Project number: T12C-714/14-R

RESEARCH GRANTS COUNCIL
THEME-BASED RESEARCH SCHEME (TRS)

Completion Report on Funded Project

Project start date: *01/01/2015*
Project completion date: *31/12/2020*

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1. Project Title: Genetics and Functional Genomics of Neural Crest Stem Cells and Associated Disease: Hirschsprung Disease

2. Names and Academic Affiliations of Project Team Members[#]

Project team member	Name / Post	Unit / Department / Institution	Average number of hours per week spent on this project in the whole project period
Project Coordinator (PC)	Tam, Paul K.H. / Chair Professor	Surgery / HKU	8
Co-Principal Investigator(s)	Chan, Woody W.Y. / Professor	School of Biomedical Sciences / CUHK	5
	Cheah, Kathryn S.E. / Chair Professor	School of Biomedical Sciences / HKU [1]	5
	Garcia-Barceló, Maria-Mercedes / Associate Professor	Surgery / HKU [2]	10
	Hui, Chi-Chung / Professor	Molecular Genetics / University of Toronto & School of Biomedical Sciences / HKU [1]	3
	Ngan, Elly S.W. / Professor	Surgery / HKU [3]	10

	Lovell-Badge, Robin / Professor	The Francis Crick Institute & School of Biomedical Sciences / HKU [1]	5
	Sham, Pak Chung / Chair Professor	Psychiatry / Centre for Genomic Science / HKU	4
Co-Investigator(s)	Chan, Ying-Shing / Professor	School of Biomedical Sciences / HKU [1]	5
	Cherny, Stacey / Assistant Professor	School of Medicine / Tel Aviv University [4]	5
	Cheung, Martin / Assistant Professor	School of Biomedical Sciences / HKU [1]	5
	Lam, Stephen / Director	HK Department of Health Clinical Genetic Service	3
	Li, Miao-Xin / Professor[5]	Zhongshan School of Medicine / Sun Yat-sen University	5
	Lo, Ivan / Deputy Director	HK Department of Health Clinical Genetic Service	3
	Lui, Vincent / Associate Professor	Surgery / HKU	10
	Ng, Ray / Assistant Professor	School of Biomedical Sciences / HKU [6]	3
	Sham, Mai Har / Professor	School of Biomedical Sciences / CUHK [7]	5
	Tang, Clara / Research Assistant Professor	Psychiatry / Medicine / Surgery / HKU [8]	10
	Yip, Kevin / Associate Professor	Computer Science and Engineering / CUHK	2
	Zhang, Michael / Professor	UT Dallas / USA & Psychiatry / Centre for Genomic Sciences / School of Biomedical Sciences / HKU [1]	2
Collaborators	Borrego, Salud / Professor	Hospital Virgen del Rocio, Sevilla, Spain	N.A.
	Ceccherini, Isabella / Professor	I Gaslini, Genoa, Italy	N.A.
	Hofstra, Robert / Professor	University of Rotterdam, Netherlands [9]	N.A.
	Cowan, Chad / Assistant Professor	Harvard Stem Cell Institute, US	N.A.
	Lyonnet, Stanislas / Professor	Hospital Necker-Enfants Malades, France	N.A.
	Pachnis, Vassilis / Professor	The Francis Crick Institute	N.A.
	Wysocka, Joanna / Assistant Professor	Standford, US	N.A.

Please highlight the approved changes in the project team composition and quote the date when the RGC granted approval of such changes. For changes in the project team composition, please submit a separate request, together with the justification and the curriculum vitae of the new member(s), to the RGC three months prior to the intended effective date of the change.

Remarks:

1. Departments of Anatomy, Biochemistry and Physiology were amalgamated into the School of Biomedical Sciences.
2. Dr. Maria-Mercedes Garcia-Barceló retired in June 2019.
3. Professor Elly Ngan has been promoted to Professor in May 2020.
4. Dr. Stacey Cherny has joined the School of Medicine of Tel Aviv in August 2017.
5. Dr. Miao-Xin Li has joined the Zhongshan School of Medicine of Sun Yat-sen University in January 2017.
6. Dr. Ray Ng left HKU in August 2019.
7. Professor Mai-har Sham has joined CUHK in 2020 and acts also as the Pro-Vice-Chancellor / Vice-President of CUHK.
8. Dr. Clara Tang's appointment has been promoted to Assistant Professor since October 2020.
9. Professor Robert Hofstra passed away in 2021.

3. Project Objectives

Summary of objectives addressed/achieved:

Objectives*	Percentage achieved	Remarks**
1. Identify new genetic risk factors for HSCR through genome studies.	100%	• Identified novel HSCR-associated genes, including *BACE2* and ECM-associated genes • Discovered novel HSCR-associated common variants for polygenic risk score prediction • Established new method for curating copy number variations from WGS data and identified deletions of *EDNRB* and 16p11.2 deletions in HSCR patients • Developed new method to integrate genetic & epigenomic data and identified regulatory enhancers, promoters and genes enriched with HSCR-associated non-coding variants, and corresponding transcription factors
2. Understand the functional impact of known and newly discovered HSCR risk factors by studying patient-derived iPSC, human embryonic stem cells (hESC), and animal models.	100%	• Established and characterized control, patient-specific, and mutant/corrected human iPSC lines • Defined the biological impacts of the genetic lesions in *VCL*, *RET* and *BACE2* using hiPSC-based models • Confirmed cell stage–specific regulatory roles of three top HSCR-associated regulatory elements in *RET*, *RASGEF1A*, and *PIK3C2B* loci using the hiPSC-based models • Defined common and distinctive biological pathways associated with *RET* sensitized background via

Objectives*	Percentage achieved	Remarks**
		transcriptomic studies on control and patient-specific human iPSCs • Deciphered the roles of Hh signalling in ENS development using Hedgehog (Hh) mutant mice model • Demonstrated sacral neural crest cell migration defects during ENS development in *Sox10*^{Dom} mouse model • Identified two potential causes of the male sex bias seen in HSCR using the mouse as a model; one relating to accelerated development of the ENS in females compared to males and the other to female-specific effects of a Ret mutation in the brain stem leading to their loss at birth.
3. Identify the NCC gene regulatory networks controlling generation of ENS structures through transcriptome analysis of NCCs and their derivatives, and determine the functional attributes of genetic/epigenetic variations in NCC disorders.	100%	• Obtained high resolution (single cell) spatiotemporal molecular signatures for ENCC and its derivatives spanning E9.5-E16.5 . Molecularly dissected the ENCC and non-ENCCs (niche cells) in E14.5 small intestine. • Identified a major lineage bifurcation among enteric neurons at E13.5 in mouse, with ENCC-derived early-born neurons expressing inhibitory marker nNOS and requiring RET signalling. • Provided evidence that nNOS+ early-born neurons are essential in mediating the colonization of enteric progenitors in the colon by secreting survival factor Nrg1. Implicated early-born neurons as the functional hub connecting RET and NRG1 signalling with implications for HSCR pathogenesis.
4. Obtain mechanistic insights into NCC development to enteric neural lineages and the molecular pathogenesis of HSCR.	100%	• Established the role of SUFU-GLI-SOX10 in ENS development and its implication in HSCR pathogenesis • Single-cell transcriptomic analysis revealed activation of HH signalling during early phase of iPSC-to-ENCC transition significantly improves ENCC yield and the function of ENCC-derived neurons • Demonstrated perturbed ENS-niche interaction as a novel mechanism underlying HSCR pathogenesis using VCL mutant/corrected hiPSC model

Objectives*	Percentage achieved	Remarks**
		• Defined the BACE1-APP-BACE2 pathway and neuronal death as a novel disease mechanism
5. Training of postgraduate students, postdoctoral fellows, physician scientists and technologists.	100%	A total of 10 RPg students, 11 PDF and 2 scientists have been trained or recruited.
6. Conduct workshops, symposia and public forums in genetics, genomics and NCC disorders.	100%	Five research symposiums were successfully held. In addition, TRS Retreats were held yearly to provide a platform for the students and investigators to exchange ideas and to receive invaluable feedbacks on their projects from peers.
7. Establish database of HSCR variants for open access use by the scientific community.	100%	• We established important open-access resources documenting the summary statistics of genetic findings on WGS data for the research community on HSCR • Software developed for analysing coding, non-coding and structural variations of the WGS data were published and freely available to download.

6. Research Highlights and Outputs

6.1 What are the most exciting research accomplishments of the project?

(Please list five or more of the team's best research accomplishments, such as journal and conference papers, software codes, research infrastructure, etc. For each item, please clearly justify how it has achieved international excellence (e.g. best paper award, invited presentation, citations, product licensed to industry, etc.))

This programme coalesces experts with diverse backgrounds to leverage our strengths in genomics and stem cell biology to advance the understandings of the causes of Hirschsprung disease (HSCR) and to improve clinical care. In the past six years, our multi-disciplinary research team has been dedicated to establish new platforms, strategies and tools to drive cutting-edge discoveries in disease modelling. We have successfully completed the original objectives and made significant contributions in deciphering the pathological mechanisms. Given the success of this programme, a new TRS project has been awarded to us to build upon our experience, the well-established stem cell- and organoid-based platforms and strategies to advance researches of another life-threatening congenital liver disease, biliary atresia, to improve the diagnosis and therapy. In this report, we highlight several most impactful research achievements as summarized below.

1) Establishment of human stem cell-based platform to model the functional impact of HSCR-associated genetic lesions

HSCR is caused by dysregulation of the developmental pathways in which the enteric neural crest cells (ENCCs) colonize the developing gut. Conventionally, genome editing and rescue experiments were performed in vivo using animal models to test the biological impact of loss of function of HSCR candidate genes on enteric nervous system (ENS) development and gut motility. Our team was the first to generate human diseased ENCC lines derived from induced pluripotent stem cells (iPSC) of HSCR patients for disease modelling in vitro. Three patient-derived iPSC lines, including one with a deleterious *RET* mutation (G731del), and two isogenic mutant iPSC lines with heterozygous or homozygous deletion(s) of *RET* were generated to study the functional impact of genetic lesions in patients with HSCR and in *RET* against control iPSCs. Using this stem cell-based model, we demonstrated that ENCCs derived from diseased iPSC lines recapitulated HSCR-associated defects in migration and differentiation in neuronal lineage in dish and *RET* mutations were functionally connected with the severity of ENCC phenotypes in vitro. By further integrating genetic and transcriptomic information of the patient and the disease relevant ENCCs, we identified a novel mutation in vinculin (VCL M209L) that significantly reduced focal adhesion (FA) size and functionally contributed to disease phenotypes. Importantly, both the differentiation capacity and migratory behaviour were restored in ENCCs derived from disease-corrected iPSC lines generated by correction of the *RET* G731del and *VCL* M209L mutations using clustered regularly-interspaced short palindromic repeats (CRISPR)/Cas9 genome-editing. The finding was published in Gastroenterology (2017). Taken together, our team established a novel stem cell-based disease model and a new strategy applicable to functionally dissect known and novel mutation(s) for HSCR by combining multi-omics information of the patient and the disease relevant ENCCs with genome editing in HSCR-iPSC. Our results also illustrate the value of diseased and disease-corrected hiPSCs for discovering and defining disease causative mutations in complex developmental disorders and provide mechanistic insights into the disease pathogenesis.

2) Discovery of new disease mechanisms linking dysregulation of ENS development with cell-niche interaction and neuronal death

FA is a large molecular assembly that link the cytoskeleton of a cell to the extracellular matrix

(ECM) via integrin receptors and other adaptor proteins (e.g. VCL) whereas ECM is a non-cellular scaffold that provides support to three dimensional structure of the bowel. Both FA and ECM are integral components of the intestinal neural stem cell niche that develops in concert with the ENS. Through the spatiotemporal sensor–effector coupling, the neural stem cell niche orchestrates ENS development by modulating the migration, proliferation, survival, and differentiation of the ENS precursors. Our team was the first in applying next generation sequencing (NGS) technologies in deciphering the genetic basis of HSCR (Genome Biol, 2017; Genome Res, 2020; PLoS Genet, 2020). Through genome-wide burden tests aggregating association signals across genes and gene sets, our team found irrefutable significant enrichment of rare protein-altering variants in genes encoding members of the ECM from both family-based and population-based whole genome sequencing (WGS) studies on HSCR. Short-segment HSCR patients (443 S-HSCR versus 493 controls), the commonest but less severe form of HSCR, were found to have increased burden of rare damaging mutations in two ECM-associated genes— *ITGB4* and *PTK2/FAK* (also known as focal adhesion kinase)—that interact with RET and/or ERBB2 of the two core HSCR pathways (Gastroenterology, 2018). Mutational profiling of long-segment HSCR trios (the severe form of HSCR; n=9) also revealed a significant enrichment of rare *de novo* or biallelic genotypes in the ECM pathway, with some patients having a network of interacting ECM/ECM-associated proteins (e.g. collagens (COL6A2; COL6A3; COL1A2), laminins (LAMB2; LAMA5), integrins (ITGA2; ITGB5)) and growth factors (NRG1) being disrupted by rare mutations (EJHG, 2018). Such findings were further supported by pathway enrichment analysis of rare damaging variants found in our first NGS study on Bangladeshi HSCR samples in which the mutational burden of cell-matrix adhesion, ECM organization and integrin binding are second to neuron development. The enrichment appears to be more profound in the severe HSCR patients, suggesting that ECM niche abnormality may be an additional key pathogenic mechanism in this disease subtype. Besides rare variants, other cellular niche factors acting as guidance cues for ENCC (e.g. SEMA3s, NRG1) were also dysregulated in the majority of HSCR patients through common risk allele predisposition identified by our recent trans-ethnic meta-analysis (Hum Mol Genet, 2016). Altogether these studies demonstrated, for the first times, genetic evidence in human supporting the long-standing hypothesis of the intrinsic ECM niche defects in HSCR patients. The findings also highlighted the need to understand the cell-niche interaction between the ENCCs and the ECM/cellular niche, especially when new cell-based therapies are considered as alternatives to surgery to treat the disease.

Another novel finding from the WGS study was the stark excess of rare protein-altering variants in β-secretase 2 gene (*BACE2*) in HSCR patients. *BACE2* is a homolog of *BACE1* that encodes a protease which cleaves the amyloid precursor protein (APP). BACE2 cleaves APP in the beta amyloid (Ab) region and eventually prevents Ab formation. Accumulation of Ab induces neuronal death, representing the underlying cause of Alzheimer disease. In the WGS study coupled with disease modelling of the mutations in the stem cell-based platform, our research team demonstrated that some patient-specific *BACE2* rare variants significantly reduced the APP processing activity of BACE2 and interrupted its ability of abolishing Ab production. This resulted in the accumulation of Ab and thereby apoptosis of the enteric neurons. Meanwhile, case-control association tests revealed a moderately associated locus at *PLD1* (p=4.8x10^{-7}) that encodes a catalytic enzyme physically interacting with APP. This study put forward a new disease mechanism for HSCR—neuronal death—similar to Alzheimer disease. This suggested that the two diseases, one affecting the ENS and another affecting CNS, may share common pathological mechanisms and plausibly druggable targets. This landmark study has opened up a new perspective for the development of drug-based treatment for the disease. The study received commentary in the succeeding issue of Gastroenterology titled “Even When You Know Everything, There Is Still More to Learn About Hirschsprung Disease” on this exciting discovery. Presentation of the findings received

Reviewers' Choice Abstract Award at the 66th Annual Meeting of the American Society of Human Genetics held at Vancouver in October 2016.

3) Optimization of protocol for neural crest derivation from human pluripotent stem cells and development of a human innervated colonic organoid model

To date, generation of fully functioning cells from human iPSCs (hiPSCs) remains challenging. With the aim of improving the efficiency of neural fate acquisition, our team systematically studied the hiPSC-to-ENCC transition and the subsequent differentiation towards neuronal lineage at the single-cell level to identify differentiation cues for improving lineage manipulation. We quantified the transcriptional profiles of over 400 FACS-enriched hiPSC-derived ENCCs and studied the gene expression dynamics during the hiPSC-to-ENCC -to-neuron transition. Intriguingly, we found that Hedgehog (HH) signaling is activated in the postmigratory ENCC-like cells, implying that modulating the level of HH signaling may promote the cell state progression. We further demonstrated that activation of HH (with SAG treatment; SAG-ENCCs) at the early phase of the hiPSC-to-ENCC transition robustly increased the ENCC yield, while suppression of HH by addition of a HH antagonist (cyclopamine) dramatically suppressed the hiPSC-to-NC transition. To further improve our iPSC model, in collaboration with Prof James Wells (University of Cincinnati), we successfully established a human innervated colonic organoid model that is competent to assess the function of hiPSC-derived enteric neurons based on their ability of inducing muscular contraction. Using this human innervated colonic organoid model, we directly demonstrated that SAG treatment greatly improved the functional competency of hPSC-derived neurons. In summary, we established an experimental paradigm and systematically optimized the differentiation protocol for the generation of functioning NC via activation of HH signaling pathway. Improving the neural fate differentiation efficiency of hiPSC-derived ENCCs will ultimately expedite the application of these cells in regenerative medicine of HSCR. The results were included in Gastroenterology (2019).

4) Deciphering the contribution of non-coding genetic variants in the regulatory landscape of ENS development and disease pathogenesis

WGS on the S-HSCR case-control cohort has identified ~40 million genetic variations and many of these variants fall into the non-coding regions of the genome. Non-coding variants in the enhancer regions of *RET* are among the variants most strongly associated with HSCR, alongside with other HSCR-associated variants in the promoter, intronic, and intergenic regions of *NRG1* and *SEMA3s*. To improve power of detecting association of regulatory variants, we developed a novel noncoding variant association analysis framework called MARVEL (Multiscale Analysis of Regulatory Variants on the Epigenomic Landscape). It leverages cell-type-specific chromatin epigenomic features and protein binding motif prediction to define and prioritize transcriptional regulatory variants from WGS data and jointly analysed these regulatory variants across the same gene to detect association with disease. We applied this framework on the WGS data of S-HSCR, coupled with histone modification (H3K4me1 and H3K27ac) data from ChIP-seq and chromatin accessibility data from ATAC-seq on hiPSC and hiPSC-derived ENCCs. We generated genome-wide map of accessible chromatin and identified 152,279 active ENCC enhancers critical for ENCC gene expression by cross-referencing with RNA-seq data on hiPSC-derived ENCCs. In addition to *RET*, we discovered nine novel enhancers and four genes significantly associated with S-HSCR using MARVEL. Using the established hiPSC-based platform, we confirmed cell stage–specific regulatory roles of three top novel regulatory elements in the *RET*, *RASGEF1A*, and *PIK3C2B* loci respectively and suggested plausible upstream regulators. Pathway analysis of 417 loosely HSCR-associated genes showed enrichment of functional interactions and aggregation into functional pathways of (1) chemotaxis and cell-cell signaling, (2) cell adhesion, migration, and interaction with ECM, (3) PI3K-PKC-MAPK signaling, (4) E3 Ubiquitin Ligase complexes, and (5) transcriptional regulatory factors highly related to

ENCC migration and ENS development. Overall, our work has produced a large number of novel regulatory elements, genes, upstream regulators, and transcription factors associated with S-HSCR for follow-up investigations. The method as well as the analysis results were published in Genome Research (2020).

5) Elucidating the sacral neural crest cells migration in ENS Development

Sacral NCCs, originating from the caudal region of the neuraxis, is another cell source of the ENS. Compared to vagal NCCs that migrate rostro-caudally in the gut tube to colonize the whole intestine, sacral NCCs migrate caudo-rostrally in the developing gut and contribute in a smaller but significant extent to the ENS, mainly in the post-umbilical gut segment. Our team aimed to determine whether sacral NCCs is also involved in the pathogenesis of Hirschsprung disease and determine how Sox10 regulates the migration of sacral NCCs using *Dominant megacolon* (*Sox10*^{Dom}) mutant mice, a commonly used HSCR animal model. We demonstrated that sacral NCCs exhibited retarded migration to the distal hindgut in Sox10-null embryos with simultaneous down-regulated expression of cadherin-19 (*Cdh19*). Cdh19 formed cadherin-catenin complexes which bound to filamentous actin of the cytoskeleton during cell migration. Knockdown of *Cdh19* resulted in retarded sacral NCC migration in vitro and ex vivo, whereas re-expression of *Cdh19* ameliorated the migratory defects of mutant sacral NCCs in vitro. The study suggested that *Cdh19* is a direct target of Sox10 and that sacral NCCs may also contribute to the etiology of HSCR. Therapeutic strategies to up-regulate cadherin-19 expression in local cells, or in autologous ENCCs, may help rescue defective cell migration in HSCR or other enteric neuropathies. The work was published in Gastroenterology (2021) and received commentary in the succeeding issue of Gastroenterology titled ' *"Going the Extra Mile": A Sox10 Target, Cdh19, is Required for Sacral NC Migration in ENS Development*'.

6) Spatiotemporal heterogeneity of ENCCs-during development

a) Identification of a novel ENCC subpopulation enriched with retinoic acid anabolic gene signature

To characterize the heterogeneity of developing ENCCs at single-cell resolution, we generated and analyzed the transcriptomes of 513 single vagal NCCs and ENCC derivatives at three stages of mouse development during the gut colonization: as the neural crest migration front reaches the foregut (E9.5), the proximal colon (E12.5), and the distal colon (E14.5). Neural crest descendants tagged using neural crest specific Wnt1-Cre mediated reporters (R26-tdTomato or Z/EG). In addition to eight major vagal NCC subpopulation/states, we identified a unique cell subpopulation annotated as "mesenchymal-like (M)" cells that expressed neural crest transcription factors (*Sox10, Snai2, Hand1*) and were also enriched with a signature characterized by a combination of retinoic acid anabolic genes (e.g. *Aldh1a2*, *Rdh10*), ECM genes (*Col2a1*, *Col6a1*), and mesenchymal cell genes (*Acta2*, *Actg2*). Small number of vagal NCCs with similar gene signatures were also found in published Sox10 expressing E12.5-E13.5 ENCC dataset (PMID: 28522527) as well as in published E13.5 to E16.5 and postnatal day 21 datasets from collaborators (Drs. Ulrike Marklund and Igor Ademkyo (Karolinska Institute) and Dr. Vasilis Pachnis (Crick Institute, London)). Vitamin A and retinoids play a pivotal role during ENS development. The lineage identity and transcriptome signature of the "M" implies a potential role for this novel ENCC-descendant in regulating ENS differentiation and/or maturation via maintaining a retinoic acid and ECM niche. The findings were presented in international conferences.

b) The essential interaction between vagal-origin enteric neuron and neural crest progenitors

We have provided new insights into whether there is direct interaction between differentiated and undifferentiated ENCC descendants which may impact on the colonization of the distal colon. We identified the essential interaction between vagal-origin enteric neuron and neural crest

progenitors when colonizing the distal colon. This finding highlights that crosstalk between RET and NRG1 signalling is via interactions between early-born neurons and progenitors. By molecularly dissecting the heterogeneity of 2750 ENCC-descendant single cells from mouse gut at E12.5 and E13.5, we found evidence suggesting a major lineage bifurcation among enteric neurons at E13.5. Signatures for early-born neurons, are readily detected at E12.5, showing robust *Ret* expression, together with *Nos1* (nNOS) and another HSCR gene *Nrg1*; but significant late-born neuron signatures could only be detected after E13.5, featuring signature genes like *Chodl*, *Unc5c* with low level of *Ret* transcripts. The identification of early-born and late-born neurons among ENCC descendants suggests they may have distinct functions during ENS development. Loss of nitric oxide synthase (NOS)-expressing neurons has been previously demonstrated in HSCR. Focusing on the early-born neurons we found the robust differentiation of NOS+ neurons in the colon starting from E12.5 and E13.5 in WT mice. Intriguingly, the majority of the enteric progenitors adhered to the neurites extending from the NOS+ neurons near the migration wavefront in WT foetal colon at E13.5. In *Sox10NGFP* hypomorphic mutant colons where the wavefront lags due to migration defects, these adhering neurite associated progenitors were only found at later time points adjacent to surviving neurons. The close interaction between enteric progenitors and nNOS neurites imply that the latter may facilitate survival of adhering progenitors via secreting particular neurotrophic factors. Given that Nrg1/Erbb3 signalling is essential for enteric progenitor survival), and Nrg1 is localised only close to enteric progenitors along the caudally-projecting NOS+ neurites, we suggest nNOS neurons are critical for distal colonization by the enteric progenitors. Our findings connect the epistatic interaction between RET/NRG1 signalling with neuron/progenitor interaction during the colonization of the distal colon in the context of HSCR. Collectively, our study shows the early-born neuron's indispensable developmental role in nurturing and mediating colonization by the enteric progenitors in the distal gut by connecting RET and NRG1 signalling as a functional hub, and has important relevance for HSCR. These findings are described in a manuscript (Lu et al, in preparation).

7) Exploring the sex bias seen in HSCR using the mouse as a model

a) Sex-specific differences in timing of neuronal lineage specification in the ENS during its colonisation of the gut

Bulk RNAseq was carried out on ENCCs and gut mesenchymal cells isolated and separated from male and female embryonic guts using *Wnt1-cre;R26RYFP* mice at E11.5, E13.5 and E15.5. At E11.5, most of the differentially expressed genes (DEGs) were X and Y-linked genes active in most tissues. However, at E13.5, a time when sacral neural crest enters the hindgut, almost all the DEGs, including many transcription factors and extracellular matrix/collagen genes, were female biased. By E15.5, many of these early DEGs were now also expressed in the male samples. Across all stages, gene sets relevant to neural crest migration and differentiation were significantly enriched in females suggesting advanced development in females. This was confirmed by scRNAseq, which showed earlier lineage specification towards a neuronal fate in ENCCs from females compared to males, with markers such as *Nrg1*, *Vip* and *Nos1* readily seen in female ENS cells at E13.5. Although none of these DEGs were HSCR-associated, the earlier, perhaps more robust differentiation towards neurons could protect females from HSCR under a sensitised background.

b) ENS phenotypes in mouse models of HSCR are influenced by environment more than sex, but sex-specific expression of a RET isoform in the brainstem could explain a male sex bias in diagnosed cases of HSCR.

With the long-term aim of exploring underlying mechanisms responsible for the male sex bias, eight mouse models of HSCR were investigated, with male and female embryos being scored in each for the severity of ENS defects. These included mice carrying various *Ret*, *Sox10*, and *Ednrb* mutations, two of which had been described previously to show a sex bias. Penetrance and phenotypic severities across five mouse lines in two laboratory environments showed no

significant sex differences. However, it was noted that: (i) Females were more affected in lines where penetrance was not 100%. (ii) Penetrance of phenotypes varied between environments. (iii) There was no sex bias overall, except $Ret^{9/-}$ where females were more severe. (iv) Any male sex bias in phenotype severity found was lost from one laboratory environment to another. The main conclusion from this analysis was that 'environmental factors' can have a notable effect on phenotypic severity of the ENS defects, even within a strain (i.e. with the same genetic make-up), which provided important insights into the variable incidence and severity of HSCR in humans. We also noted that $Ret^{51/51}$ females were underrepresented after birth compared to males, with a 1:3 sex ratio. By observing pups delivered by c-section, almost all $Ret^{51/51}$ females exhibited breathing distress, which was not seen in males. RET has been reported to be expressed in respiratory centres in the brain stem and to have a role in controlling breathing. There was a severe reduction in total RET expression in some of these centres in $Ret^{51/51}$ females but not males, suggesting both RET9 and RET51 isoforms are absent in the former. The basis of this sex-specific control of RET isoform expression will be investigated in future studies, but it provides an explanation for the failure of the females to survive postnatally. It also suggests that, at least some of the sex bias seem in HSCR, which is not usually suspected until at least 48 hours after birth, could be due to a female-specific lethality missed as a failure to breathe at birth or still birth.

8) Development of bioinformatic software and algorithms for genomic analyses on genome sequencing data

Besides MARVEL focusing on regulatory variant analysis, our team has developed a number of bioinformatic tools, algorithms and pipelines to facilitate the interpretation of genetic variants on NGS data not only for HSCR but also for the research community. This include RUNNER and WITER implemented in KGGseq for annotating and prioritization coding and non-coding genetic variants (Nucleic Acids Res. 2021; Nucleic Acids Res. 2019), method to predict enhancer–target networks (Nat Genet, 2017), lassosum for constructing polygenic risk score (Genet Epidemiol, 2017), DESE for estimating driver tissues by selective expression of genes associated with complex diseases or traits (Genome Biol, 2019), and CNV-JACG for curating copy number variations from WGS data (NAR Genom Bioinform, 2020). These software and methods have been extensively used worldwide to study contribution of genetic variants and regulatory landscape of diseases and traits other than HSCR.

9) Other contributions to clinical genetics and precision medicine

To accelerate the translation of genomic medicine into clinical practice in Chinese population, our team also started to lead researches on evaluating the clinical application of NGS data with high medical actionability as the first step for the realization of precision medicine. These studies included the first reports of medically actionable secondary findings in Chinese population on a panel of 59 genes recommended by the American College of Medical Genetics (ACMG) (Hum Genet, 2018; J Hum Genet, 2020) , the first study on projected prescription impact of actionable pharmacogenetic variants (PLoS Genet, 2020), and the first comprehensive catalogue of carrier frequencies for recessive genes included in expanded carrier screening (npj Genomic Med, accepted) in Southern Chinese population. These findings can inform public health policy making and resource allocation in local Chinese population. More importantly, these studies also laid the foundation for the translation of genomic findings to bedside for other complex congenital genetic disorders, including HSCR.

6.2 What was the added value of the TRS funding, rather than standard project grant funding?

This work could not have been achieved without the continuous TRS funding support. Our project sets out an innovative, multi-pronged approach to generate and integrate large-scale genomic, transcriptomic and epigenomic profiles of HSCR patients and animal models and to

understand the molecular disease mechanisms using stem cell-based platforms, which necessitates the assembly of a highly collaborative and multidisciplinary research team under a long term funding commitment. For a congenital disorder with low incidence locally and worldwide, the sustained funding support of TRS also provides a unique opportunity to carry out the powerful profiling and in-depth characterization of the clinical cohort over a longer timeframe. Such resources will be the invaluable assets for future translational researches on precision medicine of the disease. The TRS fund also facilitates the organization of research meetings and symposiums that bring together world class scientists, which provides the opportunities to establish new and to strengthen collaborations between local and universities overseas. The high level of synergy between team members is and will be the key of success to this TRS and other research projects arising from the current TRS program. These could not have been made possible with standard or short term funding support.

6.3 If the project has not met its original objectives, why?

The project has met and exceeded all of the original objectives.

6.4 (a) Peer-reviewed journal publication(s) arising directly from this project:

The Latest Status of Publications				Author(s) *(denote the corresponding author with an asterisk*)*	Title and journal/book *(with the volume, pages and other necessary publishing details specified)*	Submitted to the RGC *(indicate the year ending of the relevant progress report)*	Attached to this report *(Yes or No)*	Acknowledged the support of RGC *(Yes or No)*	Accessible from the institutional repository *(Yes or No)*
Year of publication	Year of acceptance *(for paper accepted but not yet published)*	Under review	Under preparation *(optional)*						
	2021			Chau JFT, Yu MHC, Chui MMC, Yeung CCW, Kwok AWC, Xuehan Z, Lee R, Fung JLF, Lee M, Mak CCY, Ng NYT, Chung CCY, Chan MCY, Tsang MHY, Chan MCK, Chan KYK, Kan ASY, Chung PHY, Yang W, Lee SL, Chan GCF, Tam PKH, Lau YL, Yeung KS*, Chung BHY*, Tang CS*	Comprehensive Analysis of Recessive Carrier Status using Exome and Genome Sequencing Data in 1,543 Southern Chinese. **NPJ Genom. Med.**	No	Yes	Yes	Yes

2021				Lin Jiang, Hui Jiang, Sheng Dai, Ying Chen, Youqiang Song, Clara Sze-Man Tang, Shirley Yin-Yu Pang, Shu-Leong Ho, Binbin Wang, Maria-Mercedes Garcia-Barcelo, Paul Kwong-Hang Tam, Stacey S. Cherny, Mulin Jun Li, Pak Chung Sham* and Miaoxin Li*	Deviation from baseline mutation burden provides powerful and robust rare-variants association test for complex diseases. **Nucleic Acids Research**. 2021 1–15. https://doi.org/10.1093/nar/gkab1234	No	Yes	Yes	Yes
2021				Taida Huang, Yonghui Hou, Xia Wang, Liang Wang, Chenju Yi, Cuifang Wang, Xiaoyun Sun, Paul K.H. Tam, Sai Ming Ngai, Mai Har Sham, Alan J. Burns*, Wood Yee Chan*	Direct Interaction of Sox10 With Cadherin-19 Mediates Early Sacral Neural Crest Cell Migration: Implications for Enteric Nervous System Development Defects. **Gastroenterology**. 2021 Aug 21;S0016-5085(21)03409-0. doi: 10.1053/j.gastro.2021.08.029.	No	Yes	Yes	Yes
2021				Anwarul Karim, Clara Sze-Man Tang* and Paul Kwong-Hang Tam*	The Emerging Genetic Landscape of Hirschsprung Disease and Its Potential Clinical Applications. **Front Pediatr** 9:638093.	No	Yes	Yes	Yes
2021				Yue Ji, Paul Kwong-Hang Tam* and Clara Sze-Man Tang*	Roles of Enteric Neural Stem Cell Niche and Enteric Nervous System Development in Hirschsprung Disease. **Int J Mol Sci** 22(18):9659.	No	Yes	Yes	Yes
2021				Kuil LE, MacKenzie KC, Tang CS, Windster JD, Le TL, Karim A, de Graaf BM, van der Helm R, van Bever Y, Sloots CEJ, Meeussen C, Tibboel D, de Klein A, Wijnen RMH, Amiel J, Lyonnet S, Garcia-Barcelo MM, Tam PKH, Alves MM, Brooks AS, Hofstra RMW, Brosens E*	Size matters: Large copy number losses in Hirschsprung disease patients reveal genes involved in enteric nervous system development. **PLoS Genet** 17(8):e1009698.	No	Yes	Yes	Yes
2021				Ken Hung-On Yu, Christina Huan Shi, Bo Wang, Savio Ho-Chit Chow, Grace Tin-Yun Chung, Raymond Wai-Ming Lung, Ke-En Tan, Yat-Yuen Lim, Anna Chi-Man Tsang, Kwok-Wai Lo* and Kevin Y. Yip*	Quantifying Full-Length Circular RNAs in Cancer. **Genome research**, 2021-12, Vol.31 (12), p.2340-2353	No	Yes	Yes	Yes

2020				Tanja Mederer, Stefanie Schmitteckert, Julia Volz, Cristina Martı´nez, Ralph Ro¨th, Thomas Thumberger, Volker Eckstein, Jutta Scheuerer, Cornelia Tho¨ni, Felix Lasitschka, Leonie Carstensen, Patrick Gu¨nther, Stefan Holland-Cunz, Robert Hofstra, Erwin Brosens, Jill A. Rosenfeld, Christian P. Schaaf, Duco Schriemer, Isabella Ceccherini, Marta Rusmini, Joseph Tilghman, Berta Luzo´n-Toro, Ana Torroglosa, Salud Borrego, Clara Sze-man Tang, Mercè Garcia-Barcelo´, Paul Tam, Nagarajan Paramasivam, Melanie Bewerunge-Hudler, Carolina De La Torre, Norbert Gretz, Gudrun A. Rappold, Philipp Romero, Beate Niesler*	A complementary study approach unravels novel players in the pathoetiology of Hirschsprung disease. **PLOS Genetics** 16(11): e1009106. https://doi.org/10.1371/journal.pgen.1009106	No	Yes	Yes	Yes
2020				Christina Huan Shi, Kevin Y. Yip*	A general near-exact k-mer counting method with low memory consumption enables de novo assembly of 1063 human sequence data in 2.7 hours. **Bioinformatics**, vol. 36, issue S2, pp. i625-i633, (2020). https://doi.org/10.1093/bioinformatics/btaa890	No	Yes	Yes	Yes
2020				Qin Cao, Zhenghao Zhang, Alexander Xi Fu, Qiong Wu, Tin-Lap Lee, Eric Lo, Alfred S. L. Cheng, Chao Cheng, Danny Leung and Kevin Y. Yip*	A Unified Framwork for Integrative Study of Heterogeneous Gene Regulatory Mechanisms. **Nature Machine** Intelligence, vol. 2, issue 8, pp. 447-456	No	Yes	Yes	Yes
2020				Xuehan Zhuang, Rui Ye, Man-Ting So, Wai-Yee Lam, Anwarul Karim, Michelle Yu, Ngoc Diem Ngo, Stacey S. Cherny, Paul Kwong-Hang Tam, Maria-Merce Garcia-Barcelo, Clara Sze-man Tang* and Pak Chung Sham*.	A random forest-based framework for genotyping and accuracy assessment of copy number variations. **NAR Genomics and Bioinformatics**, 2020, Vol. 2, No. 3. doi: 10.1093/nargab/lqaa071	No	Yes	Yes	Yes

2020				Alexander Xi Fu, Kathy Nga-Chu Lui, Clara Sze-Man Tang, Ray Kit Ng, Frank Pui-Ling Lai, Sin-Ting Lau, Zhixin Li, Maria-Mercè Garcia-Barcelo, Pak-Chung Sham, Paul Kwong-Hang Tam*, Elly Sau-Wai Ngan* and Kevin Y. Yip*	Whole-Genome Analysis of Noncoding Genetic Variations Identifies Multi-Scale Regulatory Element Perturbations Associated with Hirschsprung Disease. **Genome Research**, gr.264473.120.	No	Yes	Yes	Yes
2020				Jessica Aijia Liu, Andrew Tai, Jialin Hong, May Pui Lai Cheung, Mai Har Sham, Kathryn S. E. Cheah, Chi Wai Cheung, and Martin Cheung*	Fbxo9 functions downstream of Sox10 to determine neuron-glial fate choice in the dorsal root ganglia through Neurog2 destabilization. **Proc Natl Acad Sci U S A**. 2020 Feb 25;117(8):4199-4210. doi: 10.1073/pnas.1916164117. Epub 2020 Feb 6. PMID: 32029586; PMCID: PMC7049171.	No	Yes	Yes	Yes
2020				Edwin Yu-Kiu Ho, Qin Cao, Mengting Gu, Ricky Wai-Lun Chan, Qiong Wu, Mark Gerstein and Kevin Y. Yip*	Shaping the Nebulous Enhancer in the Era of High-Throughput Assays and Genome Editing. **Briefings in bioinformatics**, 2020-05-21, Vol.21 (3), p.836-850	No	Yes	Yes	Yes
2019				Jiang L, Xue C, Dai S, Chen S, Chen P, Sham PC, Wang H, Li M*	DESE: estimating driver tissues by selective expression of genes associated with complex diseases or traits. **Genome Biol**. 2019 Nov 6;20(1):233. doi: 10.1186/s13059-019-1801-5. PMID: 31694669; PMCID: PMC6836538	No	Yes	Yes	Yes
2019				Lin Jiang, Jingjing Zheng, Johnny S.H. Kwan, Sheng Dai, Cong Li, Mulin Jun Li, Bolan Yu ,KaF.TO, Pak C. Sham*, Yonghong Zhu* and Miaoxin Li*	WITER: a powerful method for estimation of cancer-driver genes using a weighted iterative regression modelling background mutation counts. **Nucleic Acids Res.** 2019 Sep 19;47(16):e96. doi: 10.1093/nar/gkz566. PMID: 31287869; PMCID: PMC6895256.	No	Yes	Yes	Yes
2019				Martin Cheung, Andrew Tai, Peter Jianning Lu, Kathryn S.E. Cheah*	Acquisition of multipotent and migratory neural crest cells in vertebrate evolution. **Current Opinion in Genetics & Development.** 57. 84-90. 10.1016/j.gde.2019.07.018.	2019	No	Yes	Yes

(b) Recognised international conference(s) in which paper(s) related to this project was/were delivered:

Month/Year/ Place	Title	Conference name	Submitted to the RGC *(indicate the year ending of the relevant progress report)*	Attached to this report *(Yes or No)*	Acknowledged the support of the RGC *(Yes or No)*	Accessible from the institutional repository *(Yes or No)*

Month/Year/ Place	Title	Conference name	Submitted to the RGC *(indicate the year ending of the relevant progress report)*	Attached to this report *(Yes or No)*	Acknowledged the support of the RGC *(Yes or No)*	Accessible from the institutional repository *(Yes or No)*
12/2020/ Virtual	Genetics and Functional Genomics of Neural Crest Stem Cells and Associated Disease: Hirschsprung Disease	TRS Symposium 2020	No	Yes	Yes	Yes
11/2020 Virtual	Expanded carrier screening panels and the prevention of inherited monogenic diseases: The first key in precision medicine evaluated using 1116 Hong Kong Chinese exome sequencing data	Joint Annual Scientific Meeting 2020 of The Hong Kong Paediatric Society (HKPS), Hong Kong College of Paediatricians (HKCPaed), Hong Kong Paediatric Nurses Association (HKPNA) and Hong Kong College of Paediatric Nursing (HKCPN)	No	Yes	Yes	Yes
	Actionable secondary findings in Hong Kong Chinese based on exome sequencing data		No	Yes	Yes	Yes
	Actionable pharmacogenetic variants in Hong Kong Chinese exome sequencing data and projected prescription impact in the Hong Kong population		No	Yes	Yes	Yes
6/2020 Virtual	Genes regulating enteric nervous system development are impacted by Copy Number loss and modify penetrance in epistasis with RET	The 53rd European Society of Human Genetics (ESHG) Conference	No	Yes	Yes	Yes
10/2019 Houston	A new machine-learning based method to accurately assess copy number variants from whole genome sequencing data and its application on the analysis of the Hirschsprung disease genome	American Society of Human Genetics (ASHG) 2019 Annual Meeting	No	Yes	Yes	Yes
	Genetic profile of a multiplex Hirschsprung disease family		No	Yes	Yes	Yes

(c) RGC funding should have been acknowledged in all publication(s)/conference papers listed in (a) and (b) above. If no acknowledgement has been made in any of the publications/ papers, please indicate and provide explanations.

Not applicable.

6.5 To what extent this project has strengthened inter-institutional collaborations and other partnerships?

The TRS research team members have had intensive collaborations for the past six years. Principal investigators' meetings were held monthly to update the progresses of collaborative researches. Regular research meeting was held every two - three months with detailed updates

on research progress for each group and to discuss new initiatives for integrative analyses. In addition to the quarterly group meetings, individual clinical, functional or genomic teams also met on regular basis to update ongoing studies in establishing genotype-phenotype correlation (Tam, Maria-Mercedes, Sham, Cherny, and Tang), integrating bioinformatic and genomic NGS data (Yip, Maria-Mercedes, Ngan, Sham, Cherny, and Tang), in functional characterization of genetic lesions in sequenced patients using stem cell-based platform (Tam, Ngan, and Hui), and elucidating regulatory network using animal models (Cheah, Chan, and Lovell-Badge). Research retreat was held yearly to enhance strategic planning of the collaborative research work. Many of these concerted collaborative efforts between TRS team members and collaborators have resulted in joint publications (see Appendix I) and successful grant applications (see Appendix IV). In particular, the strong level of synergy between team members laid the foundation and resources for a new TRS funding to tackle the clinical challenges of a life-threatening congenital liver disorder, biliary atresia.

6.6 Research students trained (registration/awards):

Name	Degree registered for	Date of registration	Date of thesis submission/ graduation
Mr. Zhixin Li	Ph.D	July 1, 2018	On-going
Dr. Kathy Liu	Ph.D	September 1, 2018	November 2021
Dr Karim Md Anwarul	Ph.D	January 1, 2017	March, 2021
Dr Xuehan Zhuang	Ph.D.	December 1, 2016	January 19, 2021
Dr. Huan Shi	Ph.D	August 1, 2015	October 2020
Dr Tingwen Zhou	Ph.D	July 1, 2015	June 2020
Mr Reeson Wang	Ph.D	September 1, 2015	August 2019
Mr Alexander Fu, XI	M.Phil	August 1, 2016	February 2019
Dr. Rosalyn Flower	Ph.D	October 1, 2014	September 2018
Dr. Aijia Liu	Ph.D	September 1, 2011	August 2015

6.7 Specific products (e.g. software or netware, instruments or equipment developed):

The genomic research team has developed a number of bioinformatic software, including KGGSeq, CNV-JACG, and MARVEL under the support of the TRS for the discovery of disease-associated variants/genes/pathways and to aid the interpretation of genomic data. In addition, new statistical methods and bioinformatic tools were developed, including lassosum for polygenic risk score prediction, RUNNER for case-only rare-variants association test, CQF-deNoise for removing noisy false k-mers in de novo sequence assembly, and GEEK for modelling gene expression from various types of protein interactions and chromatin accessibility data.

6.8 Other education activities and/or training programmes developed:
Under the TRS programme, five research symposiums were organized to promote the research interests and findings of the TRS team members, SAB members and other collaborators to other postgraduate students and researchers for knowledge exchange and to nurture international collaboration. In additional to training postgraduates, the TRS project provides invaluable training opportunities for junior scientists, including 11 postdoctoral fellows (Peter Lu, Peikai Chen, Frank Lai, Cynthia Lau, etc) and 2 scientists.

In view of the rapid advancements and clinical application of genome sequencing in diagnosis and clinical management, Sham and Tang have been contributing to the “Best Practice Guidelines in Genetic and Genomic Medicine” by Hong Kong Academy of Medicine to provide a general framework to guide and facilitate good practice in Genetics and Genomics medicine to local medical practitioners and to enhance public education and awareness in this

area.

6.9 Please highlight any deliverables indicated in the project implementation timetable endorsed by the RGC which have not been covered or achieved as per sections 6.1 to 6.8 above, and explain/ elaborate.

All deliverables in the endorsed timetable have been covered or achieved.

Project Management

6.10 Please elaborate how the PC has played his/her role in coordinating and managing the project.

Project Coordinator (PC) has played an important role in monitoring and coordinating research activities (including collaborations, research directions and divisions of works etc) and resources among different research groups and parties. In order to ensure good communications and openness, various meetings were held regularly to update our members of the progress and status of the TRS project. In addition, PC circulated information among members in between meetings to seek their comments and approvals. Furthermore, PC arranged meeting for PIs (either locally or overseas) for meetings with the SAB members to inform them our research progress and seek for their advice. For instance, three SAB meetings (with Professors Chakravarti or Pachnis) were held from April to July 2018 during the conference or overseas visit. In summary, PC is able to communicate well with all levels of members in the administrative, financial and technical areas, thus, ensuring the success of the project. (Please refer to Appendix II)

7. Awards and Recognition

7.1 Have any research grants been awarded that are directly attributable to the results obtained from this project?
The TRS team members have been productive in securing research grants within their respective disciplines. A total of 13 research grants (Total amount: ~HK$26M) directly related to the results obtained in this TRS project have been awarded to individual team members. Please refer to Appendix IV for details.

7.2 Have any project team members participated as invited speakers in or organisers of international conferences as a result of this project?
The TRS team members have been regularly invited to present plenary or symposium lectures at international conferences and workshops both locally and overseas (Total: 50). Team members have also been invited to organize international conferences (Total: 12). Please refer to Appendix I for details.

7.3 Have any project team members taken leadership positions in editorial boards, scientific and professional organisations?
Please refer to Appendix IV for details.

7.4 Any documentary proof of the application of technologies arising directly from this project?
A number of software, pipelines, and algorithm as listed in section 6.7 have been developed for genomic analyses of genome sequencing in this TRS project. These tools and the open source scripts were widely applied or utilized (total number of citations >150) in annotation and prioritization of functional variants in genome sequencing and to compute polygenic risk score for complex diseases, e.g. asthma, stroke, systemic lupus erythematosus, and coronary artery disease, etc.

7.5 Other awards and recognitions as a result of this project (please specify):
Please refer to Appendix IV for details.

8. Impacts

8.1 What are the current and expected impacts of the project on the long-term development of Hong Kong (social or economic development, e.g. patent, technology transfer, collaboration with external organisations, etc.)?

We have successfully (1) identified new HSCR associated genetic factors; (2) defined more accurately the functional attributes of known and newly discovered genes implicated in HSCR, and; (3) integrated the knowledge gained to model and to understand the disease and the sex-linked mechanisms in HSCR. Furthermore, our TRS has generated impacts and momentum in the following areas:

IP filing application has been submitted to our Technology Transfer Office (TTO) for the following inventions:

(1) Use of human pluripotent stem cells (hPSC) to establish a human organoid model of colon with innervated nervous system.

(2) A method to improve the existing differentiation protocol to generate more functional enteric neurons.

By integrating the above two inventions, a more functional innervated human colonic organoid (HCO) model was established. It is of particular importance for assessing the impact of drugs on colonic motility and that can be used for drug toxicity test and drug screening.

Knowledge transfer events have been successfully organized to further engage public

Communication of science to the general public is increasingly recognized as a responsibility of scientists. We have created a website and organized press release, public lectures and symposia to share our most recent research findings with public and, most importantly, the people who are most affected by the disease, and ensures that our work can be used to improve people's lives. Please refer to Appendix I for the details of our research outputs and press release etc.

Our TRS nurtures collaborations worldwide and make Hong Kong becomes a super connector between eastern and western scientific fields

Our TRS facilitates the following collaborations with external institutions and strengthens the super connector role of Hong Kong.

Tam, Paul K.H., Sham, P.C., Garcia-Barceló, Maria-Mercedes, Tang, Clara S.M.
Collaborator: Dr. Bjarke Feenstra (Statens Serum Institut, Copenhagen, Denmark) and Prof. Mikko Pakarinen (University of Helsinki, Finland)

New collaborations were established with two Northern European teams working on genetics of HSCR, including Dr. Bjarke Feenstra and Prof. Mikko Pakarinen. Genome-wide association analysis (GWAS) data from these two new collaborating teams were meta-analysed with the other GWAS data to leverage power for detecting association of common and low frequency variants.

Collaborators: International Hirschsprung Disease Consortium (IHDC)

Small group meeting with IHDC members, Prof. Robert Hofstra (Erasmus, Rotterdam, The Netherlands) and Prof Jeanne Amiel (INSERM, Paris, France) with Prof. Paul Tam and Dr. Clara Tang was scheduled during the 5th symposium of the Enteric Nervous System. Among

other matters, we updated our collaborators and colleagues on the progress of the meta-analysis of GWAS and consortium participants were eager to follow up the association.

Ngan, Elly S.W.
Collaborators: Prof. Urban Lendehl, Dr. Igor Adamekyo and Dr. Ulrika Marklund of Karolinska Institutet
Collaborations were established with various principal investigators in Karolinska Institute, Sweden, including Prof. Urban Lendehl, Dr. Igor Adamekyo and Dr. Ulrika Marklund. With these newly established collaborations, two additional collaborative research grants in related topics (UICP grant of HK$7.6 million and LDS fund of HK$3 million) were secured in non-overlapping fashion by Innovative Technology Fund (ITF) and LDS research center of stem cell and regenerative medicine, to establish high resolution transcriptomes (single cell RNA sequencing) of human ENCC and hiPSC-derived enteric cells. The data generated from these projects will help determine the disease relevant population(s) of ENCCs and the expression data will also be used to support the genetic and functional studies in the TRS project.

- LDS Seed Funding for Stem Cell and Regenerative Medicine Research (LDS-IS-2016/17) Impactful scheme: Neural crest cells and enteric nervous system: development, disease modeling and tissue repair. HK$3,000,000 (PI) (2017-2020) [Dr. Elly Ngan (HKU), Dr. Ulrika Marklund (KI)]
- UICP (UIM/299): Establishment of new treatment strategy for neurological diseases using patient specific induced pluripotent stem cells and high-resolution single cell sequencing. HK$7,600,000 (2017-2019) [Prof. Paul Tam (HKU), Dr. Elly Ngan (HKU), Prof. Urban Lendehl (KI), Dr. Igor Adamekyo (KI)]

Collaborator: Prof. James M. Wells (Cincinnati Children's Hospital Research Foundation, Cincinnati, US) and Maxime M Mahe (University of Nantes, France)
A new collaboration was recently established for generation of human innervated colonic organoid model. The data generated from this project has been submitted to our TTO for patent application and a manuscript is ready for submission.

Cheah, Kathryn
Collaborator: Dr. Paul Trainor, (Stowers Institute for Medical Research, Kansas City USA) for functional studies on the role of Rdh10 expressing ENCCs*, Dr. Marianne Bronner and Dr. Rosa Uribe* (California Institute for Technology, and *Rice University, USA), Dr. Vasilis Pachnis (Francis Crick Institute) and Drs. Igor Adameyko, Ulrike Marklund (Karolinska Institute) and Dr. Peter Kharchenko (Department of Biomedical Informatics, Harvard Medical School)* sharing of unpublished transcriptomic data on NCCs isolated from different stages of fetal and postnatal mice and from Zebra fish (*Bronner, Uribe*).

8.2 Others (please specify):
Nil.

9. Sustainability of the Project

9.1 Whether there are new ideas evolved directly from this project?
Upon the completion of the TRS project, we have identified novel genetic risk factors, including common regulatory and rare coding variants, underlying the genetic etiology of HSCR. Transcriptomic studies on patient-specific hiPSC models established under this TRS as well as animal models have uncovered novel NCC gene regulatory networks mediating ENS development. These new findings provide new insights and bring out new hypotheses that form the foundation of new research projects. For example, based on the novel HSCR-associated common variants identified in this TRS together with known causal variants,

we develop a polygenic risk score model that significantly predicts disease risk. Such genetic risk model can be used for genetic risk prediction in high risk families and to further elucidate the genetic landscape across different disease subtypes. Validation of the model on independent samples is on-going. In addition, new disease mechanisms uncovered in this project, including neuronal death and impaired ECM niche-cell interaction caused by genetic lesions in the BACE1-APP-BACE2 pathway and ECM-associated genes respectively, give rise to new testable hypotheses of plausible functional roles in ENS development of other interacting partners or genes under the same pathways. The genetic effects of several novel candidates are under investigation using hiPSC and animal models. Follow-up in vivo studies on the impact of loss of function of gene(s) defining the novel "M" cell population in NCCs is in progress.

9.2 Whether there are new projects evolved directly from this project?

New projects have been evolved directly from this TRS and we have sought external funding sources to continue supporting these researches after the successful completion of the TRS. Currently, we have obtained

(1) one HMRF and one non-profit patient community (REACH)-based research grants to improve, leverage and test the polygenic risk model developed based on novel genetic findings from this TRS project;

(2) one GRF and one HMRF grants to pursue further development of machine learning-based methods on structural variation detection and framework for multi-omics data analysis and to apply these methods on patient/HSCR animal model dataset to detect novel HSCR/ENS-relevant genetic risk factors;

(3) one HMRF grant (recommended for support) to functionally characterize the lineage-specific effect of loss of function of HSCR candidate genes in the neural stem cell niche and in the ENCCs using hiPSC-based platform;

(4) one HMRF Commissioned grant to elucidate the genetic architecture of the rarer disease subtypes of HSCR (e.g. long segment HSCR and total colonic aganglionosis) for the comprehensive interrogation of the full genetic landscape of HSCR.

(5) one HMRF grant to test functionally non-coding regulatory regions identified in HSCR patients.

In addition, with the emerging evidence of intrinsic genetic defects of the ECM and cellular niche in HSCR patients from this TRS, we initiate a new collaborative project aiming (i) to identify the niche factors responsible for the ENS developmental defects in HSCR patients through transcriptomic and proteomic studies and (2) to discern their underlying patho-mechanisms using the stem cell-based platform. To achieve this, we have put forward a CRF application with full proposal submitted to request for funding support to this new initiative.

9.3 Whether there are new collaborations developed directly from this project?

As detailed in section 8, new collaborations in the field of genetics, developmental biology and stem cell biology have been established directly from this project, which places the project team in a strong position in international collaborations in the research field of ENS development and to lead global research effort (e.g. transethnic meta-analysis of genomewide association) in deciphering the genetic basis of the Hirschsprung disease.

9.4 Please give details on how much money and from which sources has been obtained/requested for the specific purpose of continuing the work started under this project.

We have successfully obtained a total of ~11.8 million external research grants as listed in 9.2 to continue the new projects dedicated on work started under this TRS program. Individual groups of investigators under TRS have also jointly put in a GRF application (~1.2 million) to functionally characterized HSCR-associated structural variations in ENS development and an

RGC Collaborative Research Fund full proposal (~4.2 million) to elucidate the genetic defects in ENS-niche interaction in disease pathogenesis.

10. Statistics on Research Outputs

	Peer-reviewed journal publications	Conference papers	Scholarly, books, monographs and chapters	Patents awarded	Other research outputs (please specify)	
No. of outputs arising directly from this research project	33	50	-	2	Type	No.
					Thesis	8

11. Public Access of Completion Report

Information that cannot be provided for public access	Reasons

12. The Layman's Summary

(describe in layman's language the abstracts and research impact of the project.)

Hirschsprung disease (HSCR) is the leading cause of functional intestinal obstruction in newborn babies, with incidence being the highest among Asians. Surgery is currently the only treatment yet complications often arise and physiological dysfunction persists in many patients. Regenerative medicine, particularly stem cell therapy, has become a promising new treatment paradigm for HSCR to cure the lifelong sequelae and to improve the health-related quality of life. HSCR is a complex genetic disorder caused by abnormal development of the enteric nervous system (ENS), whereby enteric neural crest cells, a multipotent stem cell-like population capable of differentiating into enteric neurons and glia, fail to reach the distal portion of the colon. Thus far, the underlying genetic risk factors and detailed disease mechanisms of HSCR remain largely unknown. In this TRS, we have assembled a multi-disciplinary research team of clinicians and scientists and employed a multi-faceted approach to exploit the genome, transcriptome, and epigenome of HSCR patients and mouse models through next generation sequencing to comprehensively elucidate the genetic basis and molecular mechanisms of HSCR pathogenesis. We have developed and optimized the neural crest derivation from human induced pluripotent stem cells (hiPSCs) to functionally demonstrate the impact of the newly identified HSCR-associated genes on ENS development. Novel disease-associated genetic factors were identified through large-scale genome sequencing studies on HSCR patients. When coupled with genome editing on hiPSCs, we unravelled two novel disease mechanisms in which genetic defects resulting in neuronal death and impaired interaction between enteric neural progenitors and neural stem cell niche underlie the etiology of HSCR. Transcriptomic analyses on patient-derived iPSCs and animal models further (i) uncovered common and distinctive biological pathways associated with *RET* commonly dysregulated in HSCR patients; (ii) revealed novel interaction and regulatory landscape of ENS development; and (iii) confirmed cell stage–specific regulatory roles of novel regulatory elements together with hiPSC. Overall, the TRS project has strengthened international collaborations and offered training opportunities to postgraduate students, postdoctoral fellows, and junior scientists. The advances in genomics and stem cell biology of HSCR arising from this TRS are positioned in light of the current endeavour directing toward translational research and personalized non-surgical treatment of HSCR. Ultimately, the project helps to establish Hong Kong as a leading research centre in rare diseases in children.