***i*Human (*in-vitro & in-vivo* Human Platform)**

Off The Human, For The Human

Human embryonic stem cells (hESC) are **ETHICAL** [A, B], and the prototype "**GOLD STANDARD**" [a] pluripotent cells. The hESCs and progenies are ideal and promising human cell source for **WIDE** clinical, biomedical, biotech applications**, UNLIMITED** direct and indirect human use [b, c, d, e, f, g, h]:

- Human relevant function and biosafety/toxicity assessment of therapies, drugs, cosmetics, food, chemicals, materials and techniques
- Human relevant environment analysis of water, soil, air, and natural/artificial products and techniques; Gene/protein delivery to cure diseases
- Cell based reconstruction therapy including regenerative medicine
- Development and gene control of human tissue/body
- Disease study

The iHuman is to develop *in-vitro & in-vivo* Human Platform of vascularized [i, j], innervated, functional, standard and live Tissue-Organ-System from hESC as ethical and unlimited source to improve clinical service (regenerative medicine, transplantation, precision medicine, etc.) [k] and as efficient platform to upgrade human evaluation in health and medical studies [l, m, n, o].

The *i*Human (*in-vitro & in-vivo* Human Platform) technology introduces next generation of *in-vitro & in-vivo* human models with the consistent, functional, robust functionality with the unlimited availability tailored for the specific unmet needs of health and life science industry.

Background

Most our current understanding of biology has come through primary/ immortalized mammalian/ human cell cultures and various animal models. These systems have provided us with valuable insights about human development, health and disease; and have also paved way in the early preclinical development of pharmaceutical and cosmetic products. Without these systems it would have been difficult for academic and industrial researchers to have access to the human system due to various practical and ethical concerns. However, the relevance of monolayer cell culture and animal model systems to humans is questionable. For instance, cells in the human body grow in 3-dimensions through interactions with surrounding cells and extracellular matrix (ECM). However monolayer cell culture systems are predominantly grown on flat plastic surfaces and in isolation without interaction with other cell types. Secondly, primary cells cultures are limited by various issues related to donor availability, limited propagation, culture induced ageing/ senescence, and

inter-donor/ inter-batch variability. Though commonly used animal models like rodents, rabbits, and pigs provide the 3-dimensional (3D) interactions absent in monolayer cultures and resemble humans in terms of certain anatomic and/or physiologic aspects, they are quite dissimilar to humans and extrapolations needs to be carefully weighed. These drawbacks of the current biological systems have also been attributed to be one of the causes for large amount of drugs/ biological products failing in the clinical trials.

Worldwide it is estimated that the number of vertebrate animals from-zebrafish to nonhuman primates—ranges from the tens of millions to more than 100 million used annually [1]. Invertebrates, mice, rats, birds, fish, frogs, and animals not yet weaned are not included in the figures; one estimate of mice and rats used in the United States alone in 2001 was 80 million [2]. US government funded animal testing alone costs US taxpayers over US$31 billion in 2010 and increases yearly [3]. Despite the supposed stringency of animal tests on drugs deemed safe for human consumption and released onto the market, two million Americans become seriously ill and approximately 100,000 people die every year because of reactions to medicines they were prescribed [4]. This figure exceeds the number of deaths from all illegal drugs combined, at an annual cost to the public of more than US$136 billion in health care expenses. In England, an estimated 70,000 deaths and cases of severe disability occur each year because of adverse reactions to prescription drugs, making this the third most common cause of death (after heart attack and stroke) [5]. The drug company Ciba-Geigy has estimated that only five per cent of chemicals found safe and effective in animal tests actually reach the market as prescription drugs after the R&D investment of at least 10 years and over $ 1billion on each single drug candidate [6]. Even so, during 1976 to 1985 the US Food and Drug Administration (FDA) approved 209 new compounds – 102 of which were either withdrawn or relabeled because of severe unpredicted side-effects including heart attacks, kidney failure, liver failure and stroke [7]. The fact that months or years of human studies are also required suggests health authorities do not trust the results. In 2004, the FDA reported that 92 out of every 100 drugs that successfully had passed animal trials subsequently failed human trials [8].

Hence, academies, research institutes and the industries of health, drug, food, cosmetics, chemicals and environment are presently hindered by a lack of functional, healthy and standardized human platform of cells, tissues and organs.

Human ESC technology and its potential as a novel alternative evaluation platform

Human embryonic stem cells (ESCs) are pluripotent stem cells derived from the inner cell mass of a blastocyst (an early developmental stage of preimplantation embryo) derived through *in vitro* fertilization. These cells possess the ability for virtually unlimited self-renewal and differentiate to all somatic lineages in the human body. These human ESCs serve as a genetically healthy, single source from which all cell types in the body could be derived. Additionally, it is amenable to derive human ESCs representative of various diseased states.

Lineages of differentiation:

- **Ectodermal:** Keratogenic, Neurogenic and Amelogenic lineages
- **Mesodermal:** Mesenchymal, Fibrogenic, Osteogenic, Chondrogenic, Endothelial, Adipogenic, Odontogenic, haematopoietic lineages
- **Endodermal:** Hepatic, Pancreatic, Renal lineages

Due to the potential for unlimited self-renewal ability, human ESCs and their progenies are an ideal and promising cell source for wide range of biomedical and biotech applications [9].

- Human relevant function and biosafety/toxicity assessment of therapies, drugs, cosmetics, food, chemicals, materials and techniques
- Human relevant environment analysis of water, soil, air, and natural/artificial products and techniques; Gene/protein delivery to cure diseases
- Cell based reconstruction therapy including regenerative medicine
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As genuine pluripotent stem cells, human ESC serves as an unlimited source potential to develop into all cell types of human body. Hence, global pioneers and governments like EU and UK endeavor to develop a technically-simple, cost-effective and replicable system of human ESC derived live platforms in the last decade. This fast development is revolutionizing health sciences from animal-based platforms to much more accurate human-based platforms. The revolution will bring a new burgeoning industry of human ESC-derived human platform of live cells, tissues, organs and systems in the next few decades. Leading in the world, the US Congress, federal and local governments, investors and charities have been supporting 'promising' human ESC R&D through legislations, policies, guidelines and funds. US initiated the clinical trials of human ESC therapies for eye diseases and spinal cord injury since 2011. Besides various human ESC progenies, functional tissues with multiple cell lineages, unique vascularization and innervation by autologous human ESC progenies are currently being explored. The human ESC progenies, functional tissues and organs will offer ideal ***in-vitro & in-vivo*** **'clinical' platforms** of no-risk trials/tests for the basic, translational studies and applications of all human health related sciences including fundamental study of health, ageing [21, 61, 62, 65, 66], disease, prevention, diagnosis, immunity [19, 64], therapy and transplant; drug and med-tech R&D. Moreover, those standardized *in-vitro & in-vivo* human live platforms of no-risk trials/tests will be widely adopted in much more areas beyond medicine and pharmaceutics. The major other applications will be the human function and safety evaluation of food; cosmetic; daily and general chemicals; organisms; nuclear, IT, communication, electromagnetic, radiating device and technique; environment (air, water, soil, daily living and working environment); other human-contact substance, products and techniques. The platforms of human ESC progenies, functional tissues and organs will be ethically and gradually used

at reasonable and practical pace, non-clinically, pre-clinically and clinically in all human health related industries, academies and authorities.

Europe and United States have recently initiated the similar strategy. EU Embryonic Stem cell-based Novel Alternative Testing Strategies (**ESNATS, EU)** has since 2008 been developing a novel toxicity evaluation platform based on human ESC to accelerate drug development, reduce related R&D costs and propose a powerful alternative to animal tests [10]. In 2007, The UK Government decided to establish a public-private partnership to develop predictive toxicology tools for stem cell lines[11]. Department of Health UK has started the program of Stem Cells for Safer Medicines (**SC4SM**) to enable the creation of a bank of stem cells, open protocols and standardized systems in stem cell technology that will enable consistent differentiation of stem cells into stable homogenous populations of particular cell types, with physiologically relevant phenotypes suitable for toxicology testing in high throughput platforms [12]. In 2009, GE Health and Geron incorporations in US have jointly initiated the strategy to develop and commercialize cellular assay products derived from human ESCs for use in drug discovery, development and toxicity screening [13].

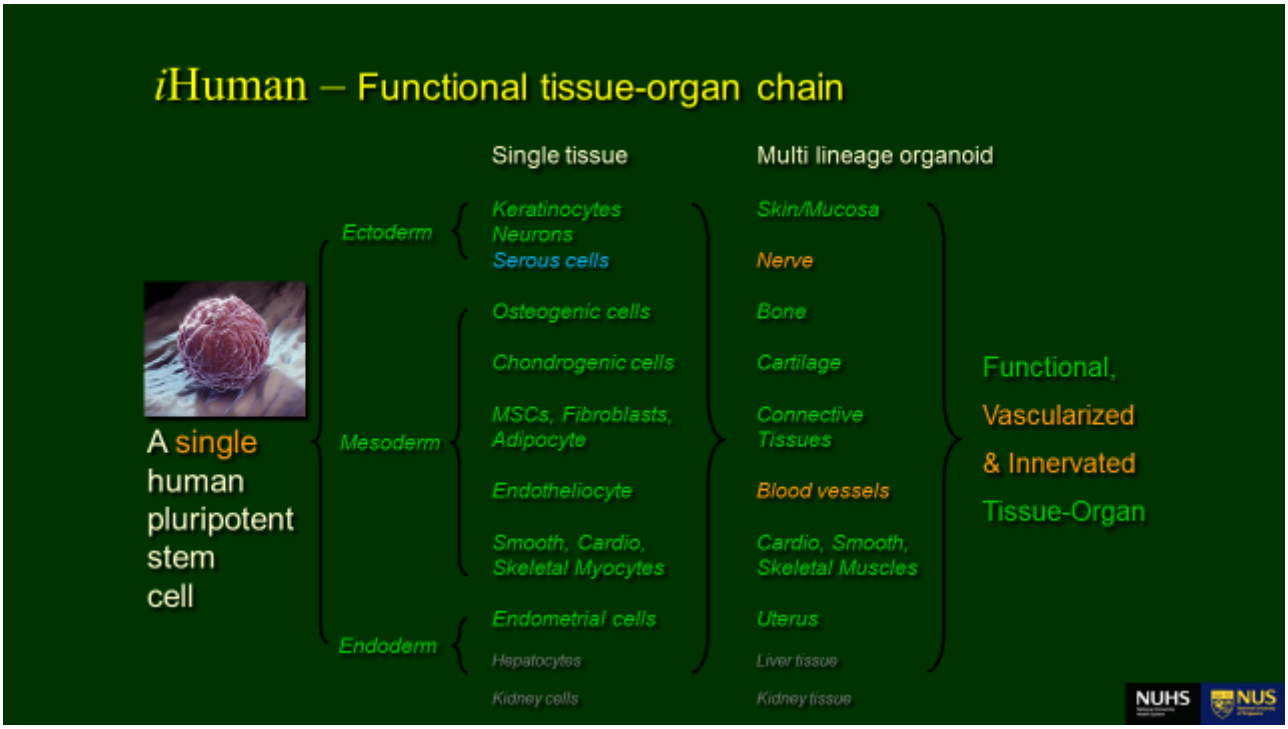
Need for human organs

Miniature human organs termed as organoids grown in the lab are potentially useful for studying human development, model diseases, evaluate the efficacy, safety and toxicity of new drugs or therapies. Hence these miniature human organs/ organoids can play a vital role in replacing the use of animal models in research. Furthermore, they can be used as a modality of therapeutic organoid graft in regenerative medicine. Harnessing the potential of 3D organoid cultures various laboratories around the world have developed miniature organoids that are representative of human skin, liver, lung, brain, esophagus, heart, blood vessels, intestine and stomach.

In addition, US and other nations are to take key actions to **reduce the organ waiting list** by facilitating breakthrough research and development [**c**].

***i*Human technology (*in-vitro & in-vivo* human platform)**

The *i*Human (*in-vitro & in-vivo* Human Platform) technology introduces next generation of *in-vitro & in-vivo* cell, tissue and organ models with the consistent, functional, robust functionality with the unlimited availability tailored for the specific unmet needs of health and life science industry. Tong Cao and his team from Faculty of Dentistry, National University of Singapore have been working for more than 10 years on human ESCs. They are currently working towards developing the *i*Human technology by combining the ability of human ESCs to differentiate to various cells in the body and 3D organoid culture platform.



The iHuman is an *in-vitro & in-vivo* platform to provide reproducible, standard and functional human cells, tissues and organs. The platform is created from permanently renewable, healthy and autogenic human embryonic stem cells (ESCs). The *in-vitro & in-vivo* human platform of vascularized, innervated, functional, standard and live Tissue-Organ-System developed from human ESCs is to be an ethical and unlimited source to **improve clinical service**. Moreover, the iHuman will be an efficient and cost effective platform to upgrade human evaluation in health and medical studies. As a result, the iHuman will largely **replace and minimize the use of animals** in all human health related industries, authorities, academies and institutes.

Self-renewable, genetically-healthy and single-sourced human ESCs exhibit enhanced biological relevance and stable predictivity over its more expansive counterparts. As genuine pluripotent and good standard stem cells, human ESCs serve as an unlimited source potential to develop into all cell types of human body. Hence, global pioneers and governments like EU and UK endeavor to develop a technically-simple, cost-effective and replicable system of human ESCs derived live cellular platforms in the last decade. This fast development is revolutionizing health sciences from animal-based platforms to much more accurate human based platforms. The revolution will bring a new burgeoning industry of human platforms of live cells, tissues, organs and systems in next decades.

iHuman – tissue, skin/mucosa models

The multilayered epithelium covering the exterior parts of the body and the oral cavity forms a protecting barrier toward mechanical damages and invasion of pathogens, toxins and antigens. From cosmetic,

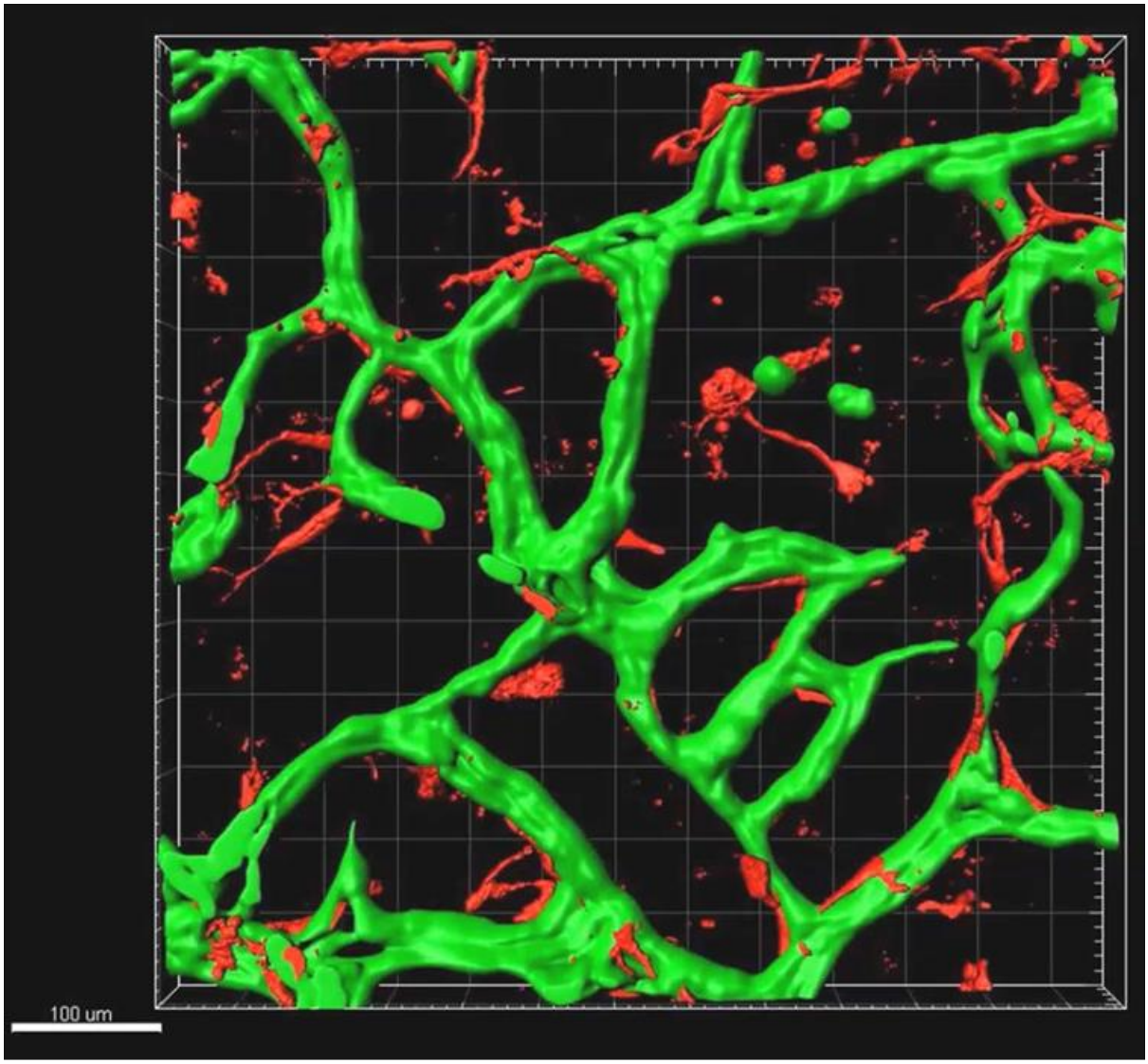
pharmaceutical and toxicology point of view, the barrier property of the skin/mucosa plays an important role. Effective delivery of therapeutic active ingredients delivered by topical application requires permeation across this superficial skin/ mucosal barrier. Currently available models use rabbit eyes, rodent skin, pig skin or human excised skin as human skin equivalents for assessment of safety, toxicity and permeability of cosmetic, pharmaceutical and industrial chemical agents. However, use of animal skin is associated with issues related extrapolation and human relevance. Though, human excised skin is the best alternative available, it is difficult to obtain sufficient amounts from donors. Secondly, they are associated with large inter-batch variations due to differences in donor site, age, gender and race [14, 15]. Hence, human-based *in vitro* organotypic skin equivalents are extremely valuable alternative. In addition to the use of organotypic skin equivalents doe cosmetic, pharmaceutical and chemical industries, they are also potentially useful for

- Understanding basic biology of skin development,
- Disease modeling, understanding and development of therapeutics for various dermal/ mucosal pathologies like psoriasis, genodermatoses, vesiculo-bullous diseases, keloids, skin cancer,
- Wound healing models and assessment of various therapeutic agents,
- Studying microbiome and its effect on health and diseased states of skin/ mucosa.

The need for these organotypic skin equivalents are magnified by changes in regulations across various parts of the world on the prohibition of use of animals for development of skin and oral care products [16] [17]. Similar to other systems, use of primary or immortailsed cells for development of skin/ mucosal equivalents are associated with limited availability and inter-batch/ inter-donor variations.

In this aspect, Tong Cao and his team from Faculty of Dentistry, National University of Singapore have developed novel methods of deriving keratinocytes [18-22, 61, 62, 70] and fibroblasts [23-27] from human ESCs and organotypic full thickness/ epidermal, skin/ mucosa equivalents using human ESC-derived keratinocytes and fibroblasts, which has been patented recently [28]. Use of human ESC technology provides an unlimited access to keratinocytes and fibroblasts that are used for the generation of organotypic full thickness/ epidermal skin/ mucosa equivalents. Secondly, the both keratinocytes and fibroblasts are derived from the same source of human ESC and hence, reflects the use of cells derived from a single donor i.e., human ESCs. Further, human ESCs could be derived from embryos of different gender and races, and hence would pave way for generation of skin/ mucosal equivalents reminiscent of skin/mucosa of different gender and race. This has great potential value as the properties of skin/ mucosa differs among different gender and race, which is need for development of cosmetic and therapeutic products. Hence, the iHuman-skin/mucosa model serves as a valuable tool for academic and industries involved in development or safety, toxicity and therapeutic assessment of cosmetic and pharmaceutical products.

***i*Human - vascularized tissue equivalents**



Regulated process of blood vessels development involves proliferation, migration and remodeling of endothelial cells in presence of neighboring pre-formed blood vessels (angiogenesis) or directed emergence of blood vessels occurs de novo from the early embryo in presence of mesoderm precursors (Vasculogenesis) [29]. Various studies on embryonic blood vessel development and angiogenesis in the adulthood have shown the supportive role of vascular smooth muscle cells/ pericytes (commonly referred to as mural cells). Without the presence of these supportive mural cells, micro vessels formed by endothelial cells undergo regression.

There are various limitations in studies related to angiogenesis that include use of monolayer cultures of primary cells, lack of 3D *in vitro* angiogenesis (co-culture) models, and animal models. Umbilical vessels, adipose tissue and skin are excellent sources of endothelial cells and mural cells; however their availability and proliferation potential in culture are limited and are associated with inter-batch and inter-individual

variations. Additionally, it is quite difficult to procure sufficient quantities of endothelial cells and mural cells from the same donor.

Currently available methods for preclinical development of novel angiogeneic and antiangiogenic compounds is based on assessment using monolayer cultures of primary endothelial cells, *in vitro* Matrigel tube formation assay, mouse Matrigel plug angiogenesis assay, and hind limb ischemia rodent model [30-32]. Other models used include mouse ischemic retinal angiogenesis assay, incorporation into retinal vasculature of diabetic rats, myocardial ischemia model, stroke and vascularization in dermal wounds [33-41]. All these models are primarily based on monolayer primary cell cultures and animal models. Additionally, *in vitro* angiogenesis assays are based on the behavior of endothelial cells without the presence of supporting mural cells (which play a vital role in the formation and maintenance of blood vessels).

From a tissue engineering/ regenerative medicine perspective, one of the major obstacles challenging successful tissue engineering is vascularization of the tissue engineered scaffold/ graft that can support the survival of implanted cells upon transplantation *in-vivo* [42]. Studies have shown that prevascularization of the engineered construct allows faster engraftment of the implanted tissue construct by anastomoses of graft vasculature with host vasculature [43, 44]. However, we do not have efficient methods to enable vascularization of tissue engineered constructs. Hence, there is a great need for a novel source of endothelial and mural cells, and development of 3D *in vitro* angiogenesis assays that could replace/ reduce the use of animal models. Further, a platform to create blood vessels within 3D matrices that could be used as prevascularized graft and hence enable faster and better engraftment of the tissue engineered graft.
Using human ESC technology, researchers around the globe have developed novel protocols to derive endothelial cells and vascular smooth muscle cells. Specifically, Tong Cao's team from National University of Singapore, have developed a novel protocol to efficiently differentiate human ESCs to endothelial cells and vascular smooth muscle cells in a culture environment that is almost devoid of animal-derived components [45-47, 73]. Further, using the iHuman technology to grow the human ESC-derived endothelial cells and vascular smooth muscle cells within a fibrin-based 3D scaffold, they have created network of blood vessels that resemble a capillary network within the tissues in the body [46-48, 71]. These blood vessels networks have a patent lumen and are functional in terms of their ability to respond to permeability increasing agents like histamine. Further, they have developed a miniaturized, high-throughput platform for generation of 3D vascularized tissue equivalents of a volume of less than $10mm^3$. This miniaturized, high-throughput iHuman-vascularized tissue equivalent platform opens up potential opportunities to identify novel angiogeneic and anti-angiogenic compounds in a high-throughput format. The advantages of this platform include the use of human cell source, blood vessels in 3D, rapid *in vitro* assay for blood vessel formation, ability to monitor blood vessel formation in real-time over a period of 3-4 weeks, high-throughput capabilities and reduced cost.

***i*Human - vascularized tissue, skin/mucosa equivalents**

In human body, most of cells can diffuse nutrients and oxygen via angiogenesis within a maximum space of up to 150-200µm [49]. Vasculature is one of the most important criteria during the development of tissues/organs and to keep them in function. Directly or indirectly, vasculature is the only means from which all the parts of body receives nutrition and oxygen [50]. Till today in the field of tissue engineering, limitation of mass transfer is one of biggest challenges. Limiting factor of the *in vitro* developed tissue constructs is survivability for longer period after subsequent *in-vivo* implantation into the body. Due to the restricted/absence of angiogenesis, nutrition exchange is limited and leads to failure of implanted graft, finally it is left as non-functional tissue construct [51]. Thus, the concern of optimal and functional vascularization was highlighted for better survival rate of implanted tissue engineered constructs. Along with the vasculature, the size of tissue constructs, tissue type, nutrients diffusion rates and location of tissue integration should be taken into consideration.

Recent reports have showed the development of *in vitro* three dimensional vascularized soft tissue in both micro as well as nano scale levels, which is reported under the name *"ArtiVasc 3D"*. *ArtiVasc 3D* is bio-engineered tissue which is being developed using either primary cells or immortalized cells and decellularized porcine intestine as the source of cell and matrix. *ArtiVasc 3D* promises potential applications in studying cell-cell interactions, solving tissue engineering challenges, regeneration of soft tissue and also development of pre-clinical platform to assess the pharmaceutical implications of specific drug chemicals [52]. However, *ArtiVasc 3D* might show strong disadvantages since the cell source used might come up with few challenges in procuring sufficient number of cells (primary cells) and maintenance of actual human *in vivo* state (immortalized cells). Secondly, it based on the process of infusing the endothelial cells into a piece of decellularized porcine intestinal mucosa. The relevance of this model is questionable if for instance, a vascularized skin/ oral mucosa need to be reconstructed. Further, it depends on the use of animal-derived tissue. Hence, there is need for developing an alternative platform that uses human-based cells and matrices.

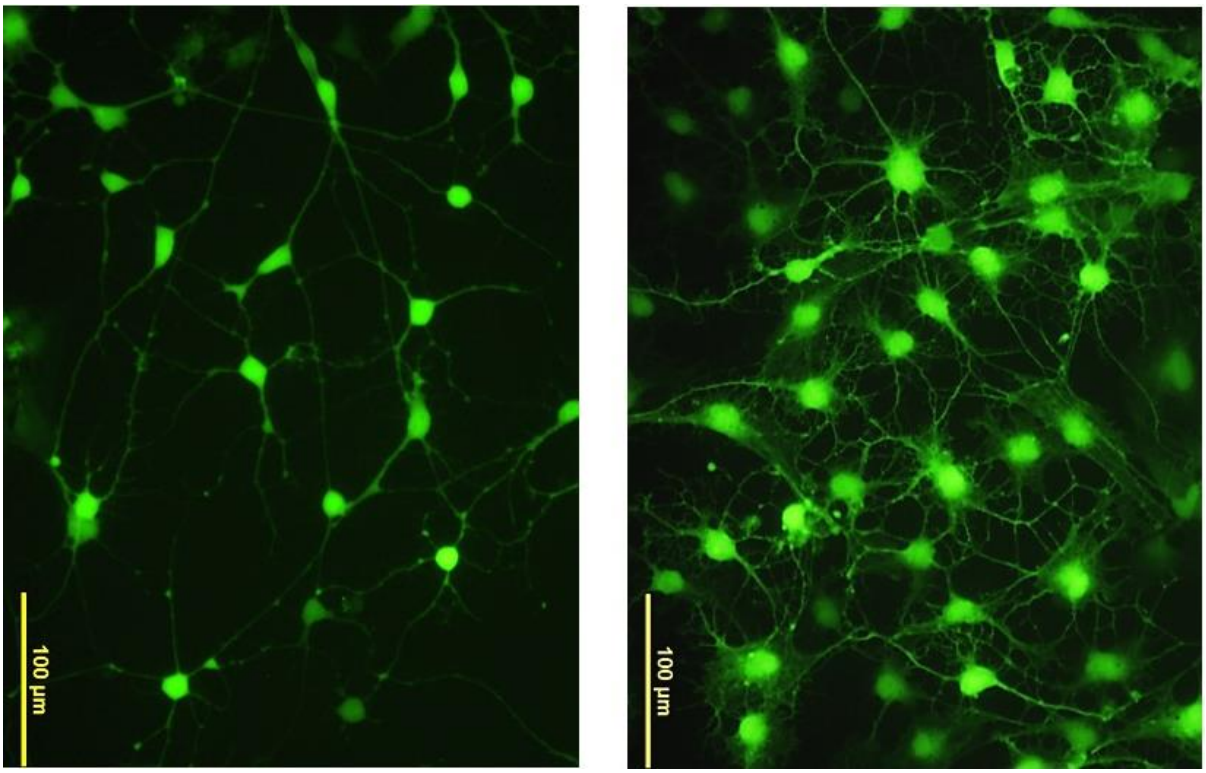
Tong Cao's team from National University of Singapore, has developed the expertise in differentiation of human embryonic stem cells into [18-22], fibroblasts [23-27], endothelial cells and vascular smooth muscle cells [45-47, 73], which are subsequently capable enough to form functional epithelium and blood vessels *in vitro* under serum and animal component free conditions. Using the *i*Human-vascularized tissue equivalents platform to create *in-vitro & in-vivo* vascularized tissues and they have developed *i*Human-vascularized skin/ oral mucosa platform. This iHuman-vascularized skin and oral mucosa models consists of a vascularized dermal equivalents with human ESC-derived endothelial cells, vascular smooth muscle cells and fibroblasts and a stratified squamous epidermis (keratinized for skin & non-keratinized for the oral mucosa model) contributed by human ESC-derived keratinocytes.

Primary feature of the iHuman-vascularized skin/mucosa is the origin of all the cells (keratinocytes, fibroblasts, endothelial cells and vascular smooth muscle cells) from a single cell source i.e., human ESCs.

In a clinical setting, obtaining all these cells from a single donor is quite difficult considering the amount of donor tissue needed for their isolation. On other hand, due to the virtually unlimited self-renewal capacity and differentiation to all the above lineages, human ESCs are an unlimited source of donor cells.

In vitro vascularized models provides realistic 3D micro-environment which can reflect the human *in vivo* tissues/organs, and can be an essential platform in drug screening, toxicity evaluation, penetration studies of specific drugs, nano-particles permeability studies, host-microbial interaction investigation as well as it could be a used for *in vitro* disease, cancer and tumor [67] modeling platform. Since 2017, Tong Cao's team has collaborated with global corporate **Evonik Industry** to develop technology, product and **international partnership** in other countries in the area of iHuman.

***i*Human - neuron**



During late 1990's Neural Stem Cells (NSCs) were first isolated from sub-ventricle zone of mouse brain and also descried to have an self-renewing as well as multipotent capacity [53]. NSCs are considered to have the potential to differentiate themselves into neurons, astrocytes and oligodendrocytes [54]. Neurons have shown a vital role in Parkinson's disease and Alzheimer's disease. Understanding neurons and the signaling cascade is one of major global challenges. Tong Cao's team from National University of Singapore have initiated promising piece of research on Neurons by differentiating from human ESCs under *in vitro* conditions. Existing available models describe the mechanism of neural tissue development during embryogenesis. Existing models proposed induction of neural tissue by BMP secreted through notochord. Additionally, role of FGF and Wnt signaling have also impacted on the differentiation of neurons from human

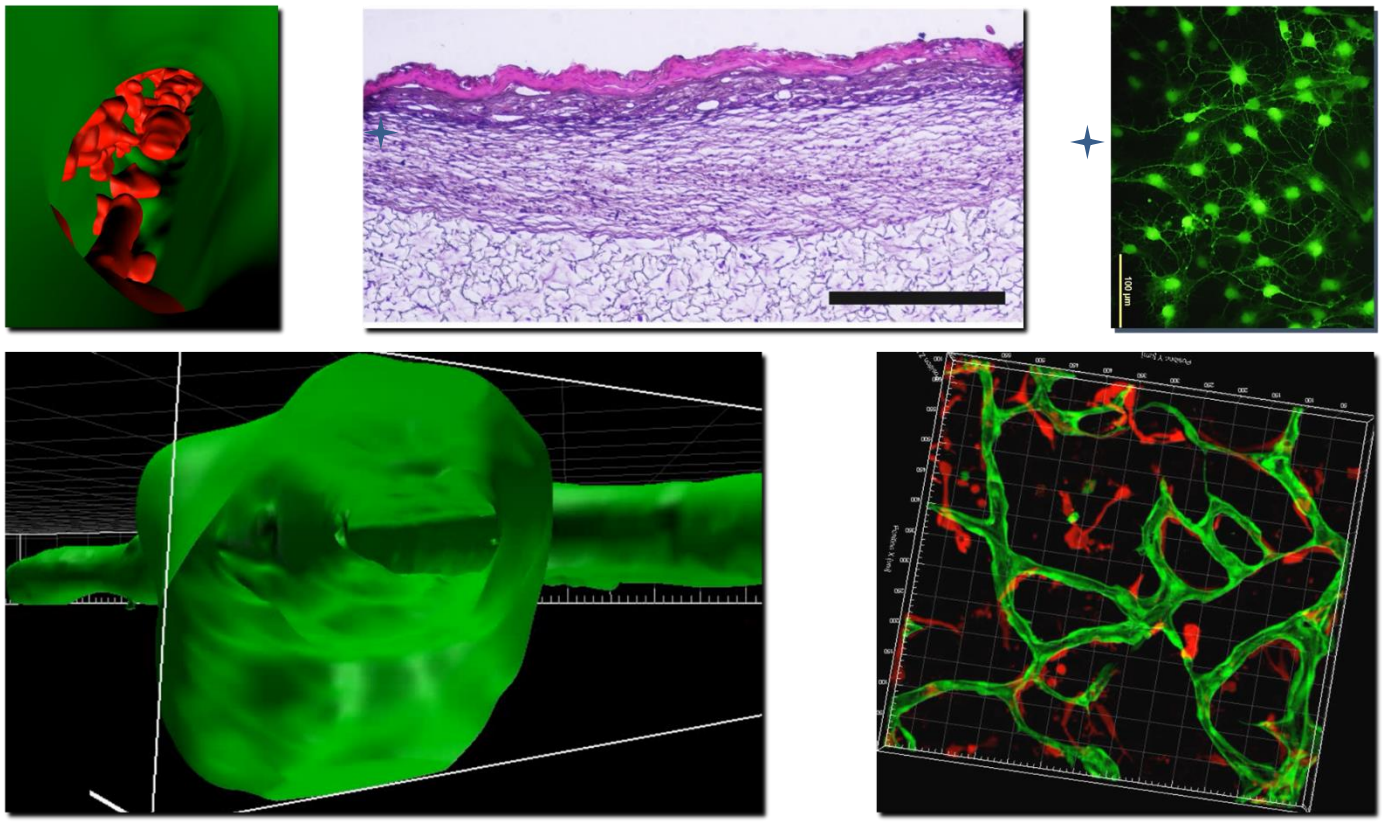
ESCs. Tong Cao's team have also evidenced the role of FGF to stabilize lateral plate and paraxial mesoderm subtypes [55]. Hereby, Tong Cao's team are successful in differentiating dopaminergic neurons [56, 62, 70], neural crest and peripheral neurons [57, 68, 69] models under *in vitro* conditions. We have aimed to establish *i*Human-neuron concept which provides a potential *in vitro* platform for studying neuronal signal mechanisms and also disease models. *i*Human-neuron also has huge potential in drug screening as well drug discovery research areas.

_i_Human - innervated tissue, skin/mucosa models

Skin and oral mucosa are quite complex tissues which are basically composed of an overlying stratified squamous epithelium and an underlying vascularized dermis. In addition, skin also possesses innervation with predominantly non-myelinated peripheral sensory neurons, sympathetic neurons and various appendages like hair follicle, sweat glands and sebaceous glands. Similarly, the oral mucosa is innervated with the sensory neurons, autonomic nervous system, and minor salivary glands. Considering the mentioned complexity in the structural organization of skin and oral mucosa, current models of *in vitro* skin/ oral mucosa equivalents are highly simplified form of the complex *in-vivo* form. So, as a first step towards moving closer to the *in-vivo* reality, researchers have tried to incorporate peripheral neurons within full-thickness skin equivalents [58]. The significance of innervation in tissue engineering has previously been demonstrated for pharmaceutical testing purpose [59]. Furthermore, bioengineering of innervated tissue can be developed to promote peripheral nerve regeneration and spinal cord repair [60]. The innervated skin models represent a humble beginning to mimic the complex *in-vivo* structure. However, to innervate the tissue-engineered skin rat dorsal root ganglion cells are used. Currently, there are no human based platform available as it is difficult to obtaining human dorsal root ganglion and also not possible to grow the peripheral neurons in culture.

Members from Tong Cao's lab in National University of Singapore have developed differentiation protocols to derive central neurons [56, 62], neural crest and peripheral neurons from human ESCs [57, 68, 69]. Hence, using human ESCs as a cell source, it is feasible to obtain and grow peripheral neurons in culture. Further, they are currently working towards incorporating these peripheral neurons within *i*Human-skin/ oral mucosa model to develop an innervated skin/ oral mucosa model. Furthermore, by combining with *i*Human-vascularized skin/ oral mucosa models, it is feasible to generate innervated and vascularized skin/ oral mucosa models. By the simulation of tissues with nervous system in three dimensions, innervated *i*Human can be projected into both preclinical and clinical application. Based on the work of iHuman central neuron and ongoing peripheral neurons, it is expected that the development of Innervated iHuman will broaden our outlook on neural development and tissue level study.

iHuman organs, skin/mucosa with vascular and neural networks



With the establishment of 3D models of innervated skin-mucosa and vascularized skin-mucosa, the integration of both these models together to generate human ESC-derived *in-vitro & in-vivo* 3D skin-mucosa organoids with integrated vascular and neural networks.

Validation and application of developed vascularized skin-mucosa organoids, innervated skin-mucosa organoids and integrated skin-mucosa with vascular and neural networks in the selected pilot studies of health, ageing, safety, disease, toxicology, diagnosis, prevention, drug testing and biomaterial/device. Library of drugs/ small molecules would be used along with human ESC-derived vascularized and innervated skin-mucosa models for various downstream applications that include toxicity evaluation, and permeability studies [72].

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