. Therefore, an informatic simulation of the SVS of an ideal, human thyroid lobe can be achieved (Figure 8).

We have also planned to calculate the fractal dimension (or similarity factor) *d* of a real human thyroid SVS on bidimensional images of parenchymal phases of thyroid angiograms, taken from our ethnicity-, sex-, age- and BMI-matched archive of normal human thyroids (detailed in the previous section). Using the box-counting method (112), each radiograph can be "scanned" throughout its entire extent by sequentially covering its different portions with a grid of L square boxes. Then, the number of boxes containing any part of the image outline Nb (L) is counted. A log-log plot of the Nb(L) vs 1/L is finally drawn, and the point are interpolated by a straight line. The slope of this line represents *d* for the vascular distribution under study. Although the radiographic image is a 2D representation of a 3D vascular arrangement it has been shown that orthogonal projections of fractals onto a lower-dimensional subspace retain the fractal element of their dimension (116). This allows for their use to calculate the mean *d* of an entire thyroid SVS. In addition, since $d = - \log (n) / \log (s)$, i.e. *d* is directly related to the recursion depth *n* and the scale factor *s* of the fractal, a suitable comparison of a real SVS with a corresponding informatic simulation becomes possibile, as suggested by our preliminary results on fractal reconstruction of an ideal thyroid SVS (111).

In conclusion, we expect that combining these mathematical approaches with data on post-mortem vascular casts, digital and possibly also conventional, angiotomographic images of the intraparenchymal arteries will enable us to understand the geometrical properties of the human thyroid SVS. This should permit us the necessary informatic simulation to lay the groundwork for physical realization of a bionic scaffold with compatible biomaterial as a guide for seeded autologous thyrocytes and endothelial precursor cells to generate a mature thyroid gland.

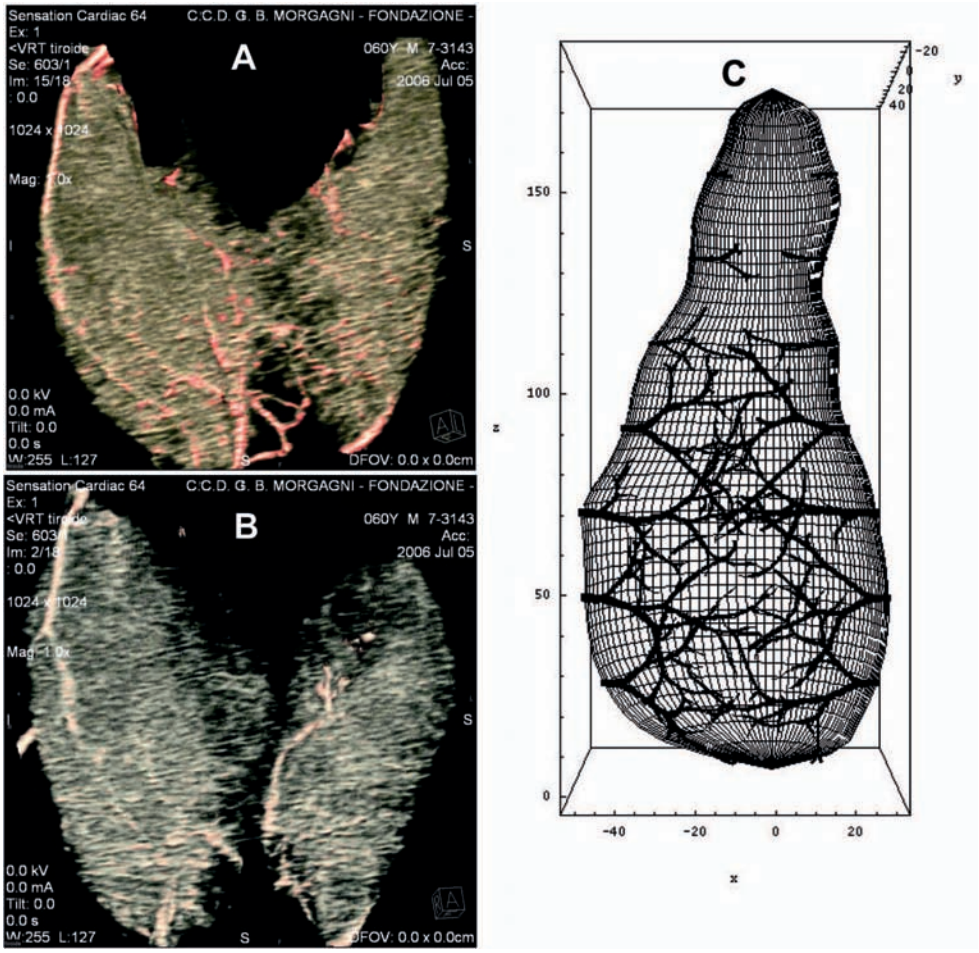

Figure 8. Techniques for in vivo visualization and computer reconstruction of the stromal/vascular scaffold (SVS) of the human thyroid. A) Digital multilayer angiotomography (Siemens Somaton Sensation Cardiac 64) of an adult human thyroid displaying 1st and 2nd order arterial branches at the surface of the gland (red color) and their finest, perifollicular ramifications visualized as texture of the lobe (brown color); B) Following graphic "bleaching" of the image, a reduction in texture density of the gland allows for better appreciation of a number of follicular cavities (black holes); C) graphic result of an informatic simulation of the 3D fractal growth of a SVS inside the contour of a human thyroid lobe, obtained using a probabilistic approach (*Diffusion Limited Aggregation*). Note that the branching structure strongly resembles that of an adult, human thyroid gland, depicting 2nd to 4th order arteries. Mean arterial caliber, as determined from the literature (118, 119), has been the sole anatomical parameter imposed on the algorithm, whereas the origin of 2nd order branches has been restricted to a conventional number onto the surface of the lobe profile with identical distance between each branch. The similarity of the final SVS to that of a real thyroid lobe suggests that the general algorithm closely replicates that achieved by digital multilayer angiotomography

Thyroid-compatible biomaterials and scaffold-related growth of human thyrocyte and endothelial progenitors

The ability of mammalian thyrocytes to grow on a fibrous scaffold in primary culture has been long known. Studies by the Nobel laureate Alexis Carrel at the beginning of the 20th century showed that coagulated plasma offered natural fibrillary support for proliferation of thyrocytes obtained from dog thyroid fragments (120, 121). Further studies on vascular explants showed that natural fibers, such as silk, were particularly suitable for permitting continuous growth of connective and endothelial cells (122). Remarkably,

these studies came about as a result of the attempt by Carrel and associates to establish a way to maintain alive *ex situ* thyroid tissue for transplantation in humans (123). Thus, Carrel's lesson on thyroid explants might be seen as an inspiration to the current vision of a bioartificial thyroid.

In particular, it has been recently shown that a 3D environment is fundamental for mammalian (124, 125) and human (126, 127) thyrocytes in primary culture to acquire a functional follicular phenotype, and exhibit a correctly polarized secretion as in the native thyroid gland. This phenomenon can be explained by the fact that epithelial cell polarization is guided by strain properties of ECM components, becoming very different from 2D to 3D settings (128, 129). In 3D cultures, thyroid folliculogenesis evolves through an intermediate "epithelial-mesenchymal" transition during which spurious apical-apical polarity of monolayers (130) is temporarily lost in favour of a mobile fibroblast-like appearance (131), to be eventually regained as a basal-lumen-basal polarity typical of thyroid follicles (130). In addition, it is now clear that collagen type I, assembling as fiber bundles in the native thyroid stroma (93), serves an important function in directing rodent, porcine and human thyrocyte ability to produce basement membrane-related matrix in culture, thus directing the correct functional polarity of growing follicles (124, 125, 130). Finally, it has been shown that stromal and vascular growth preceeds and addresses follicular formation during tissue regeneration in the course of subacute thyroiditis in man (132), supporting the concept that the SVS is essential to guide epithelial assembly in the growing normal thyroid.

Based on these premises, we have designed a biothecnological scenario for growth and differentiation of human thyroid follicular tissue *ex situ*, seeded onto a biocompatible SVS. Although it is still unclear which biomaterial could be ideal for such a task, a number of options seems feasible. In particular, it is reasonable to choose polymers that can be both easily moulded into a 3D shape with a specific branching morphology, as that dictated by our SVS informatic simulation, and exhibit controlled properties of strength, metabolic degradation rate, microstructure and immunogenicity. Additionally, a certain degree of nanofiber porosity might be favourable, since it has been shown to increase tissue vascularization (133) as well as proliferation and cytoskeletal formation of stromal cells (134). Thus, primary choices would include the thermoplastic and biodegradable polyesters of α-hydroxy acids including polyglycolic acid, polyacetic acid and polylactic-co-glycolic acid, succesfully used to construct a number of different organ silhouettes in genitourinary bioengineering (76). Similarly, esters of hyaluronic acid (hyaluronan derivatives) have been synthesized as a biodegradale 3D knitted fabric with thick microfilaments, that favour growth of endothelial/vascular elements (135), representing an alternative biomaterial source. Very recently Kaplan and colleagues (136) have provided new insights for the use of biomimetic composites from spider silk, that are able to undergo very complex 3D arrangements in physiological conditions, and Stupp et al. (137) have bioengineered a nanofiber that may mimick ECM-mediated cell adhesion, widening the number of potential biopolymers available. Mechanical, immunogenic and degradation features of all these biocomposites have not yet been tested when in contact with the thyroid parenchyma. Nevertheless, the recent advent of computer-assisted, chip-array screening of biocompatibility for biopolymers (138) could subtantially speed up the identification of those products suitable to interact with the microenviroment where thyrocyte and endothelial precursors are expected to be seeded.

As seen above, 3D growth of both mammalian thyrocytes and endothelial cells relies upon a type I collagen-rich microenvironment (130). Therefore, to limit host versus graft immunoreactions, collagen shoud be autologous, i.e. either be extracted directly from the thyroid tissue of the bioartificial thyroid recipient (139) or obtained in the form of an acellular matrix from the donor tissue, as in the case of genitourinary regeneration (140). Major advantages in using thyroid-derived, acellular matrices might include high proliferation rate of grafted cells and formation of matrix adhesive structures, as in the native tissue (33). A primary source of thyroid stroma could be found in fragments of surgically-removed, normal thyroid lobes after total thyroidectomy, in patients with monolateral papillary cancer (see next section for discussion on the rationale of this choice for thyroid

tissue supply). In any event, collagen and/or collagen-rich matrices should be used as a material for embedding the 3D biocompatible scaffold in a way to offer an ideal biopolymer-ECM-tissue interaction for eventual growth of seeded thyrocytes and endothelial precursors.

Sources of human thyrocyte and endothelial progenitor cells and strategies for their enrichment in culture

Thyrocytes

The next fundamental step in the bioengineering of a bioartificial thyroid gland is the identification and collection of viable thyrocytes, able to grow to a sufficient rate to colonize a biocompatible SVS. Similar to collagen, thyroid epithelial cells should ideally be autologous, in oder to avoid rejection and immunosuppression therapy. An abundant supply of autologous normal thyroid tissue might be identified in the "potentially normal" contralateral lobe of patients totally thyroidectomized for monolateral papillary cancer (141, 142). In fact, more than 90% of cases with unilateral, multifocal, lesions exhibit monoclonal growth (143), rendering it easy to identify potentially mutated cells in a primary culture of the unaffected contralateral lobe. This, also in the case of an amplified growth of cancer cells or malignant trasformation of normal cells *in vitro*, as a result of the loss of *in vivo* immune regulation (144) and disregulation of iodide trapping (145). All malignant cells, in fact, should exhibit the same, or very similar, pannel of cancer biomarkers (146). Therefore, screening of primary thyrocyte cultures for these molecular products would provide a strategy to quickly identify cancer cell contamination.

Once isolated a normal autologous thyrocyte population, cells should be able to grow to a sufficient rate to colonize the biocompatible SVS. However, it is well known that normal human thyrocytes have a very low proliferation rate. In the adult mammalian gland and in the absence of TSH, less than 1% of all cells divide autonomously (91). This phenomenon has been ascribed to the capacity of few, actively growing thyrocytes to inhibit recruitment of surrounding, quiescent elements (147, 148). In contrast, autonomous growth may rise up to 30% of all cells in the fetal period (149), suggesting existence of numerous thyrocytes with innate ability to expand, overwhelming the minor growth potential of neighbouring cells. The mechanism triggering this heterogeneous, microclonal proliferation during development is still unknown (90). However, we expect that a peculiar spatial relationship, i.e. a sort of microdomain or niche, might exist between "mother" cells of microclones and the SVS, including the modulatory action of local nerve fibers coursing on the SVS branches (54). These architectural arrangements would play a role in dictating follicular growth, as reported to occur in other epithelial endocrine glands like the pituitary (150) and pancreas (151).

Similar to the intact gland, primary cultures of adult human thyrocytes display a very low growth rate and exhibit a short life span, rendering their survival in long-term monolayers difficult (152, 153). Recently, a normal human thyroid cell line has been established (154), confirming the natural occurence of thyrocytes with a high growth potential, dispersed among a large majority of quasi-quiescent cells (90). These growth-prone thyrocytes are considered thyrocyte "progenitors" (here named thyroid progenitor cells or TPC) (155), or to act as bipotential precursors for both thyrocytes and parafollicular calcitonin-producing cells or C cells (156-158). Transdifferentiation from one cell type to another, in fact, is a well known function of epithelial endocrine elements sharing a common procursor, as for pituitary thyrotroph to somatotroph transdifferentiation under tissue regeneration conditions (159). However, whether TPC are local, quiescent, stem cells assuming specific phenotypes, or terminally differentiated elements able to reenter the cell cycle and change their phenotype, is an issue still unsolved (91). Nevertheless, these TPC are belived to be involved in the formation of new follicles in cases of acute disruption of the thyroid tissue, as during subacute thyroiditis (130). Under these conditions, they would become very sensitive to paracrine/autocrine growth-regulating signals, as a likely result of loosing their native 3D assembly (127). It has been suggested that TPC would be more inclined than other thyroid cell types to respond to growth-activating agents (90), allowing for their selective expan-

sion in primary monolaryer culture under appropriate stimulation.

Concerning which factors might represent an appropriate stimulus to TPC expansion, TSH has been shown to maintain expression of PAX-8 and TSH receptor (TSHR) genes during thyrocyte differentiation of mouse embryonic stem cells (160) and, through these gene products, to control transcription of genes for thyroid hormone synthesis, including the Na^+/I^- symporter (NIS), TPO and Tg (147, 161). Thus, TSH would appear a natural candidate for TPC expansion in primary culture. However, TSH is not required to promote growth of human fetal thyrocytes *in vitro* (147), a condition where TPC are likely present in high number (149). Furthermore, Tg synthesis proceeds autonomously in serum-fed, monolayer cultures of human thyroid tissue in the absence of TSH (162), indicating intrinsic capacity of human thyrocytes to maintain functional differentiation *in vitro*. Therefore, factors other than TSH are likely required to induce selective expansion of normal TPC. The cytokine interleukin-1 (IL-1), for example, has been shown to increase proliferation of rat clonal, FRTL-5 adult normal thyrocytes (163) while inhibiting growth of human papillary thyroid cancer cells (164). We propose that IL-1 and perhaps other cytokines released by autocrine/paracrine mechanisms (130, 144) as well as sex steroid hormones (165) might represent an array of modulators that favour expansion of normal human TPC *in vitro*.

During preparation of a primary human thyrocyte culture, "contamination" by other parenchymal and stromal cells types has been reported (160). However, we belive that for our bioenginnering purposes absolute purity of a thyrocyte culture is not necessary. In rodents, for example, co-culture of thyrocytes with native mesenchymal, endothelial, immune and C cells has been suggested to stabilize and regulate thyrocyte growth and differentiation (130). In addition, the higher growth rate of appropriately-stimulated TPC is expected to overcome that of other co-cultured cell types. Indeed, this kind of growth pattern is observed in other epithelial endocrine tissues like the anterior pituitary. We have shown that a small fraction of adult, anterior pituitary cells in primary monolayer culture are responsive to the addition of the N-terminal fragment$_{53\text{-}74}$ of the thyrotropin-releasing hormone prohormone, pFT22 (166-168), to the cell media (Figure 9). Since in the adult rat, basal growth of stromal and endothelial cells is almost undetectable in culture at early stages of incubation, as opposed to that of epithelial elements (62), and pFT22 stimulation increases the cell-cycle G1/S transition of elements whose size is compatible with that of hormone-secreting cells (Figure 9), it is likely that enhanced recruitment of epithelial precursors occurs from the diploid, quiescent pool G0/G1. This raises the possibility that pFT22 activates pituitary progenitor cells (62), entering replication with a selective cell phenotype (168).

In summary, the evidence that progenitor cells would be more inclined to respond to growth factors, primarily when deprived of their native 3D architecture, might be exploited as a possible strategy to isolate and expand normal human thyrocytes in primary monolayer culture irrespective of the existence of other "contaminating" tissue cells, before seeding them onto a biocompatible SVS. As shown in rat models, in fact, thyrocytes in monolayer exhibit continuous proliferation as an epithelial pavement, but readily revert to a follicular phenotype once re-cultured in a 3D environment (130). Thus, following expansion in monolayer culture, we also expect that human TPC would pursue a polarized, follicular differentiation after 3D seeding on a biocompatible SVS.

Endothelial/vascular cells

We belive that an important preliminary step in achieving optimal colonization of a biocompatible SVS by enriched TPC is the presence of endothelial/vascular precursors (here named endothelial-vascular progenitor cells or EVPC) in the same 3D growth setting. Stromal and vascular development preceeds follicular formation during *in vivo* tissue regeneration after subacute thyroiditis in man (132), aberrant vascularization is associated to thyroid dysgenesis in cases of deletion of the "stem cell 3D organizer" Sonic Hedgehog gene (54) both in mouse (28) and man (169), and growing blood vessels direct differentiation of epithelial endocrine tissue during pancreatic islet development (27, 170). Therefore, 3D co-culture of EVPC with TPC on the same biocompati-

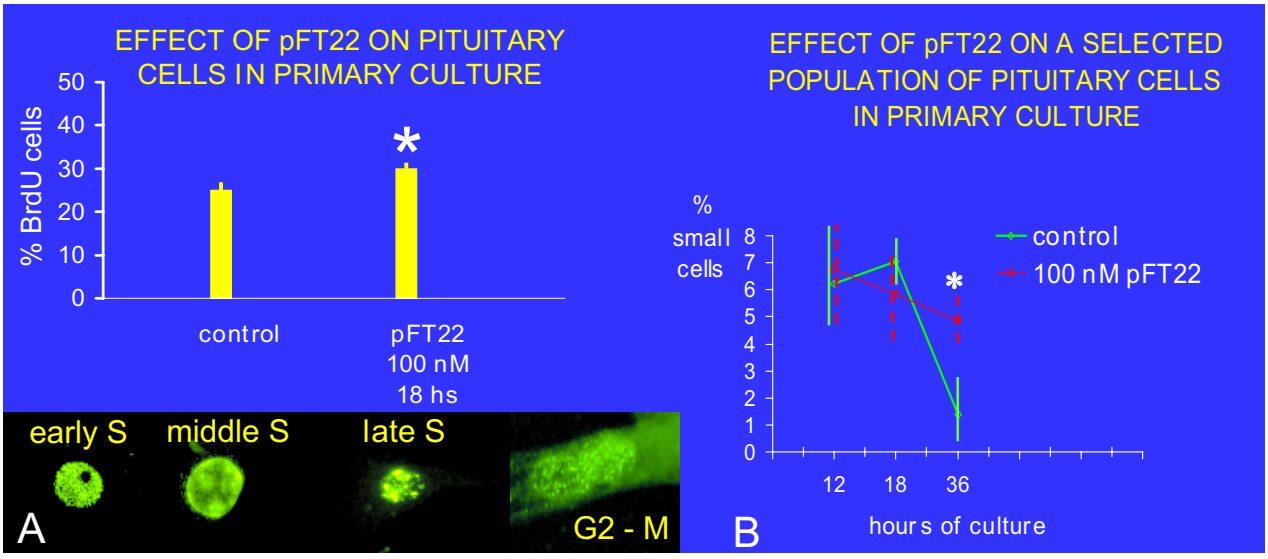

Figure 9. Capacity of a specific factor to favour growth of a subpopulation of differentiated, epithelial cells in culture. A) Results of light microscopic morphometry of asynchronously growing, 2 month-old male rat, anterior pituitary cells from 3 different primary monolayer cultures. Viable cells were plated at 40 000/ cm^2; after 72 hours (resting period) a culture medium change was made and cells were incubated with 100 nM $preproTRH_{53-74}$ (pFT22; Peninsula) or buffer for up to 18 hours, and 100 µM of the synthetic thymidine analogue BrdU / 10 µM FldU during the last 12 hours of plating. Following fixation, cells were labeled with monoclonal antibodies to BrdU (1:25), and either the avidin-biotin complex / diaminobenzidine as a chromogen or fluorescein isothiocyanate-conjugated IgGs (1:50), allowing for recognition of 3 different BrdU-associated, intranuclear staining patterns (bottom part of the figure). These include intial (early S-phase), intermediate (middle S-phase) and final (late S-phase) steps of DNA synthesis, as well as of cells in mitotic division (G2-M phase). Using an unbiased counting method (Gundersen grid principle), a statistically significant difference was found in the percentage of S-phase cells between control and treated cultures (statistical calculations performed after arcsin transformation of percentage values), suggesting that pFT22 may act as a growth factor for anterior pituitary cells. Bars express the mean ± SD of more than 100 counted optical fields; B) Kinetics of Foreward Scatter cytometric profiles of growing pituitary cells in primary culture after incubation with pFT22 as detailed in A). Note that after 36 hours after administration, pFT22 increases the cell-cycle G1/S transition of small elements, compatible with a selected population of hormone-secreting, pituitary cells. Values represent the mean ± SD of 5-8 different cultures for each time point. Statistical calculations were performed after arcsin transformation of percentage values (From Mosca S, Zamai L, Martelli AM, Vitale M, Cocco L, Toni R, 1991-1994, unpublished results). BrdU = bromodeoxyuridine, FldU = 5-fluoro-2-deoxyuridine , * = $p < 0.0001$

ble SVS seems essential to provide adequate *ex situ* assembly of a viable, human thyroid bioartificial construct.

Small amounts of autologous, human EVPC have been isolated from the mononuclear fraction of peripheral blood CD34-, Flk-1-, AC133- and Tie2-antigen-positive cells (171). These EVPC have been expanded in culture under stimulation with auto-serum for 7 days, possibly after granulocyte-colony stimulating factor treatment of the donor favouring mobilization of $CD34^+$ cells from bone marrow to peripheral blood (172). Alternatively, addition of human vascular endothelial growth factor (VEGF) 1, basic fibroblast growth factor 2, epidermal growth factor and insulin-like growth factor 1 has been used, since all these peptides promote endothelial lineage differentiation (171, 173, 174). Remarkably, the same array of growth factors has been found to be extensively expressed in endothelial cells, fibroblasts, macrophages, mast cells and thyrocytes during regenerative angiogenesis in subacute thyroiditis (175, 176). This supports our concept that specific growth-promoting factors may be used as a tool to address proliferation and differentiation of precursor cells in primary culture, before their seeding onto a biocompatible SVS.

Using these approach, totally differentiated (177), life span limited (2-3 months), and enriched EVPC colonies (172) have been obtained for clinical

application. We anticipate, therefore, that isolation and enrichment in primary culture of human autologous EVPC will be possible by exploiting the above mentioned strategies and once obtained, there will be 80- to 90-fold expansion of the original EVPC number (172) to co-seed with TPC on collagen type I-rich and -layered, biocompatible SVS branches.

Authentication of lineage-dependent differentiation and function of cells in the bioartificial thyroid

Both in the course of monolayer expansion of an enriched TPC culture and after 3D follicular growth on a biocompatible SVS, lineage-specific differentiation and secretory activity of human thyrocytes needs to be assessed. Analysis by fluorescence-activated cell sorter (FACS) of specific markers for the thyroid hormone synthesis machinery should include immunolabeling of the TSHR, NIS, TPO and Tg (161). In the recent past, in fact, we have shown that both growth activity and differentiation of epithelial endocrine cells in monolayer culture, like adult rat anterior pituitary cells, can adequately be studied by combining FACS analysis and light microscopic immunocytochemistry (62).

Similar to TPC, also the endothelial phenotype of EVPC in primary culture should be authenticated, by demonstrating expression of specific biomarkers. Endocytosis of acetylated low-density lipoprotein and binding of *Ulex europaeus* agglutinin 1 have been shown to be peculiar endothelial properties of these cells (178). In addition, expression of endothelial genes such as KDR, VE-cadherin, CD31, CD34, vWF, ecoNOS, and presence of specific antigens, like the VEGF receptor 2, all have been demonstated in primary cultures of EVPC, by either reverse trascriptase (RT)-polymerase chain reaction (PCR) or FACS analysis, respectively (172). Therefore, in collaboration with Marco Vitale in the Department of Human Anatomy of the University of Parma, we have initiated a feasibility study to determine the utility of FACS and RT-PCR analysis to identify differentiation markers of human endothelial and thyrocyte lineages.

We are also considering the possibility of authenticating the activity of the bioartificial thyroid tissue by peptide profiling using mass spectrometry, especially after growth of both TPC and EVPC cells on the biocompatible SVS, and ultimately in the blood of animal hosts following experimental transplantation of an entire bioartificial gland. Thousands of small, low molecular weight peptides, most of which are fragments of larger precursor proteins, have now been detected by mass spectrometry in human serum (179). Therefore, the pattern that they create may provide a specific reference to assess function of a bioartificial thyroid tissue. Indeed, utilizing a MALDI-TOF mass spectrometry platform, the thyroid proteomics working group at Memorial Sloan-Kettering Cancer Center (MSKCC) in New York, USA is already underway in identifying a multitude of peptides that may create a thyroid-specific pattern. Using a hierarchical clustering technique, Villanueva et al. (180) identified 98 statistically relevant potential peptides that appear to distinguish the serum of 27 thyroid cancer patients from that of 32 normal control samples. In addition, a pilot study by Suriano et al. (181) has begun to identify a potential panel of thyroid specific peptides using SELDI-TOF mass spectometry to distinguish common thyroid disorders from each other prior to tissue sampling (183). In order to accurately extract relevant clinical information from the multitude of data present in the serum peptide profile, sophisticated bioinformatics software is required (182). (for thyroid proteomics see A Martorella A and RJ Robbins, this Issue).

A final step in assessing the quality and survival potential of a bioartificial thyroid construct would be the implantation of bioartificial composite fragments in the athymic mouse, and their retrival at different time points (up to few months) to determine *in vivo* maitainance of thyrocyte and vessel organization, as in a native thyroid tissue. Experiments with bioengineered genitourinary tissue have shown that this strategy is important to determine the biocompatibility of the bioartificial graft (183). In particular, since *in vivo* implantation of crude tissue volumes larger than 3 mm^3 would expose the core of grafted cells to the risk of necrosis (184), best results for larger bioartificial tissue volumes are obtained using biomaterials scaffolds with a branching pattern and high porosity (185). We belive, therefore, that the 3D branching of the biocompa-

tible SVS may represent an ideal structure to induce formation of an anastomotic network between vessels intrinsic to the bioartificial construct and capillaries of the host tissue. Similar to recent experiments with *ex situ*-engineered, urinary bladder-shaped constructs (186), transplantation in animal models of an *ex situ*-formed entire thyroid gland will be required to prove the anatomical and functional viability of a bioartificial thyroid for clinical applications.

Summary

Theoretical and experimental basis for the model

In this article we have put forth the theoretical and experimental basis to bioengineer *ex situ* a human thyroid gland, morphologically and functionally adequate for potential transplantation replacement. Some of the methodological steps required for this task are now becoming an investigation priority of our research group to make such a possibility a distinct reality in the near future.

The fundamental theoretical assumption choosen to realize a human, bioartificial thyroid is the *topological arrangement* (i.e. the invariance of symmetries with respect to transformations in 3D coordinates) of the gland tissue components. The developmentally-programmed thyroid morphology design includes a constancy of relationships among thyroid cells, vessels, nerves and stroma. Cell adhesion molecules and ECM mediate these relationships, as expected following the Topobiology theory (40, 47). Consequently, the thyroid parenchyma can be seen as sort of deformable "putty", moulded over an elastic SVS that leads to the mature morphology of the gland.

In addition, experimental data suggests that 3D symmetries in the geometrical coordinates of this system are kept invariant during adult life and in pathophysiological conditions. As a result, growth trajectories of thyrocytes in such a space become strictly associated to the geometrical properties of the gland shape that, in turn, are dependent upon its SVS. Therefore, we have suggested that the geometry of the SVS provide pivotal, epigenetic information to address thyrocyte and endothelial/vascular proliferation and differentiation, *making organ form a pre-requirement for organ function*.

The importance of keeping a specific topological arrangement in tissue architecture for a satisfactory physiological performance has also emerged from recent studies on spontaneous aggegation of endocrine cells growing in a 3D collagen matrix. In these conditions, cells spontaneously form constructs with a metastable (i.e relatively stable although in a non-equilibrium state) toroidal topology, whose geometrical properties are maintained from 2D to 3D arrangements (187). This has led us to hypothesize that in the case of thyrocyte growth on a biocompatible SVS, a toroidal construct might be the primary growth pattern, leading the mature gland to retain at least some topological properties of the original self-assembly (Figure 10). Remarkably recent engineering theory of quantistic computers (for quantistic computations see also R Lupacchini, this Issue) implies that a topological arrangement of functional units used for calculation (i.e. the subatomic particles) would reduce the number of errors during computation (188). In a similar manner, a topological assembly of the functional particles responsibile for thyroid activity (i.e the thyroid cells) might be thought necessary to reduce the number of "computational errors", i.e. performance failures of the gland (for the concept of computation in neuroendocrine structures see R. Toni et al, this Issue).

Various experimental strategies have been explored to obtain a biocompatible matrix, 3D preformed as the thyroid gland. Among these, we have choosen to reproduce a real SVS with a fractal growth. Besides a fractal simulation, however, other informatic approaches could eventually result appropriate: a probabilistic cellular automaton (189), for example, might generate a 3D model of auto-organizing SVS, starting from an initial state sets on probabilistic constraints, represented by nodes of a graph (190), topologically consistent with the basic branching pattern of an injection cast of the intrathyroidal vasculature.

Once the template of a biocompatible and degradable SVS has been achieved, the major aspect has been recognized in the *ex situ* satisfactory growth of thyroid tissue. Techniques to harvest and expand in primary culture autologous TPC and

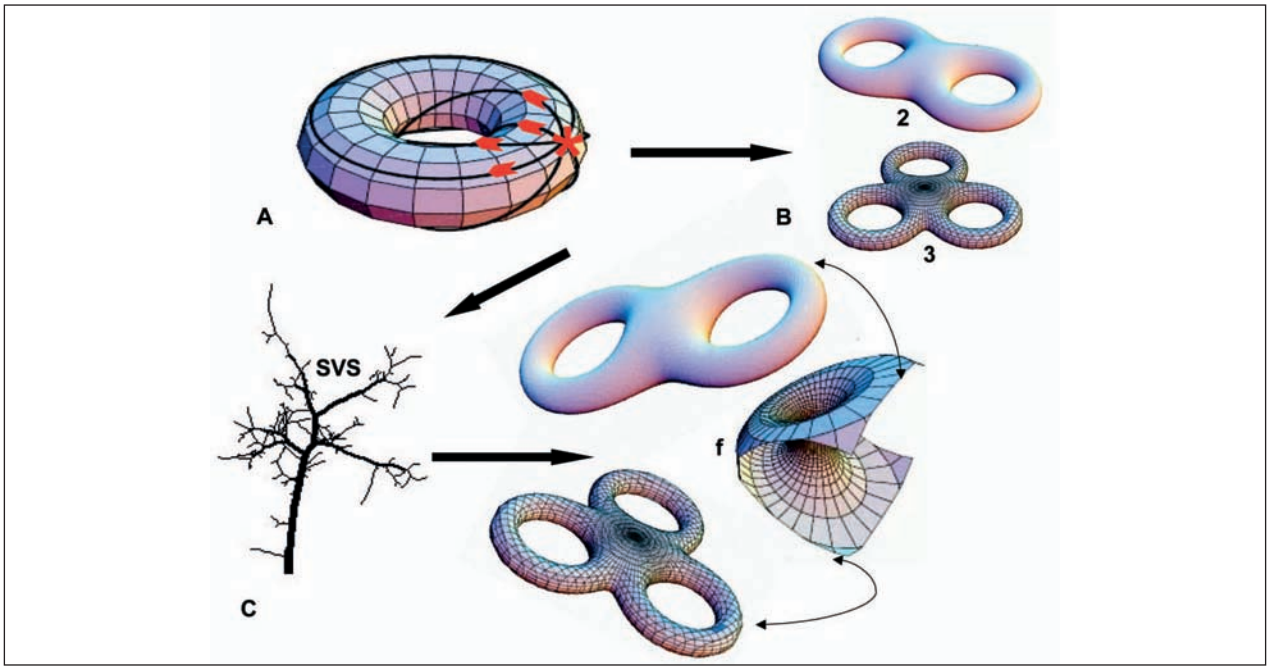

Figure 10. A simplified hypothesis of toroidal and fractal configuration for the initial assembly of growing thyrocytes in a bioartificial thyroid. A) The geometry of a regular *torus*, or tubular ring, shows that constituting cells (red asterisk) may travel in a 3 D space following orthogonal or oblique, circular pathways (arrowheads) (189); B) Progressive contiguity of thyrocytes assembled as toroidal constructs might give rise to more complex thyrocyte geometries, or *n*-tori here exemplified by double (2) and triple (3) ring-like constructs C) Finally, 3 D expansion of the thyrocyte *n*-tori could be superimposed onto a fractal dimension, provided by the biocompatible stromal/vascular scaffold (SVS) of the gland favouring their 3D fusion into a lumenous module like the follicle (f). The overall process would allow for growth of thyrocytes following multidimensional trajectories, ultimately ascribing a key role to the SVS topology in the morpho-functional maturation of the bioartificial gland

EVPC from human donors, to *in vitro* and *in vivo* authenticate their lineage differentiation, and to establish in animal hosts the viability of the bioartificial thyroid construct have been proposed, offering some clues to the interpretation of intriguing and still obscure aspects of cell and molecular biology of the thyroid tissue.

Potential clinical applications

From a broad pespective, we belive that the biothecnological scenario proposed to realize a bioartificial thyroid might offer a reference paradigm to eventually construct *ex situ* other, more complex and clinically-relevant, bioartificial endocrine organs such as the human pancreatic islets and the liver. Bioengineering of these structures, in fact, is far from satisfactory and still is based on isolated cell delivery and transplantation of fetal buds (191). On a speculative basis, our model of an "organ-shaped SVS" could provide a new horizon for brain bioengineering. As we have analyzed elsewhere (see R. Toni et al on brain computation and neuromorphic chip performance, this Issue), even the highest brain activities can now be interpreted as computational procedures, whose efficiency depends upon specific nervous tissue architectures. However, these architectures are dictated by 3D physical scaffolds established by fibrillary/vascular components of the nervous tissue (12, 22-26). To this purpose, studies on neocortex development in vertebrates show that 3D assembly of vasculature dictates cortical telencephalic architecture (192). Even more astonishing is the fact that a core mechanism for neocortical activity, called *framing* (193), has been suggested to be based on "thought scaffolds" or *frames* (194). Thus, knowledge of the "3D physical frames" in which neurons are settled, i.e. the *brain scaffolds*, seems an inescapable requirement for any future attempt to re-

produce *ex situ* a neural stem cell-based, bioartificial structure with physiological function, primarily the neocortical areas. Indeed, very recent achievements in spinal cord regeneration point towards this direction (195).

Conclusions

A modelling of human bioartificial thyroid gland has been presented, and potential clinical applications of its general principles have been suggested. We believe that this modelling introduces a new frontier in endocrine organ bioengineering for transplatation replacement. We also hope that benefits of this method can become available on a large scale for a number of different endocrine organs, finally fulfilling the original prophecy expressed by Alexis Carrel almost a century agò: *"Thus, while the problem of the transplantation of organs has been solved from a surgical point of view, we see that this by no means suffices to render such operations of definite surgical practicability, and it will only be through a more fundamental study of the biological relationships existing in living tissues that the problems involved will come to be solved"* (123).

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